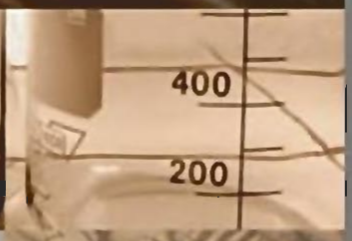
HANDBOOK OF ALCOHOLIC BEVERAGES

Technical, Analytical and Nutritional Aspects

Editor Alan J. Buglass



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Handbook of Alcoholic Beverages

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

Handbook of Alcoholic Beverages

Technical, Analytical and Nutritional Aspects

Volume I

Edited by

ALAN J. BUGLASS
Department of Chemistry, KAIST, Republic of Korea

WILEY
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Technical, Analytical and Nutritional Aspects

Volume II

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WILEY
A John Wiley and Sons, Ltd., Publication

This edition first published 2011
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John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, United Kingdom

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Library of Congress Cataloging-in-Publication Data

Handbook of alcoholic beverages : technical, analytical and nutritional aspects / edited by Alan J. Buglass.
p. cm.
Includes bibliographical references and index.
ISBN 978-0-470-51202-9 (cloth)
1. Alcoholic beverages–Handbooks, manuals, etc. I. Buglass, Alan J., 1946–
TP510.H35 2011
663′.1–dc22

2010036849

A catalogue record for this book is available from the British Library.

Print ISBN: 978-0-470-51202-9
eBook ISBN: 978-0-470-97651-7
oBook ISBN: 978-0-470-97652-4
e-Pub ISBN: 978-0-470-97665-4

Typeset in 10/12pt Times by Aptara Inc., New Delhi, India.
Printed in Malaysia by Ho Printing (M) Sdn Bhd.

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General Acknowledgments and Thanks

The following is a thank you note to individuals who have provided help and inspiration in one form or another and have made the writing of this book all the more enjoyable.

David Ando (publishing consultant), Dartford, UK

Roger Bennett (head brewer), Holden's Brewery, Woodsetton, UK

Winrich von Bierbrauer of Bühler GmbH, Braunschweig, Germany

Cindy Burchfield of Alaskan Brewing Co., Juneau, Alaska, USA

Philip Darby and Niven Balfour of the Nottingham Brewery Co Ltd, UK

Jenny Cossham, Richard Davies, Amie Marshall, Zoe Mills and Jonathan Peacock at John Wiley and Sons Ltd, Chichester, UK

Andrew Davis and Laura Harter of Sierra Nevada Brewing Co., Chico, USA

Bob Douglas, Ken Morrison and Nick Sewter of Magpie Brewery, Nottingham, UK

Alison Duke of Vancouver Island Brewery, Canada

Jerôme Dupont of SARL Domaine Familial Louis Dupont, Victot-Pontfol, France

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Christine Logan of Lady of the Isles, Islay, Scotland

Jess Longhurst of Black Sheep Brewery Ltd, Masham, UK

Corrie W. Martin of Farnum Hill Ciders and Poverty Lane Orchards, New Hampshire, USA

Iain Masson, former head brewer of (now defunct) Hardys and Hansons Brewery, Kimberley, UK

Derek Pritchard of Dunkerley Vineyard, UK

Kelly Roberts of Coopers Brewery, Adelaide, Australia

Andi Rose of New Belgium Brewing Co., Fort Collins, USA

Jonathan Scott of Ian Macleod Distillers Ltd (Glengoyne Distillery), Scotland

Sandra Schmid of G. Schneider & Sohn GmbH, Germany

Jamie C. Smith of Firestone Walker Brewing, Paso Robles, USA

Julian Temperley of Royal Somerset Distillery, Kingsbury Episcopi, UK

Karen Varty and Peter Laws (former head brewer) of Jennings plc, Cockermouth, UK

Donna Warner of Heritage Brewing and Scotch Irish Brewing, Canada

Anthony Wills of Kilchoman Distillery, Islay, Scotland

Part 1
Introduction, Background and History

John A. Hudson and Alan J. Buglass

'Drink! for you know not whence you came, nor why;
Drink! for you know not why you go, nor where."
—Omar Khayyam.

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

1.1

Alcoholic Beverages of the World: An Introduction to the Contents of This Book

In many parts of the world, alcoholic beverages are an important part of day-to-day life. Their moderate consumption in a social environment is seen as a boon for both body and soul. Many are the proposals of marriage, forging of business partnerships and fruitful scientific discussions that have taken place through the centuries over a drink or two in a bar, or over dinner at home. It is not by chance that Francis Crick and James Watson celebrated their discovery of the double helix structure for DNA over pints of ale in the bar of The Eagle, an old coaching inn close to the centre of Cambridge. Indeed, public drinking places are more often than not meeting places: focal points for merriment, discussion, liaison and the sharing of dreams. From the cosy English country pub, the sunny German Biergarten and the elegant Parisian café to the tavernas of Italy and Greece, the small taverns high in the Andes, the bars of South Africa and the jumak of Korea, the multitudes of alcoholic drinks consumed in all these places are potent social lubricants.

There is truly a fantastic range of alcoholic beverages to enjoy – something to suit almost everyone for almost every occasion. At the heart of all alcoholic beverages is fermentation (Part 2), particularly alcoholic fermentation, whereby sugars are converted to ethanol and many other minor products (Chapters 2.1 and 2.2). Many different fungi are able to promote at least some conversion of sugar to ethanol, but in doing so they are often able to stamp their personalities on the beverage by producing characteristic flavor profiles (Chapter 2.2), thus contributing to the rich diversity of alcoholic drinks. Likewise malolactic bacteria, by performing malolactic fermentations under the right conditions, are able to positively influence the character of alcoholic beverages (Chapter 2.3). Fermentations caused by certain other bacteria or fungi can have undesirable influences on aroma and flavor (Chapter 2.4), but these are usually suppressed in favor of the action of desirable microorganisms by the use of antiseptics and preservatives such as sulfites (Chapter 2.5).

Beers are enjoyed all over the world: they are brewed according to many different recipes and procedures, giving many hundreds of different brands or styles, from pale Pilsners and wheat beers to brown ales, porters and stouts (Chapter 2.6). Beverages brewed from cereals other than barley or wheat, such as maize, millet and rice (and without the use of hops) are enjoyed by millions of people in Africa, Asia, and Central and South America (Chapter 2.7). Some general information on beer and cereal beverages, and where they are brewed can be found in Figures 1.1.1 and 1.1.2.

Cider and perry are produced in many countries in a wide range of styles and flavors by a variety of methods (Section 2.8). Although from a biochemical and sensory viewpoint, cider and perry are closer to wine than

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2003 or 2004 Approximate beer production figures for selected European countries and the Russian Federation (in hl × 10^3): Germany (106 300), Russian Federation (70 000), U.K. (58 000), Spain (28 000), Poland (26 000), Netherlands (25 000), Czech Republic (19 000), France (18 000), Belgium (17 400), Austria (9000), Denmark (8 300), Ireland (8 000), Slovakia (4700).

Hops are grown all over Europe, the best known areas being Bavaria (Germany), Bohemia (Czech Republic), Hereford (UK), Kent (UK) and Saxony (Germany).

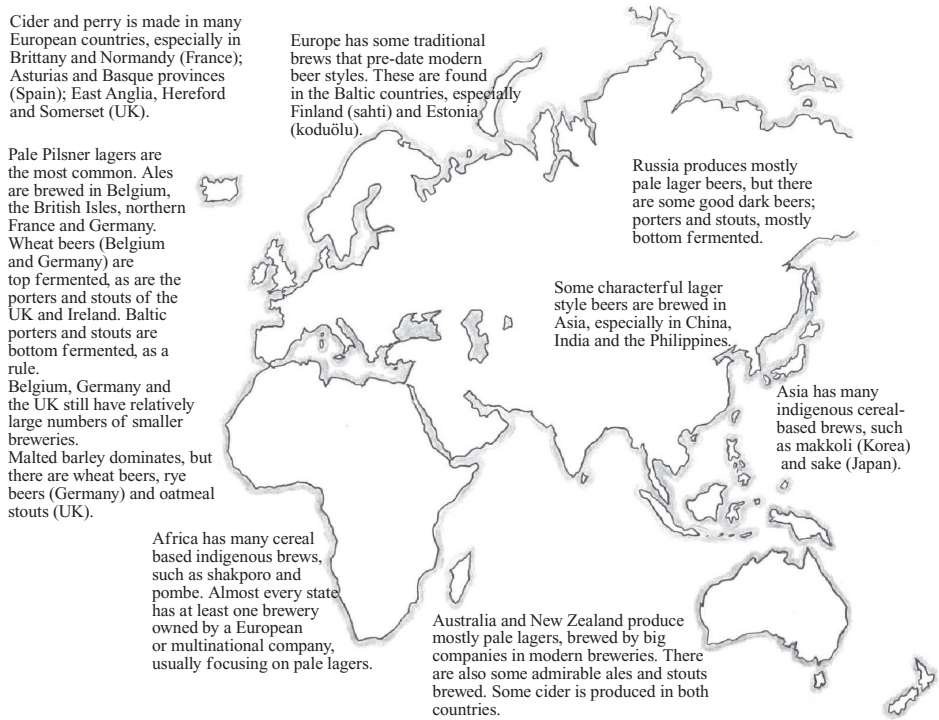


Figure 1.1.1 *Beer and Cider in Europe, Africa, Asia and Australasia. Thanks are due to Belhaven Brewery Ltd, Coopers Brewery Ltd, Domaine Familial L. Dupont, Hite Co., Jennings plc, Keo Ltd, Brasserie Lindemans, Nottingham Brewery Ltd, G. Schneider & Sohn GmbH and Whin Hill Cider for permission to use label and bottle images*

Like USA, Canada's beer market is dominated by pale lagers brewed by a handful of large companies. However, again like USA, there has been an upsurge in the brewing of ales and special lagers by a rapidly growing number of microbreweries and brewpubs since the 1990s. Cider and perry are made in nearly all the states, especially Quebec and British Columbia.

Hops are grown extensively in Oregon, Washington State and British Columbia. They are also grown in some eastern states, such as Vermont.

The Carribean islands produce some characterful lagers and stouts.

USA can be considered to be the world's prime brewing nation. Not only is the country the biggest producer of beer and the home of one of the largest brewing companies (Anheuser Busch), but also has the widest range of beer styles (although these are not necessarily universally available). Many of these styles are derived from European styles and are brewed mostly by smaller regional and (especially) by the hundreds of microbreweries and brewpubs that have sprung up since the 1980s. Although pale Pilsner style lagers dominate the everyday market, the beer drinker will find a huge range of ales of all strengths, colors and flavors, as well as wheat beers and high quality lagers. Many of these beers, however, are not widely distributed. Light beers are popular in USA and Canada. Cider and perry are produced in many states, by both large and small companies. Good cider is made from indigenous apples, but also nowadays from European cider apples.

Central and South America produce mainly pale lagers from a relatively small number of large breweries. Mexico is one of the biggest exporters of beer: much of it going to the USA. There are many indigenous brews, such as chica, usually made from maize and other grains. Two of the biggest drinks companies (which includes brewing companies) are partly Brazilian-owned. Cider is produced in some South American countries.

Figure 1.1.2 *Beer and cider in the Americas. Thanks are due to the Alaskan Brewing Co., Farnham Hill Cider, Firestone Walker Brewing Co., McAuslan Brewing Inc., New Belgium Brewing, Red Hook Ale Brewery, Scotch-Irish Brewing, Sierra Nevada Brewing Co. and Vancouver Island Brewery for permission to use label images*

beer, socially they are closer to beer, and so for the latter reason general information on these two drinks can be found in Figures 1.1.1 and 1.1.2, alongside beers.

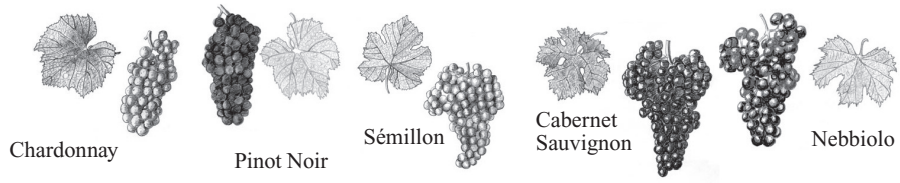
Wines are similarly enjoyed throughout the world, produced in a wide range of styles from many vine varieties by numerous methods. They range from light sparkling wines and still, dry white wines through rosé, red and sweet, white table wines (Chapter 2.9) to fortified wines (Chapter 2.10) and fortified/flavored wines (Chapter 2.12). These are all made from grapes and some general facts concerning their production and styles are given in Figures 1.1.3 and 1.1.4. Wines from fruit other than grapes or even from vegetables, flowers, honey or sap (Chapter 2.11) are made in many parts of the world: general information on these drinks can be also be found in Figures 1.1.3 and 1.1.4.

Apart from the multitude of biochemical reactions that occur during fermentation, important biochemical, chemical and physical processes occur at other stages during the manufacture of alcoholic drinks. Included here is the boiling process in the brewing of beer (Section 2.6.3); different wine maturation processes, such as those for fino Sherry (Section 2.10.3) and Madeira (Section 2.10.6); clarification processes (centrifugation, filtration and fining) and pasteurization (see for example Sections 2.6.9, 2.8.5, 2.8.6, 2.9.4, 3.2.5); and blending and packaging (see Sections 2.6.10, 2.10.2 and 3.2.5).

Certain alcoholic beverages are brewed specifically for distillation, which converts them into distilled beverages or spirits (Part 3). These are produced using a variety of distillation techniques and a range of types of stills (Chapter 3.1). Thus malted cereal beverages are used to produce Scotch whisky (Chapter 3.2) and other whiskeys (Chapter 3.3), as well as a number of other distilled drinks (Chapter 3.4) such as akvavit, gin and vodka. Canes, roots, tubers and saps can also be used to make distilled beverages, such as arrack, rum, schnapps and tequila (Chapters 3.5 and 3.8). Brandy is distilled wine and is produced in nearly all wine-producing countries (Chapters 3.6 and 3.7). Likewise, fruit wines such as plum wine or cherry wine can be converted to fruit brandies such as slivovitz and kirsch (Chapter 3.8) and spirits like brandy, gin or Scotch whisky can be infused with fruit, herbs or spices and (often after redistillation), sweetened with sugar or honey to make liqueurs (Chapter 3.9). Figures 1.1.5 and 1.1.6 give some general information on the wide variety of distilled beverages and where they are made.

Science and technology now play major roles at all stages in the production of most alcoholic drinks – from genesis in the barley field, hop garden, orchard or vineyard, through manufacture by fermentation, distillation and other processes to maturation, clarification, blending and packaging. Quality assurance and control have elevated the overall quality of many beverages to consistently high levels (Section 1.2.5). The analytical methods that are used for the maintenance of quality are described in Part 4. Many of these techniques also increase our knowledge and understanding of the various materials and processes involved in alcoholic drinks production, as well as helping the brewer or winemaker to maximize the quality of his or her product. The biological and chemical natures of the raw materials (Sections 2.6.2, 2.6.3, 2.8.2, 2.11.2, 2.12.2, 3.2.2, for example) are of prime importance in the making of alcoholic beverages, as is the chemical composition of the finished product, particularly with regard to sensory analysis (Chapter 4.7). The quality and authenticity of a beverage can often be judged by the absence or presence (and relative quantity) of certain constituents, as determined by one or more of the numerous methods described in Part 4: by chromatographic (Chapter 4.3), spectroscopic (Chapter 4.4) or electrochemical methods (Chapter 4.5), for example.

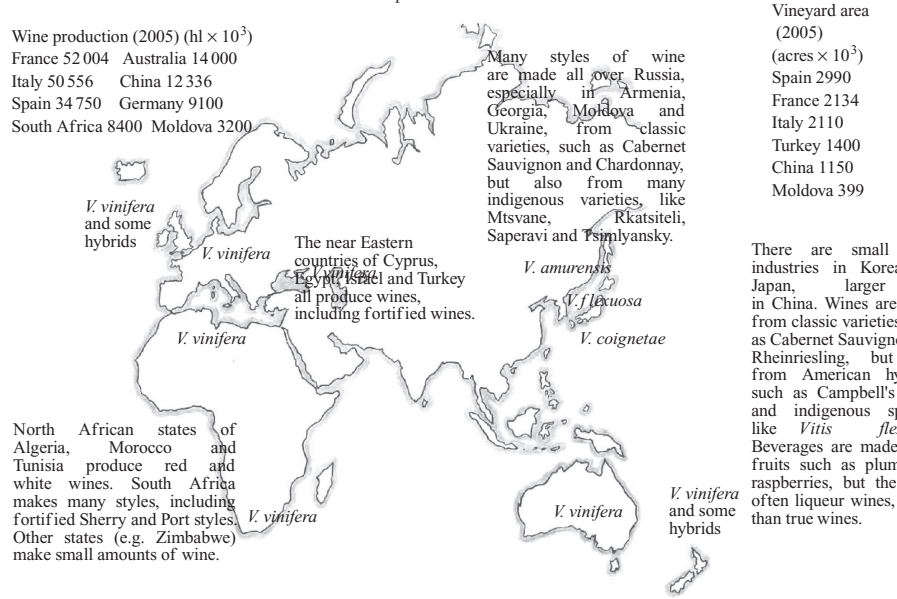
The levels of many alcoholic beverage components are now subject to legislative restriction, which means samples must be checked by government or approved laboratories from time to time to ensure such components are present below their maximum allowed levels or maximum residue levels (MRLs). Many producers also perform analyses throughout the production process, as part of their quality assurance programs. These components include some that are added deliberately as part of the production process (additives; see Chapter 5.9 and plant flavorings; see Chapters 2.12 and Sections 3.9.3 and 5.11.2), as well as some that are derived from the basic raw materials (e.g nitrogenous allergenic substances, Section 5.11.3) and some,



There are small wine industries in the UK, Belgium, Ireland, the Netherlands and even southern Scandinavia. Good wines are made, often in state of the art wineries from *Vinis vinifera* crosses and hybrids that have been bred for cool climates. These include Bacchus, Huxelrebe, Kerner, Müller Thurgau, Schönburger and Seyval Blanc. Classic varieties like Pinot Noir and Chardonnay are used to make very good sparkling wines by the Champagne method. Fruit wines are made in several northern European countries.

Wine production (2005) (hl × 10^3)
France 52 004 Australia 14 000
Italy 50 556 China 12 336
Spain 34 750 Germany 9100
South Africa 8400 Moldova 3200

Vineyard area (2005) (acres × 10^3)
Spain 2990
France 2134
Italy 2110
Turkey 1400
China 1150
Moldova 399



There are small wine industries in Korea and Japan, larger ones in China. Wines are made from classic varieties, such as Cabernet Sauvignon and Rheinriesling, but also from American hybrids, such as Campbell's Early and indigenous species, like *Vitis flexuosa*. Beverages are made from fruits such as plums and raspberries, but these are often liqueur wines, rather than true wines.

France produces the biggest quantities of the world's finest wines; nearly all the great wine styles have French origins. This includes Burgundy (Chardonnay, Gamay Noir and Pinot Noir), Bordeaux (Cabernets, Merlot Noir, Sauvignon Blanc and Sémillon), the Rhone valley (Syrah for red, Marsanne, Roussane and Viogner for white), Alsace (mainly dry white wines), the Loire valley (light reds to sweet whites). Vermouth and fortified wines (e.g. Muscat de Lunel) are also made. Spain and Portugal produce a wide range of wines, from dry white to sweet red, including the great fortified wines, Madeira, Port and Sherry. Italy is the home of some great red wines, like Barolo in the north and Brunello di Montalcino further south. Italy also produces the unique styles of reciotto and ripasso. Vermouth and the fortified Marsala are also made. Germany, along with Alsace, make great wines from the Rheinriesling. Other countries of central, southern and eastern Europe also make good wines in many styles (e.g. Tokay of Hungary). Fruit wines are made all over Europe, but much of it is distilled (see Figure 1.1.5).
Australia has a large number of wineries that produce some great red wines, like Penfold's Grange, made from classic varieties such as Cabernet Sauvignon and Shiraz (Syrah). There are also good white wines made from Chardonnay, Rheinriesling, Sémillon and others, as well as great fortified Muscat wines. New Zealand makes some especially flavory white wines from Gewürztraminer and Sauvignon Blanc, and also lighter red wines from Cabernets and Pinot Noir. Hybrids are still grown in New Zealand.

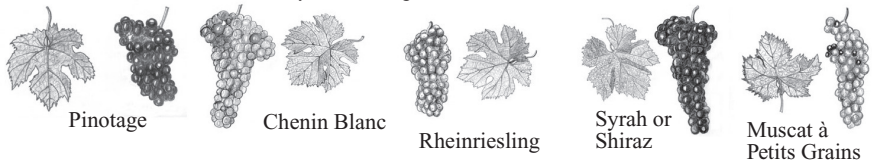
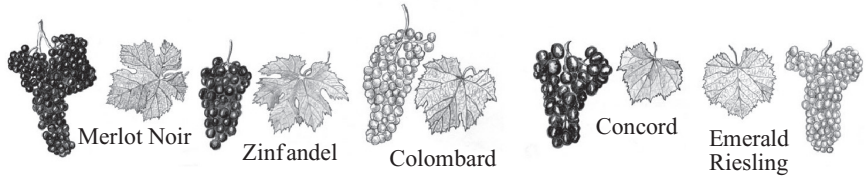


Figure 1.1.3 *Wine in Europe, Africa, Asia and Australasia*

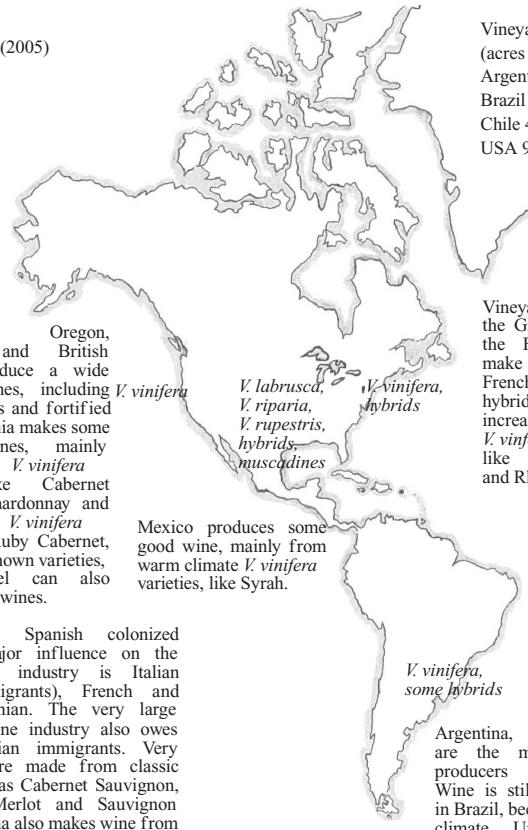


Fruit wines, from pomegranate, through cherry to black raspberry are made throughout Canada and USA, even in states with extensive vineyard areas, such as California and New York.

Many eastern, central and southern states produce wine from native American varieties such as Catawba, Concord, Delaware, Niagara, Noah and Scuppernong. Wines are also made from French-American hybrids, such as Baco Noir, Cascade, de Chaunac and Marechal Foch.

Wine production (2005)
($hl \times 10^3$)
Argentina 15 222
Brazil 3200
Chile 7890
USA 28 750

Vineyard area (2005)
($acres \times 10^3$)
Argentina 520
Brazil 170
Chile 465
USA 935



California, Oregon, Washington and British Columbia produce a wide range of wines, including sparkling wines and fortified wines. California makes some world class wines, mainly from classic *V. vinifera* varieties, like Cabernet Sauvignon, Chardonnay and Pinot Noir. *V. vinifera* crosses, like Ruby Cabernet, and less well known varieties, like Zinfandel can also give very good wines.

V. vinifera

V. labrusca, V. riparia, V. rupestris, hybrids, muscadines

V. vinifera, hybrids

Vineyards close to the Great Lakes or the Finger Lakes make wine from French-American hybrids and increasingly from *V. vinfera* varieties, like Chardonnay and Rheinriesling.

Mexico produces some good wine, mainly from warm climate *V. vinifera* varieties, like Syrah.

Although the Spanish colonized Chile, the major influence on the Chilean wine industry is Italian (through immigrants), French and lately, Californian. The very large Argentinian wine industry also owes much to Italian immigrants. Very good wines are made from classic varieties, such as Cabernet Sauvignon, Chardonnay, Merlot and Sauvignon Blanc. Argentina also makes wine from Malbec, as well as Barbera and other Italian varieties. Some South American states produce wine from other fruits, such as blackberry.

V. vinifera, some hybrids

Argentina, Brazil and Chile are the most important wine producers of South America. Wine is still made from hybrids in Brazil, because of the hot, humid climate. Uraguay makes good wine from less common *V. vinifera* varieties, like Gewürztraminer and Tannat.

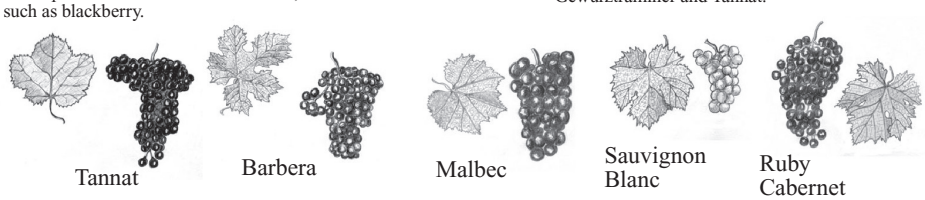
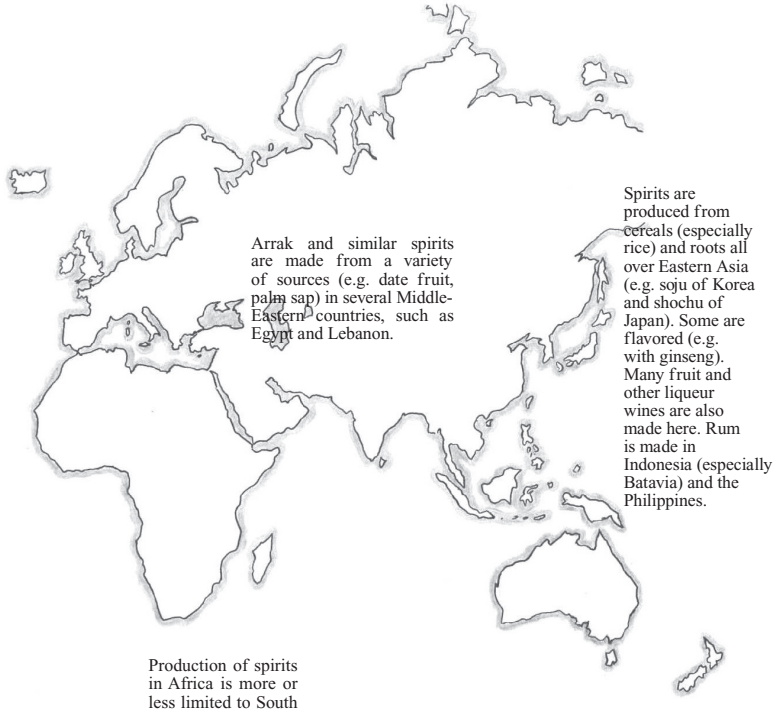


Figure 1.1.4 *Wine in the Americas*

Brandy (distilled wine) is the major spirit of mainland Europe. Cognac and Armagnac are the finest and best known, but brandy is made in nearly all wine growing countries, from Spain to the Balkan countries of Greece and the former Yugoslavia. Grape pomace spirit is made in France (marc) and Italy (grappa) in particular. Fruit brandies and liqueurs (many brandy-based) are produced all over Europe. The former from Alsace are especially fine, but good fruit brandies are also made in Greece and the former Yugoslavia.
In northern Europe, akvavit (also called schnapps) is popular. It is distilled from grains, roots or even fruit and is usually flavored with herbs.
Vodka is a similar drink that is produced in Finland, Poland, other Baltic states and Russia. It is made mainly from cereals and is sold mostly unflavored, often being used in cocktails and mixes. There are flavored versions of vodka. Gin is made in the Netherlands, as are some liqueurs (e.g. cherry brandy). Russia produces brandy in the south of the country.



Scotch whisky is the most noted spirit of the British Isles: it is exported all over the world, mainly as blends and single malts. The styles range from the light Lowland whiskies, through fuller bodied highland whiskies, to the heavily peated Island whiskies. Irish Whiskey also finds world wide popularity and can be increasingly found as single malts. The Isle of Man and Wales also produce small amounts of whisky. Gin is distilled in London and Plymouth. Tiny amounts of brandy and apple brandy are made. There is small range of liqueurs, good examples being sloe gin and the Scotch whisky-based Drambuie.

Arrak and similar spirits are made from a variety of sources (e.g. date fruit, palm sap) in several Middle-Eastern countries, such as Egypt and Lebanon.

Spirits are produced from cereals (especially rice) and roots all over Eastern Asia (e.g. soju of Korea and shochu of Japan). Some are flavored (e.g. with ginseng). Many fruit and other liqueur wines are also made here. Rum is made in Indonesia (especially Batavia) and the Philippines.

Production of spirits in Africa is more or less limited to South Africa, where good brandy is made.

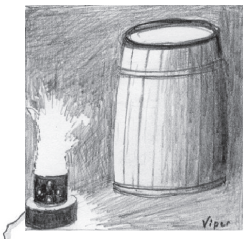
Figure 1.1.5 *Spirits in Europe, Africa, Asia and Australasia. Thanks are due to Familial Dupont, Kilchoman Distillery Co. Ltd., Keo Ltd., Glengoyne Distillery and The Rum Story, for permission to use label and bottle images*

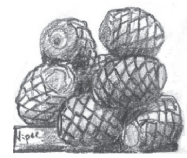
such as ethyl carbamate (Section 5.11.5) that are sometimes formed during the manufacturing process. Also included here are pesticide residues and various other contaminants (Chapter 5.10).

It has long been known that alcoholic beverages possess nutritional and health values, in both positive (beneficial) and negative (detrimental) senses (Part 5). As public awareness and interest in nutrition and health issues have grown during the past two or three decades, so scientific interest in the nutritional value

During the last decade there has been a marked growth in microdistilleries, especially in the USA. These distilleries produce a wide range of spirits, although mostly from grains: gin, rum, vodka and whiskeys of various styles, mostly using batch column or pot stills.

Whiskey (whisky in Canada), is distilled all over North America, from mainly mixed grain mashes, although maize (corn) dominates the whiskeys of USA; wheat is more important in Canada. Bourbon is made from at least 51% maize, with barley and rye. The whiskeys of Kentucky, Tennessee and Virginia are probably the most famous, but much Canadian whisky is consumed in the USA. Straight rye and corn whiskeys are making a modest resurgence in the USA and Canada (rye). Distilling is mostly by batch and continuous column stills, but a few pot stills are in use.





Tequila is the major spirit of Mexico, although brandy and rum are also produced. Tequila and mezcal are made from fermented *Agave* pulp and are much exported, especially to the USA.

Many of the South American states are wine producers and so also make brandy. Perhaps the best known brandy is pisco, made from Muscat wine in Peru, and from other wines in Chile.

The Carribean countries produce much of the world's bestrum. Light rum for mixed drinks and cocktails is made throughout the area, but is typified by those from Cuba and Puerto Rico. Likewise, Heavier darker rums are produced everywhere, but those of Demerara, Jamaica and Martinique are the best known. Both pot stills and continuous stills are used.

Cachaca is the cane spirit of Brazil. It is still produced by hundreds of small to medium distilling companies and its exports are rising. Pot stills, as well as batch and continuous column stills are used. Brazil grows much of the sugar cane used for Carribean rum.

Figure 1.1.6 *Distilled spirits and liqueurs in the Americas*

of alcoholic drinks and their components has increased (Chapters 5.1–5.5). Several alcoholic drinks are important for their macronutrients such as carbohydrates (Chapters 5.3 and 5.7) and many possess significant quantities of micronutrients (Chapter 5.4) and 'prebiotic' components, such as oligosaccharides, lactic acid bacteria and yeast. Ethanol, although it has a high calorific value itself, its presence in the diet can negatively influence uptake and metabolism of nutrients (Chapter 5.5). The past 10 years in particular have also witnessed a rapid growth in scientific interest in the health values of specific components of beverages, notably ethanol (Chapter 5.6), carbohydrates (Chapter 5.7), phenolic compounds (Chapter 5.8), additives (Chapter 5.9)

and trace components (Chapter 5.11). Similarly, growth in public interest in health, safety, pollution and environmental issues has catalyzed increased scientific activity in the study of pesticide residues and other contaminants of alcoholic beverages and the raw materials from which they originate (Chapter 5.10).

The various alcoholic drinks industries are important parts of the food and drinks industry: they contribute to a nation's economic prosperity to a significant degree. Indeed in some countries, such as several in the Caribbean area, the contribution is a major one. Moreover, the alcoholic drinks industries support numerous other diverse industries, like glass, plastics, cork, engineering and tourist industries. Additionally, agriculture has important ties with the alcoholic drinks industries: the growing of barley and other cereals for beer and spirits production, hops for the brewing of beer, apples for cider making, grapes for the production of wine and brandy, sugar cane for making rum and so on. Forestry provides cork, oak or chestnut wood to make casks for the maturation of wine or spirits and other kinds of timber to produce stakes for the support of crops such as grapevines.

According to the Wine Institute of California, in 2008, the wine industry in that state provided 309 000 jobs, produced an annual $51.8 billion in economic value for that state, generated $125.3 billion for the US economy, paid $10.1 billion in wages in Calfornia ($25.2 billion nationwide), made $13 billion in state and federal tax payments, attracted 19.7 million tourists per annum and generated wine-related tourism expenditures of $2 billion in California. The Californian wine industry also gives $115 million in annual charitable contributions and offers numerous intangible benefits to local communities, such as (amongst other things) enhancing the general quality of life, bringing positive visibility to the community, building local pride, offering cultural attractions, supporting local businesses, promoting responsible farming and winemaking, and providing scenic pastoral landscapes. The Californian wine industry can reasonably be regarded as a microcosm of the alcoholic beverage industry as a whole, where wineries, breweries and distilleries all over the world play similar roles in support of their local and national communities. Although the alcoholic drinks industry per se is not discussed in detail in this book, its presence obviously permeates the text of Parts 2 and 3 in particular, and also to a lesser extent that of Part 5.

1.2

History and Development of Alcoholic Beverages

1.2.1 The Beginnings (From the earliest Times to ca. 1100 AD)

Serendipity is defined as the art of making fortunate chance discoveries. In prehistory a number of such serendipitous events occurred, all of which were to have enormous consequences for humankind. We can only speculate as to how these discoveries were made, and they probably took place on several separate occasions. Amongst them was the observation that clay could be permanently hardened by the application of a high temperature. This discovery was probably made by a sharp-eyed individual while examining the remains of the fire around which the family had sat a few hours previously, and from this observation there ultimately arose the art of making pottery. The first tiny beads of a smelted metal may likewise have been observed in an old fire ring where hot charcoal in the fire had come into contact with a stone that contained an appropriate ore, and as a result humans eventually progressed from the Neolithic Age to the Bronze Age. The alcoholic beverages industry has over the years employed the products of both the potter and the metalworker, but a serendipitous discovery of more immediate concern to us is that of fermentation, by which sugar is converted to alcohol by microbial action. As in the cases of pottery manufacture and metal extraction, we can speculate as to how this discovery might have taken place, but in this instance there is a charming legend that describes the event.

According to the legend, the King of Persepolis is supposed to have tired of one of the ladies of his harem. Finding herself banished, the young woman decided to end her life, so she drank some liquid from a jar labeled 'poison' in the palace storeroom. The jar had originally contained grapes, the king's favorite fruit, and was thus labeled to prevent others from eating his special delicacy. The grapes in the jar had broken down and the yeast on the grape skins had caused the juice to ferment, with the result that the woman found the drink to be most pleasurable. She took a cup to the king who was likewise delighted, and the young lady was duly restored to her position in the harem (Pellechia, 2006).

The one possible glimmer of truth in this legend is that it places the accidental discovery of vinous fermentation in a location in present-day Iran. Archaeological evidence for the very early production of wine has come from excavations carried out at a site in Iran in the Zagros mountains, north west of Persepolis. Jars have been unearthed which have been dated at 5400–5000 BC and which have been found to contain residues that almost certainly indicate that they were used for storing wine. Using a combination of analytical

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
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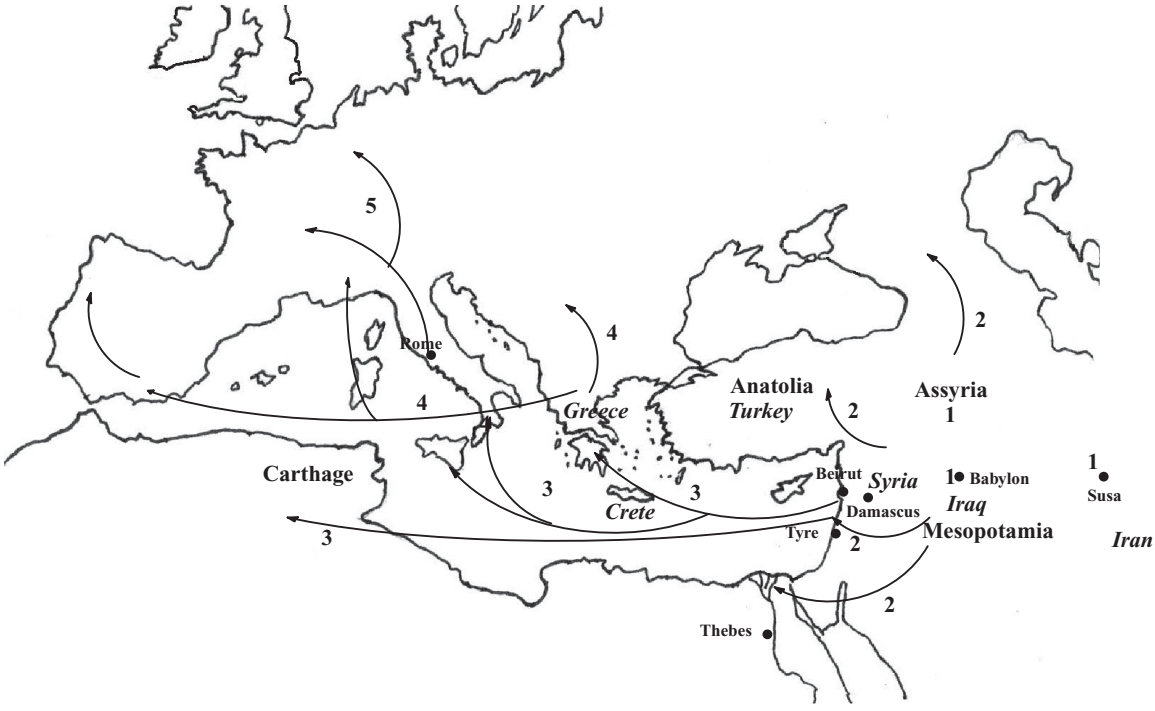
methods – wet chemical analysis, infrared spectroscopy and high performance liquid chromatography – the jars were found to contain traces of tartaric acid and calcium tartrate. The only likely source of these compounds is wine, and furthermore the jars also contained traces of terebinth tree resin (Winepros, 2007). It is known that early winemakers used terebinth and other resins to help preserve wine, which would also have added a characteristic taste. The tradition of adding resin to wine survives today in the Greek *retsina* (Section 2.12.3), although this is now done solely for flavoring purposes. It should be noted that there are other contenders for the location of the first production of wine, for the wild grape grows on the slopes of mountains surrounding the Mesopotamian region, in areas in modern Syria, south eastern Turkey, Georgia and Armenia, as well as northern Iran. These mountainous areas surround the so called 'Fertile Crescent,' which is the region where it is thought that humans first made the transition from a hunter-gatherer existence to a more settled way of life in which crops were grown and animals domesticated.

The production of wine implies the deliberate cultivation of grapes, and an important early development must have been the selection of a suitable variety of vine. Plants of the wild grapevine, *Vitis vinifera sylvestris*, carry either male or female flowers, and pollination of the female flowers would therefore be a somewhat chancy business. It is surmised that one or more of the early grape growers noticed a vine that produced grapes much more consistently than was usual. This would have been a mutant that produced both male and female flowers, and was thus self-pollinating. By cultivating vines from the seeds of this hermaphroditic variety, now known as *Vitis vinifera sativum*, the early viticulturists were able to establish vineyards that gave reasonable crops.

Knowledge of wine and winemaking was spread principally by trade and sometimes by conquest. Grape cultivation was introduced into the Nile delta in Egypt in the third millennium BC, and winemaking scenes are depicted in scenes on tomb walls of the Old Kingdom period (2650–2152 BC). Dark deposits from the bottom of jars of the New Kingdom Period (1550–1070 BC) have been shown by LC/MS/MS (Sections 4.3.3 and 4.4.5) to be derived specifically from red wine (Guasch-Jané *et al.*, 2004). Traces of tartaric acid were discovered in these deposits and alkaline fusion of deposit samples produced syringic acid, a well-known product of hydrolysis of polymeric tannins derived from malvidin-3-glucoside, the major pigment of black grape skins. Although little wine was produced in Babylon, we know there must have been a flourishing import trade (probably by boat or raft down the Tigris or Euphrates), for a set of laws called the Code of Hammurabi was drawn up around 1800 BC which specified severe punishments for dishonest wine sellers. Somewhat later, between 1200 and 800 BC, the Phoenicians, a seafaring race living on the coast of what is now Lebanon and Israel, were instrumental in spreading knowledge of grape cultivation and wine production further afield. Their influence spread along the north African coast, where they founded the city of Carthage, and reached as far as the Iberian peninsular. The route by which wine became known to the ancient Greeks is unclear, but it was certainly familiar to the preceding Minoan and Mycenaean civilisations. Subsequently the Romans spread knowledge of wine even further afield into parts of Europe north of the Alps. Some ideas relating to the spread of viticulture and winemaking are illustrated in Figure 1.2.1, where dates become more uncertain the further back in time one goes.

An alcoholic beverage that almost certainly predates wine is beer, although in this case there is no attractive legend to account for its discovery. The production of beer from grain is a multistage process, and serendipity probably played a part on more than one occasion in the development of brewing. The modern procedure involves allowing grain to germinate (sprout), stopping the germination by roasting, at which stage the material is called *malt*, and then grinding the malt to produce *grist*. The grist is then mashed with hot water, the resulting extract is filtered and is then boiled with sugar and hops. The hops are strained off, the liquid (called 'wort') is cooled and run into a fermenting vessel, when the yeast is pitched in. After fermentation the beer is clarified and distributed in casks (draught beer) or in bottles or cans. During the germination process the complex polysaccharides in the seed are partially broken down into simple sugars, and these are then turned into alcohol in the fermentation stage. The first step in the process may have been stumbled upon

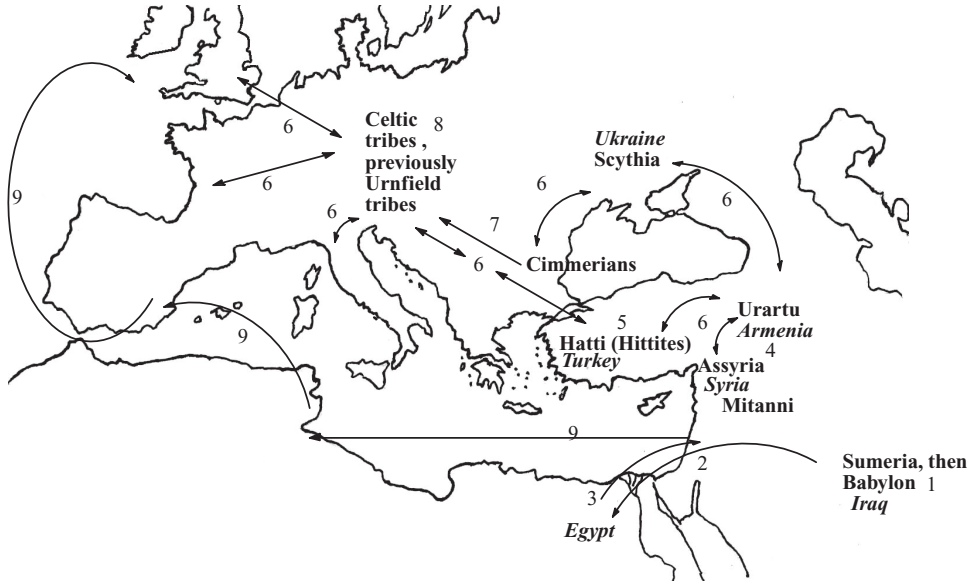


1. Records of viticulture and winemaking in the various empires of western Asia (now consisting of Caucasian states, Iran and Iraq) from ~6000 BC
2. Export of vines, viticultural and winemaking techniques to Phoenicia and Egypt and maybe other countries from ~3000 BC
3. Introduction of winemaking into Greece, Italy, North Africa, and Sicily by Phoenicians and their descendants ~2000-1000 BC
4. Greeks took their ideas of viticulture to Andalusia, Italy and Provence ~1000–500 BC
5. Roman invaders took vines and winemaking technology to northern Europe, as far as Britain from ~500 BC until the fall of the Roman Empire

Figure 1.2.1 *Early movements of viticulture and winemaking. Dates are approximate only and modern names of countries are given in Italics*

during the hunter-gatherer stage of human existence. It is possible that wild cereal seeds were collected, and once they had sprouted they were carefully heated. This primitive malt would have kept better than untreated seed, and would also have had a sweeter taste. Another accidental discovery might have occurred when malt was left in contact with water, thus allowing the production of a crude beer when fermentation of the unfiltered mash occurred as a result of infection by airborne microorganisms. All this is of course pure speculation, for as with the origin of winemaking our detailed knowledge of the discovery of brewing is nonexistent.

As with winemaking, brewing could not become properly established until an agricultural way of life had been adopted. Just as the early farmers selected a vine for cultivation that was more productive than the wild variety, so in the case of cereals, higher yielding varieties were chosen. In this case the selection was probably entirely accidental. The seeds of wild cereals are easily removed from the stem and dispersed by the wind, but in some plants there is a genetic trait in which the seeds are more firmly attached to the plant. When humans began to collect and sow seeds, more of the firmly attached seeds would have been available for collection, and gradually this gene would have predominated in the cultivated cereals. We do not even



1. Beer brewed from baked bread in Sumerian and Babylonian empires: *ca.* 4000–2000 BC
2. Beer exported to Egypt, where later it was brewed in a similar manner
3. Beer and expertise exported to Palestine and other nearby countries: 3000 BC onwards
4. Barley and wheat grown–beer brewed: *ca.* 1000 BC
5. Emmer, einkorn (forerunners of wheat) and barley grown, beer brewed: 3000 BC onwards
6. Interactions between peoples sometimes leading to possible spread of beer culture
7. Migration to central Europe, with beer culture: *ca.* 500 BC
8. There is evidence that Celtic tribes (*ca.* 500 BC onwards) were seasoned brewers, brewing from grains, not bread
9. The Phoenicians were great sailors and traders; it is possible they spread beer culture: 1000 BC onwards

Figure 1.2.2 *Origins of beer. Major early civilizations of which there are records of brewing are indicated by names and numerals. Arrows denote migrations, travels or exports. Double arrows denote interactions, including displacements and conquests, relating to the latest of the approximate dates indicated. Modern names of countries are in italics*

know which cereal (barley, wheat or oats) was used to make the first beers, or whether the first cultivation of cereals was for bread making or for brewing. Even the location of the first beer production is uncertain, and while somewhere in the Fertile Crescent is most likely (Figure 1.2.2), beer was certainly produced at an early date in Egypt. Whichever came first, brewing and baking technology developed side by side. It was known that the froth from the fermenting vessel could be used to make dough rise, and that pieces of lightly baked bread could be used to start fermentation. Neither practice necessarily implies that one technology was the forerunner of the other.

The original beers would have been very different from today's beverages. They were probably just fermented mash. They would not just have been cloudy, they might have been something more like porridge and could have been more of a food than a drink. They would have been more nutritious than the parent cereal on account of the partially broken down carbohydrates and proteins that they contained, and they would have had a pleasing inebriating effect. These original beers were probably not unlike the present day indigenous cereal beverages of Africa – boozah (Egypt), pombe and others (Section 2.7.2). Sometimes when early beer was consumed as a drink it was sucked into the mouth using a straw, and Egyptian pictograms show it being

consumed in this way. Irrespective of whether bread or beer was produced first, humans gradually perfected the art of making leavened bread and the technique of producing beer that was less like a glutinous mass and more like the drink we know today.

Barley is the most widely used cereal in beer production today, but it is very likely that in the early days oats, rye and wheat were employed as well. Indeed, maize, millet and sorghum are still used in fermented African and South American beverages (Section 2.7.2), and rice is the base of many Asian alcoholic drinks (Section 2.7.1). In Europe, brewing technology would have gradually improved, especially in respect of drawing off the fermented material from the mash tun to yield a liquid, which although still cloudy, was very different from the likely consistency of the earliest beers. This process of clarification would have been aided by the introduction into agriculture of hulled cereals. The hulls of the cereal grains would have provided an effective filtration medium through which the beer passed when being drawn off the bottom of the vessel. Both bread making and beer production would have advanced westwards from their birthplace in the near East as humans adopted a settled agricultural way of life. Some archaeological evidence suggests that a kind of beer may have arrived spread westwards much earlier than the dates indicated in Figure 1.2.2, which are based on documentary evidence. In northern Europe the analysis of pollen grains and fragments of leaves, etc., found in drinking cups from the Bronze Age would seem to indicate that alcoholic beverages were prepared at that time using malted grain and a variety of additional sources of sugar such as honey and fruit juices (Hornsey, 2003a).

From the period when the Celts had conquered most of northern Europe (i.e. from about 500 BC), we have documentary evidence concerning the drinks that were being consumed, for although the Celts left no written record, some Roman authors gave accounts of Celtic habits and practices. The general impression created by these authors is that wine was only drunk by the rulers and upper classes, being imported from the Mediterranean region, whereas the ordinary folk drank beer (Figure 1.2.2). It was possible to transport and trade good wine, but it was usually necessary to consume beer as soon as it was brewed at a place very close to the location of its production.

When the Romans started to establish vineyards in their newly conquered territories, they were so successful that wine production in Italy itself was being threatened. As a result the emperor Domitian issued a decree late in the first century AD that limited the production of wine in the provinces and forced the grubbing up of many vineyards. The decree was not revoked until late in the third century after which new vineyards were developed in Gaul, and to some extent in Britain. The remains of a 35-hectare Romano-British vineyard have been found near the village of Wollaston in Northamptonshire. After the collapse of the Roman Empire, viticulture had to wait another 1000 years before further expansion into new areas of the globe.

So far the focus has been on the Middle East and on Europe, and we have considered beverages formed by fermenting a solution of sugars obtained either directly from grapes or indirectly from cereals such as barley, wheat or oats. However, a large number of traditional alcoholic beverages have been produced from other sources by indigenous peoples in various parts of the world, and many of these are still made today. In general they are either akin to wine, in that a solution of sugar is obtained from a plant, which is then fermented, or they are like beer, in that complex polysaccharides in a plant are first broken down to simpler sugars, and then fermentation is allowed to occur. Three examples will be quoted.

Rice wines, the common nondistilled alcoholic beverage of eastern Asia, probably originating in China. They include the Korean *makkoli* and the Japanese beverage *sake* and have been known since antiquity (Section 2.7.1). References to sake are made several times in the Kojiki, Japan's first written history, compiled in 712 AD. It is usually described as rice wine, but in reality it is more akin to beer. Traditionally, the process was started by chewing the rice and then spitting it into a pot, the enzymes in saliva causing the complex carbohydrates to start breaking down into sugar. Inscriptions from the fourteenth century BC mention Chinese millet wine, xiao mi jiu, being made the same way. Today the rice is treated with a mould/yeast starter (called

nuruk in Korea, *koji* in Japan and *men* in Vietnam) that contains appropriate enzymes to catalyze carbohydrate breakdown and to perform fermentation (Wikipedia, 2007a). The indigenous peoples of South America likewise consume a drink called *cauim*, made from starchy roots or from maize, with the breakdown of the polysaccharides also being initiated by an enzyme in saliva (Wikipedia, 2007b). In Mexico the traditional beverage is *pulque*, which is a kind of wine made by fermenting the sweet liquid obtained from the flower stems of the maguey plant (Wikipedia, 2007c) (Section 2.7.2). Such beverages are part of the culture of the regions in which they are made, but it is likely that primitive peoples used many other sources of sugar to make alcoholic beverages. Quite passable drinks can be made by allowing the juice of many wild fruits to ferment, and all that is needed to make a wine from a fruit such as the blackberry is a suitable container. This is where the craft of the potter would have been first used in alcoholic beverage production, but even animal skins might have served the purpose, and drinks made from wild fruits may have preceded settled agriculture. Nowadays, many palatable drinks are made by the fermentation of juice from wild or cultivated fruits (Chapter 2.11). It is probable that a fermented drink was made from the juice of the wild apple, but in the United Kingdom, which today has the world's highest per capita cider consumption, large-scale production did not commence until the Middle Ages. A nonplant source of sugar that was used in many cultures from 1000 BC or earlier is honey, which was (and still is) fermented to make mead (Section 2.11.5). It was also added to wine as a sweetener. There is a considerable body of evidence to show that honey has been used in these ways for many centuries. For example, analysis of the pollen grains in the deposit found in a bronze bowl from a rich celtic woman's grave (late Hallstadt period) in lower Bavaria revealed a wide pollen diversity, suggesting that the bowl contained mead that was produced from the honey of wild bees (Rösch, 2005). In contrast, the pollen found in the deposits in early medieval Coptic amphorae from Šaruna (Middle Egypt) was of narrow diversity, suggesting the honey was derived from domesticated bees. Whether the amphorae originally contained mead or honey-sweetened wine is not known (Rösch, 2005).

Returning to the drinks with which we are principally concerned, namely wine and beer, it was the monasteries that kept alive much of the knowledge of methods of beer and wine production in northern Europe in the centuries following the fall of the Roman Empire. The monasteries were self-contained entities, running their own farms, and as far as vineyards were concerned, the monks and nuns had the discipline to take the necessary care to maintain them in good order. Furthermore, the Christian religion demanded a supply of wine for use in Holy Communion.

The first possible evidence of distillation per se comes from the discovery of still-like clay pots, dating from the second millennium BC, in what was once Babylonia, although it is not known for certain what was distilled. Although the first drawings of stills date from the Hellenistic school of alchemists (second century AD), from written and archaelogical evidence it was probably the Arab alchemists who perfected the art of distillation to an extent necessary to obtain fairly concentrated alcohol from a fermented beverage. This was despite the teaching of Islam, which from its inception in the seventh century AD forbade the consumption of alcoholic beverages. The Arabs became the world's leading scientists, and were the first to use distilled alcohol in medicine. The earliest piece of distillation apparatus (from Iran) that has survived is Islamic and is dated between the tenth and twelfth centuries. Water cooling was not used, but it is likely that careful distillation would have produced a solution of alcohol sufficiently concentrated to burn (Hudson, 1992).

The Arab scientists originally referred to ethanol as distilled wine (خمر مصعّ), but later the word arak (عرق), meaning sweat (referring to the beads of distillate on the cooler parts of the still) came into general usage (al-Hassan, 2009) and is still used for certain distilled drinks today (see Sections 3.5.5 and 3.5.6). Early names for this spirit in European literature were *aqua ardens* (the water that burns) or *aqua vitae* (the water of life). The word *alcohol* itself was not used until the sixteenth century, and has an extraordinary derivation. It comes from the Arabic *al koh'l*, the name given to antimony sulfide, a material used by ladies to darken eyebrows and eyelashes. It was purified by sublimation, a process bearing obvious similarities to distillation, and in

time the name *alcohol* became associated with the process rather than the material. Eventually, anything purified by sublimation was known as *alcohol of*, and the spirit obtained by distillation of fermented grape juice was known as *alcohol of wine*. In time this name was shortened to *alcohol* (Flood, 1963). Now the name applies not only to ethanol (ethyl alcohol), but also to a wide range of chemical compounds bearing the OH functional group. It was not until the Middle Ages that distilled alcoholic beverages were manufactured and consumed (Section 1.2.2).

Microorganisms cannot survive in concentrated alcohol, which can be used as a preservative. However, undistilled beverages are prone to spoilage, and its prevention has always been a matter of prime concern for brewers and wine makers. We have seen how resins were used as preservatives in the earliest wines. Other strategies that were adopted at a later period included the floating of a layer of olive oil on top of the wine, and the tight stoppering of amphorae, both measures being designed to exclude the air. Sulfur dioxide, obtained by burning elemental sulfur, was used as a fumigant in ancient times, and the Romans were the first to use it as a preservative for wine (Robinson, 1995) (Section 2.5.2). The most important substance added to beer for preservation is hops, although this strategy does not seem to have been employed until about the ninth century, when a reference occurs to its use in Germany. At this time, most beers would have been either unflavored or flavored with various herbs and plants, such as juniper, sweet gale and yarrow. Although many of them would have been very strong (~8% ABV), most would be consumed while they still possessed a distinct prickle of carbon dioxide and probably within 24 h of pouring the beer from the yeast lees.

By 1100 AD Europe was beginning to emerge from its so-called 'Dark Ages.' This period is certainly dark in the sense that there are few extant records concerning alcoholic beverage production or indeed almost any aspect of human activity. However, from the Middle Ages onwards we are able to form a clearer picture of our brewers, winemakers and distillers.

1.2.2 Medieval Times and Beyond (*ca*. 1100–1750)

It is in this period that we get the first indication of the quantities of beverages consumed. In northern Europe the principal drink was beer, and at first glance the amounts brewed seem very large. Documents detailing the consumption of brewing materials during a 40-day siege of Dover Castle in 1216 indicate an allowance of half a gallon of beer per man per day (Hornsey, 2003b). Overall consumption in England and the Low Countries in the late Middle Ages is estimated at a more modest (but still considerable) 65 gallons per head per year (Wikipedia, 2007d). Beer also provided a significant proportion of the calorie intake of the population of northern Europe. In southern Europe, wine, and no doubt other fruit juices, both fermented and unfermented, remained the principal beverage.

The ecclesiastical establishments of monasteries continued to be extremely important during the early Middle Ages. They often owned large tracts of land, and controlled many aspects of local economic affairs. Among the activities of northern European monasteries was the brewing of beer, and they did good business supplying the local population. Alongside the monastic breweries, beer was brewed in smaller quantities in individual dwellings, often by the lady of the house. In some cases the product may have been solely for family consumption, but often it was sold or traded as well. In England the dissolution of the monasteries by Henry VIII put an end to their brewing activities, but by this time, many of the home breweries had been replaced by establishments that not only brewed the beer, but also provided accommodation for its consumption. It became a requirement for these establishments to be licensed, and a distinction was made between ale houses, taverns (which sold both ale and wine) and inns, which also provided food and beds. In 1577 there were 19 759 licensed premises in England, which equates to one for every 187 members of the population (Hornsey, 2003c).

With brewing being something of a cottage industry, it is hardly surprising that standards varied enormously and that in consequence attempts were made to introduce some kind of quality control. In England, the official charged with this duty was called the ale conner or ale taster. It was his job to assess the quality of ale before it went on sale, and the vendor was expected to send for the official when the brew was ready. In some areas the brewer erected an ale stake (a long pole with branches tied to the end) on the outside of the house to indicate that the ale was ready to be sampled. No doubt the ale conner relied primarily on his sense of taste, but according to legend, the conner used to pour some of the ale on to a wooden settle, and then he used to sit in the pool of ale (he wore leather breeches for obvious reasons). If at the end of about half an hour he could stand up without his breeches sticking to the settle, the ale was judged to be of a satisfactory quality. In modern terms, the rationale behind this primitive method of analysis is that stickiness in the beer would be caused by a significant quantity of residual sugar, meaning that fermentation was incomplete and hence the alcohol content was low. The ale stake also served as an advertizement to the public of the impending availability of the brew. In time different establishments started to put their own distinguishing symbols on their ale stakes, and this is probably the origin of the pictorial signs found on pubs in Britain today.

The practice of adding hops in the brewing process became much more widely adopted in the Middle Ages. Hopped beers keep for a longer period, so the use of hops gave beers of relatively low alcohol content a reasonable lifetime. Originally the term *beer* meant that the brew had been hopped, the unhopped beverage being referred to as *ale*, but now the terms are used interchangeably. In the early history of brewing, a great many different substances were added to ale to enhance the flavor, and one author has identified 175 plants or plant-derived products that have been used as flavoring, preserving or adulterating agents (Hornsey, 2003d). Probably the most widely used additive before the general adoption of the hop was *gruit*, a Dutch term that referred to a mixture of herbs whose precise composition varied, but which usually contained bog myrtle, marsh rosemary and yarrow among the ingredients. Like these plants, the hop grows in the wild in northern Europe, and its preserving effect on beer was probably discovered by accident. Hopped beers were first exported from Bremen and Hamburg in the early Middle Ages, after which the practice of hopping beer gradually spread to other parts of northern Europe. It is likely that initially hops were imported into Britain, for there is no firm evidence for the deliberate cultivation of the crop before the fifteenth century.

The introduction of hops into brewing in Britain was accompanied by considerable controversy, and at various times edicts were issued that sought to prohibit their use. The use of hops meant that the brewing process became more complicated, for the filtered wort now had to be boiled with hops. The result was the slow emergence of larger breweries, called common brewers, which supplied several retail outlets

We have seen that in the early days of brewing, not only could a number of cereals be used for making beer, but many different herbs and spices could be added as preservatives or flavoring agents. In 1516, when Duke Wilhelm of Bavaria passed the *Reinheitsbebot* (purity law), he enacted a piece of quality control legislation that remains the oldest food regulation still in force anywhere in the world. The Reinheitsgebot decreed that only barley, water and hops were to be used by commercial brewers in making beer. Over the preceding millennia, the term beer had encompassed a considerable variety of beverages; now a consensus was emerging as to what the term actually meant.

Turning our attention to the East, the history of sake, a typical rice wine, bears some resemblance to that of beer. In tenth century Japan, temples and shrines began to brew sake, and were the main centers of production for next 500 years. The *Tamon-in Diary* (1478–1618), written by monks of the Tamon-in temple, records many details of brewing in the temple. The diary shows that a process akin to pasteurization and the addition of ingredients to the main fermentation mash in three stages were established practices by this time (Section 2.7.1). During the Maiji Restoration (*ca.* 1866), the Law allowed anyone with the money and skill to construct and operate his own sake brewery, so the brewing of sake shifted away from the temples. About 30 000 breweries were established around the country within a year. However, through the years, the

government levied higher and higher taxes on the sake industry and within a few years the number of breweries had fallen to 8000. Most of the breweries that survived this period were set up by wealthy landowners, who grew rice crops and had rice left over at harvest time. Rather than letting the excess rice go to waste, they set up breweries and distilleries, thereby creating valuable extra income from sales of rice wines and spirits (*shochu*).

In southern Europe, wine remained the principal drink, being consumed by all social classes. This is not to say that beer was unknown. Brewing was undoubtedly carried out on a domestic basis, but beer did not become an article of commerce as it did in northern Europe. Methods of wine production south of the Alps remained essentially the same as in Roman times. However, the expanding population increased demand, and although beer was the common drink in northern Europe, the upper classes had a distinct taste for wine. Increasing demand, and the difficulties of transport, resulted in ever higher prices in northern Europe for wine imported from southern regions, with the result that local wine production was stimulated. Varieties of grape that grew well in northern areas were cultivated, and regional centers of excellence developed, examples being Bordeaux, Burgundy and the Rhine. The Church played an important role in this expansion, and monastic orders such as the Benedictines helped to expand the northern European wine industry.

The Greeks and Romans had transported their wine in pottery amphorae. In more northern areas, with their abundant oak forests, containers for storage and transportation were made from wood. The construction of barrels, casks and vats that were completely impervious to liquids became a highly skilled task, and the cooper became a vital craftsman in wine producing areas. Another northern European invention was the wooden press, in which the juice is gently squeezed from the crushed grapes through the slatted sides. This is in contrast to earlier techniques in which the grapes were crushed by a stone, or by human feet. Some of the bigger European wineries or estates possessed enormous wooden presses to cope with big harvests. Two very fine examples from the eighteenth century can be seen at the Stellenbosch Wine Museum, in South Africa (Figure 1.2.3). Casks are still used to age some of the finest wines, but as a result of the development of heavier and stronger glass, the bottle gradually became the standard container for the storage and transport of wine from about the seventeenth century, although many wines, including red Bordeaux and Port, were often shipped in cask to be bottled in a foreign country. Nowadays, most fine wines are bottled from casks at the domaine or in the region of production, and the bottles are then transported to the point of sale. Although some of the early bottles were sealed with wax, the use of cork stoppers soon became widespread. Cork provides an excellent seal, but over a prolonged period a cork can dry out, thus allowing air to enter the bottle.

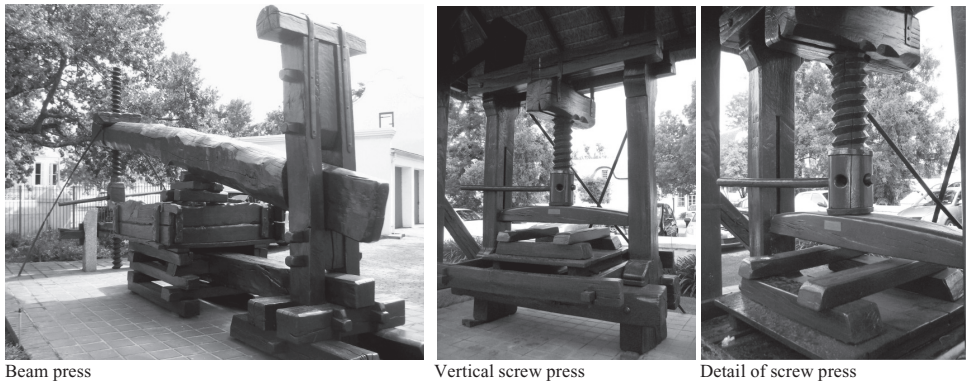


Figure 1.2.3 *Eighteenth century wine presses at Stellenbosch Wine Museum.* Photographs taken by Marianne Mackay

Figure 1.2.4 *Early wine bottles and an early corkscrew. These port wine bottles from (left to right) early, mid and late eighteenth century, early nineteenth and twentieth century demonstrate the changing shape of the wine bottle. Bottles and corkscrew are not drawn to scale*

The first wine bottles were bulbous in shape with a flat base, and could only be stored in an upright position. It was not until the latter half of the eighteenth century that cylindrical bottles with a narrow neck were made (Figure 1.2.4). These could be stored horizontally, and hence the cork did not dry out. Although some of the first corks may have been flanged or tapered, for upright storage of the bottles, later cylindrical bottles were fitted with straight corks for storing horizontally. This development gave rise to the need for a tool to extract the cork – the corkscrew. It is uncertain when the first corkscrews were used, but the first mention of the 'bottle scrue' was in 1720 (Young, 1979) and probably the earliest patent was taken out in 1795. A sketch of an early corkscrew (date unknown) is shown in Figure 1.2.4.

A further improvement in glass technology enabled the process of making champagne to be perfected. In the cool champagne region, fermentation proceeds slowly, so when the wine is bottled in the autumn some sugar remains. Fermentation recommences in the bottle in the following spring when temperatures begin to rise. The early glass wine bottles were unable to withstand the pressure of carbon dioxide, and most of the wine was lost. The seventeenth century Benedictine monk Dom Pérignon experimented with stronger bottles made from a newly invented type of glass that incorporated lead oxide, with the result that the production of the famous sparkling wine became a reliable process. Nevertheless the wine was still cloudy owing to the presence of dead yeast cells. The traditional clarification process is supposed to have been invented by a widow, Madame Cliquot. The dead yeast cells are concentrated in the neck of the inverted bottle, that portion of the wine is frozen, the bottle is opened to expel the frozen portion, and then finally recorked.

In the eighteenth century, wines fortified by the addition of brandy increased in popularity, especially in Britain. In the Douro region of Portugal, fortification was originally employed at a monastery to arrest the fermentation process, but a local aristocrat, the Marquês de Pombal, who had been Portuguese ambassador to London, realized there would be a market in Britain for the sweet, high-alcohol red wine (port), and promoted its export (Section 2.10.7). In the Jerez region of Spain, fortification was practiced for a different reason. A second type of yeast, known as *flor*, occurs in the region and forms a bread-like film on the surface of the wine maturing in the casks. It prevents the oxidation of the wine, and also imparts a characteristic flavor, but needs an alcohol content of around 15.5% to grow, hence the need for fortification. The final product, known as *Sherry* from an English corruption of the word Jerez, is a blend of the products of fermentations from several different years (Section 2.10.2). Yet another fortified wine to achieve popularity in Britain and its American colonies at this time was Madeira (Section 2.10.5). The Portuguese colony had been exporting sweet Malmsey wine to Britain for some time when the practice of fortification was introduced, partly to

improve the wine's keeping properties, and partly to satisfy the British palate. It was then found that if the fortified wine was kept in a warm environment (around 40 °C; 104 °F) for some months its quality improved and it became fully stabilized – it could keep almost indefinitely. Legend has it that the effect of warming was discovered accidentally when consignments were confined in the warm holds of ships bound for North America or the East Indies. At one time more Madeira was imported into the American colonies than any other wine.

A beverage that grew in popularity in England during the Middle Ages was cider. Although the Romans established some apple orchards, there is no evidence of cider production before the Norman conquest. The Normans brought with them the tradition of apple growing and cider making, and introduced varieties of apple such as the Permain and Costard, which were particularly suited to cider making. It was once again the monasteries that were the prime manufacturers and retailers of cider, although cider making was also practised in manor houses and on farms.

The first spirits (i.e. distilled alcoholic beverages) were almost certainly originally imbibed for medicinal rather than social reasons. The Italian city of Salerno was the location of the first medical school to be established in western Europe, and it was here that many Greek, Latin and Arabic medical texts were translated. A compendium of medicines from the Salerno school in the early Middle Ages refers to *aqua ardens* (burning water). Certainly spirits produced at this date would have been somewhat 'rough' by modern standards, and their restriction to medical applications is understandable.

Over the years distillation techniques improved, a particularly important innovation being the introduction of water-cooled condensers, but in general the principal practitioners of distillation remained doctors and apothecaries. Then in sixteenth century France the large-scale distillation of wine commenced, although it was the Dutch who provided the chief stimulus to the expansion of production. Not only was there a demand for the distilled beverage in the Netherlands, but the Dutch, being a great seafaring nation, needed large quantities of the spirit to service their ships, both because it helped to stop the drinking water going brackish and because it was a more efficient way of transporting the alcohol required for a long voyage. It was the Dutch who set up distilleries in the wine producing regions of France, calling the product *brandewijn* (burnt wine), from which the English word *brandy* derives.

At about the same time, a number of other spirits began to be made in quantity. Any fermented solution can be concentrated by distillation, and in consequence a great many kinds of spirit are made. The taste depends upon those compounds, apart from ethanol and water, which come over in the distillation process, and any flavorings that are added during the process of manufacture. A spirit that was first produced on a large scale in the late sixteenth century is vodka (Section 3.4.3), which today is almost solely ethanol and water, although various flavorings have been used in the past. Many materials can be fermented to produce the alcohol prior to distillation, but modern European legislation states that if vodka is prepared from anything other than cereals or potatoes, this must be stated on the label (Rogers, 2007). Vodka now has a world-wide popularity, but the traditional vodka belt stretches from the Nordic countries, through Poland and the Ukraine, to the far east of Russia. Another spirit originating in the sixteenth century is Akvavit (Section 3.4.3). A drink of the Scandinavian countries, it is based on alcohol distilled from potato or grain, and flavored with a variety of herbs, most commonly caraway. The name *Akvavit* is a Nordic corruption of the Latin *aqua vitae*. Gin, which was first produced in the Netherlands in around 1650 for medical purposes, is made mostly by redistillation of grain spirit, to which a mixture of spices ('botanicals') has been added. Notable amongst the botanicals in gin is the juniper berry or its essence (Section 3.4.2). In France the new spirit was called *eau de genièvre* (juniper water), which the English abbreviated to *geneva* and then to gin.

The whiskies of Scotland and Ireland are highly complex, both in their constitution and production. According to legend, whiskies have been made in these countries since Celtic times, but as with other spirits, larger-scale production commenced towards the end of the Middle Ages. The fermented malted barley is

subjected to two distillations in copper stills of traditional design, which result in the spirit containing a complex variety of compounds that contribute to the flavor. One major difference between Scotch whisky (Chapter 3.2) and Irish whiskey (Section 3.3.1) is that in the former the malted barley is dried over smouldering peat, whereas in the latter case sealed ovens are used. The distilled spirit is matured for many years in oak casks (Whisky.com, 2007). The name *whisky* or *whiskey* is an English corruption of the Gaelic *uisgebeatha*, which means water of life.

Rum (Section 3.5.2) is made by the distillation of fermented by-products of the cane sugar industry, e.g. molasses or sugar cane juice, before being matured in oak casks. Production was already established in Jamaica when the island was captured by the British from the Spanish in 1655. The British, like the Dutch, had been in the habit of issuing brandy to their sailors, but their new conquest provided them with a more reliable source of spirit, and the long association between rum and the Royal Navy commenced. Every sailor received a daily tot of rum until the practice was abolished in 1970. No doubt in part because of its naval associations, much rum was imported into Britain, via ports such as Whitehaven in Cumbria. Soon after the production of rum commenced in the Caribbean, demand in the American colonies led to the establishment of rum distilleries there, the first being in Boston in 1664. Another spirit manufactured in the American colonies was applejack from cider. The traditional method of production was not by distillation, but by freezing, when ice crystals formed leaving a more concentrated alcoholic solution behind – a process known as 'jacking.' Distillation was also used both in America and in the Calvados region of Normandy, where the famous apple brandy is still produced (Section 3.8.2).

A liqueur (Chapter 3.9) is a distilled alcoholic beverage with added flavoring and a relatively high sugar content. It was early in the sixteenth century that the first liqueur was prepared by a monk at the Benedictine Abbey of Fécamp in northern France. The monks had previously made a medicine by infusing brandy with various herbs, but it had an unpleasantly bitter taste. By adjusting the recipe the monks made a liqueur that was highly palatable (Classicliquors.com, 2007). Today Benedictine is still produced to the original recipe, although no longer by monks. Four different combinations of herbs and spices are prepared (27 ingredients being used altogether) and each mixture is soaked in brandy. Each brandy is then distilled, aged in oak casks and then the four are blended together and aged again. Another famous brandy-based liqueur, first prepared by Carthusian monks in the seventeenth century and still made by them today, is Chartreuse. Supposedly over 100 herbs are used in its preparation. A third liqueur that also has an ancient pedigree is Drambuie, which is based on whisky rather than brandy. Like many liqueurs the mixture of flavorings used is complex, and includes heather honey and a variety of herbs. The recipe is supposed to have been given by Bonnie Prince Charlie to Captain John Mackinnon in gratitude for the latter's assistance in helping the Prince evade capture by the pursuing English forces after the battle of Culloden in 1746. Drambuie was first produced on the Isle of Skye, but it is now made near Edinburgh by a company owned by one of John Mackinnon's descendants (Rampantscotland.com, 2007).

In eastern Asia, soju (Section 3.4.4) was possibly the first distilled beverage, distilled for the first time probably around 1300 AD, during the Mongol war with Korea. The Mongols had learned the technique of distilling *arak* from the Persians during their invasion of central and western Asia around 1256. The technique was then taken up by the Chinese and Koreans (and other Asian nations), whence the latter set up distilleries originally around the city of Kaesong. Indeed, in the area surrounding Kaesong, soju is still sometimes known as *arak-ju*. It is most likely that regular distilling of cereal mashes began in China and Korea at about the same time, although it is possible that distilled drinks were produced before that time in certain areas of China that were more closely linked with the trade routes to western Asia and the Arab countries.

In what is now Japan, awamori (Section 3.4.4) was probably the first distilled spirit, made from rice wine in the Ryukyu Islands in the fifteenth century, possibly from techniques acquired from Thailand, where in turn, the methods were probably picked up by traders or travellers from further west.

1.2.3 The Industrial Revolution and the Influences of Science and Technology (*ca*. 1750–1900)

Britain was the first nation to industrialize, and the so-called industrial revolution had a dramatic impact on the production of all manner of articles of commerce. The technological advance that was to transform the brewing industry was James Watt's improvement in the steam engine. This was patented in 1769, and the new engines were manufactured by the firm of Boulton and Watt from 1776. Watt's design was much more fuel efficient than the earlier Newcomen engine, which only found application in pumping coal mines where the fuel was readily available at a low price. The first steam engine to be installed by a brewery started work in London 1784 and by 1801 there were 14 in the capital. Breweries in the larger cities outside London started to acquire steam engines from 1793. Steam engines were used to grind the malt and for various pumping operations around the works. Even before the advent of the new engine, a relatively small number of breweries had grown into large concerns at the expense of their smaller competitors; now it was only the larger organizations that could afford the new machines and this further accelerated the growth of the large breweries at the expense of the small fry.

Brewing benefited from many other technological innovations during the period 1750–1900, but two in particular stand out as deserving a mention. The introduction of an effective means of cooling by refrigeration, which commenced in the late 1860s, meant that beer could now be produced all the year round. The boiled wort had to be cooled before fermentation could commence, and traditionally this had been done by simply allowing the liquid to cool in open vessels, with the consequence that in the warmer summer months there was a greater danger that spoilage would occur at this stage. Interestingly, one of the important contributors to the science of thermodynamics, which provides the theoretical basis for refrigeration, was James Prescott Joule (1818–1889), who was born into a brewing family and worked in his private laboratory adjacent to the brewery in Manchester. The other new technology that had a huge impact on the brewing industry was that of the railways. By 1860 the basic railway network of most of Britain and much of Europe had been laid out, with the consequence that breweries could transport and sell their products many miles away. This further consolidated the position of the larger breweries. When the Midland Railway opened its new London terminus at St Pancras in 1868, the cast iron pillars supporting the roof of the undercroft were spaced so that barrels of beer from Burton on Trent could be rolled between them.

Whilst Joule's scientific work was not carried out with the intention of developing a technology that would find application in breweries, other laboratory workers performed experiments that had more obvious connections with the alcoholic beverage industries. The fermentation process was studied by the famous French chemist Antoine Laurent Lavoisier (1743–1794), who weighed the sugar consumed and the alcohol and carbon dioxide produced, and found them to be equal. This was the first experimental demonstration of the law of conservation of matter, or as Lavoisier stated in 1789 '. . .in all operations of art and nature, nothing is created; an equal quantity of matter exists both before and after the experiment. . .' There was, however, uncertainty for many years over the precise nature of the fermentation process. By the late 1830s it had been established that fermentation occurred in the presence of living yeast cells, which multiplied as the fermentation progressed, and it was also known that if the yeast was killed by heating, then fermentation ceased. However the idea that a living organism played an essential role in a chemical process was contrary to contemporary chemical theory, and the idea was ridiculed in an anonymous article that appeared in 1839 in a journal edited by the famous German chemist Justus von Liebig (1803–1873).

The matter was resolved by the Frenchman Louis Pasteur (1822–1895), who in 1857 demonstrated conclusively that fermentation occurred as a result of the metabolic activity of yeast. Pasteur's first researches in this area were conducted as a result of problems experienced by a manufacturer who was producing industrial alcohol by fermenting beet sugar. Lactic acid was being formed as well, and Pasteur demonstrated

that this was caused by another microorganism (actually a bacterium). Pasteur soon succeeded in separating the microorganisms responsible for several different kinds of fermentation – alcoholic, lactic, acetic, butyric, etc. He was then able to show that many of the problems encountered in the wine industry were caused by contamination with foreign organisms during the production process. This explanation led to methods prevention of wine spoilage, among them the process of rapid heating and cooling the liquid, i.e. the process known as pasteurisation.

Pasteur also investigated problems associated with the brewing of beer, and during his research he visited Whitbread's brewery in London. As in winemaking, Pasteur demonstrated that spoilage was caused by foreign microorganisms, either added with the yeast, or introduced from the air. As a result of his work, breweries soon began to use the microscope to examine their yeasts and their beers.

The labors of well-known figures such as Watt, Lavoisier and Pasteur were of enormous benefit to producers of alcoholic beverages, but eventually the industry began to realize the advantages of employing its own chemists to work in on-site laboratories. The first breweries recorded as appointing chemists were the London firm of Truman, Hanbury and Buxton in 1831, and the firm of Allsop's in Burton-on-Trent in 1845 (Russell *et al*., 1977). By 1876 there were enough brewery scientists employed in Burton for them to form an informal dining club called the Bacterium Club (Hornsey, 2003e). The brewery chemists analyzed the quality of the water supply, monitored various aspects of the brewing process by means of instruments such as the thermometer and hydrometer, and combated problems of infection and spoilage. Probably the best-known brewery laboratory in the world was that founded by Carlsberg in Copenhagen in 1876, although from the outset it was as much a research institute as a laboratory directed towards process control in the brewery (Wikipedia, 2008). Its first director was Johan Kjeldahl (1849–1900) who devised a method of measuring the quantity of protein in the grain used by the maltsters. The technique has been much used by biochemists and food scientists ever since. His successor was Søren Peder Lauritz Sørensen (1868–1939), who devized the pH scale for measuring acidity and alkalinity. At first, some companies preferred to use consultants rather than appoint chemists as full-time employees. An example is provided by the famous English chemist (Sir) Edward Frankland, who in the mid 1850s was acting as a consultant for Watney and Co. (Russell, 1996). Not surprisingly, some chemists encountered hostility when they introduced their new ideas into a long-established industry. One new appointee in the late nineteenth century was greeted with the comment 'Mr Chemist, you know everything. I only know one thing – I know you are wrong.' (Institute of Brewing, 1986).

During the eighteenth and nineteenth centuries the major beer styles acquired the characteristics and the names by which they are known today. Traditional English beers are produced by top fermentation, i.e. the yeast floats on top of the fermenting liquid. English bitter and pale ale, two well-hopped beer styles (Section 2.6.13) are produced by this method. Porter (Section 2.6.13), which was especially associated with London breweries, has fallen out of favor, but in recent years some breweries have revived it. Porter is usually of a medium dark color as a result of the extent to which the malt is toasted. The Guinness brewery in Dublin manufactured a darker product, which from 1820 was known as extra stout porter, subsequently abbreviated to stout. However, the most popular kind of beer in the world today is lager, produced by bottom fermentation. This type of beer was first brewed in cool caves in Europe about 500 years ago. Its production on a large scale only became feasible after the introduction of refrigeration in 1876, as the fermentation is conducted at a temperature of 5–10 °C, at which temperature the yeast sinks to the bottom. The beer is then allowed to age for 60–90 days in a store designated by the German word *lager*, and hence all bottom-fermented beers are known as lagers. There are varieties of yeast that are specially adapted to bottom fermentation, and another major achievement of the Carlsberg laboratory was the isolation of one of these, *Saccharomyces carlsbergensis*, by Emil Christian Hansen in 1883. Lager beers have subsequently become enormously popular in Europe, the USA and Australia. The town of Pilsen, in the Czech province of Bohemia, gave its name to a golden colored

bottom fermented beer variously known as Pilsener, Pilsner or Pils, and other varieties of lager are associated with České Budějovice (Budweis), Dortmund, Munich and Vienna.

The winemaker, like the brewer, is faced with the problem of avoiding unwanted processes occurring during production and storage, which will spoil the final product. It has already been noted that the Romans used sulfur dioxide as a sterilant. The technique was to burn a sulfur candle in the vessel before adding the wine. Sulfur dioxide acts both as an antimicrobial agent and as an antioxidant, and today it can be used at all stages of the wine making process, from crushing to bottling. Fortunately the yeasts that cause fermentation are less susceptible to sulfur dioxide than the spoilage organisms. Another practice dating from ancient times was the addition of lead compounds to wine. Lead in solution inhibits the growth of microbes, and if lead oxide is added to wine in which acetic fermentation has occurred, or in which the ethanol has been oxidized significantly, it will react with the acetic acid to form lead acetate. This has a sweet taste, so lead oxide not only arrested the spoilage of wine, but restored its taste. However, lead is poisonous to humans, and wine that contained lead was responsible for a range of illnesses amongst consumers, which on occasions were fatal. It was not until the eighteenth century that the dangers of adding lead compounds to wine were generally accepted and the practice was outlawed.

Apart from Lavoisier and Pasteur, a number of other French scientists performed important work of relevance to the alcoholic beverages industry, especially winemaking. In 1801 Jean-Antoine Chaptal (1756–1832) published a book on winemaking, which had a significant impact, advocating that some traditional practices should be discarded in favor of a more rational approach. Among his recommendations was that sugar could be added to grape juice if without it the resulting wine contained too much acid relative to the alcohol to be palatable. Although this idea had earlier been suggested by others, it was Chaptal's advocacy that resulted in its adoption, and the process is known today as *chaptalisation.*

In Europe in the nineteenth century, a series of disasters befell the wine industry. In the 1840s, powdery mildew, a fungus native to the USA, was first found on European vines. It had the effect of reducing yields and producing off flavors in the affected grapes. The problem gradually increased, especially in France, where the vintage of 1854 was disastrous. Sulfur is an effective fungicide against powdery mildew, but it was only after the crisis of 1854 that most French growers adopted the remedy. But much more serious was the phylloxera epidemic, which commenced in the 1860s. Phylloxera are tiny yellow sap-sucking insects that attack the roots of the vine, causing damage which, coupled with the secondary fungal infections introduced by the insects, is lethal to the plant. Phylloxera was endemic in North America, where the native grapes species were naturally resistant, but the European *vinis vinifera* was highly susceptible. The effect was devastating, especially in France, where total wine production fell from 84.5 million hl in 1875 to 23.4 million hl some 20 years later. Many remedies were tried, which ranged from the rational (injecting insecticide into the soil) to the superstitious (burying a dead toad under each vine), but the eventual solution was to graft European grape varieties on to North American rootstocks. Suspicions that the taste of the grapes might be affected proved unfounded. The two scientists most closely involved with developing this remedy were Charles Valentine Riley (1843–1895), an entomologist working at the US Department of Agriculture, and Jules Émile Planchon (1823–1888), head of botanical sciences at the University of Montpellier. As a result the European wine industry recovered, but nevertheless many vineyards were never replanted. The solution to phylloxera brought another problem in its wake, namely that of downy mildew, which was introduced on the imported rootstocks. An effective agent to combat this fungus, a mixture of copper sulfate and hydrated lime, was developed by Pierre-Marie-Alexis Millardet (1838–1902), professor of botany at the University of Bordeaux. It is still used as a fungicide under the name *Bordeaux mixture*.

Just as the term 'beer' had come to acquire an agreed meaning, it was during this period that regulations were first established concerning what practices were permissible for a certain type of wine and what nomenclature could be adopted. An early demonstration of the importance of having agreed procedures associated with a particular product was provided by port wine in the early eighteenth century. By 1730 the English thirst for

port had risen so much that the producers were unable to meet the demand so they began to blend inferior wines with the genuine ones from the Douro. They also added extra sugar for sweetness and elderberry juice to obtain the required color, and they flavored the wine with pepper, cinnamon and ginger. The consumers soon detected the reduction in quality, and in consequence the amount of port shipped to England fell by more than 50% between 1728 and 1756, and the price fell by more than 80%. The Portuguese government reacted by designating the area within which Douro wine could be made, thus creating the world's first officially controlled wine-growing region. The wine-making process was also supervised. These measures were extremely effective, and by the 1770s exports to England were about 50% higher than they had been before the malpractice commenced.

Legislation similar to that pioneered by Portugal now exists in many wine growing countries for their high quality wines; an example being France which awards the designation Appelation Contrôlée (or Appelation d'Origine Contrôlée). This delimits the geographical boundaries of the area in which the named wine can be grown, and governs matters such as which grape varieties can be planted, how they should be pruned, when the harvest may begin, etc. However, with the exception of Portugal, such legislation was not introduced into most countries until the twentieth century, but well before that time distinctive styles of wine had come to be associated with certain countries or regions. Thus the Bordeaux region of France became associated with a style of high quality red wine called claret by the British, although the region is also famous for its white wines. Other regions of France associated with particular wine styles are Burgundy (Bourgogne) and Champagne. In 1855, prior to the Universal Exhibition in Paris, Napoleon III requested that a quality ranking be given to the best Bordeaux wine-producing châteaux whose products were to be on display to visitors. The result was the *cru* system of classification, in which the 61 best red wines were placed in five categories ranging from Premières Crus (first growths) to Cinquièmes Crus. All the wines were from the Médoc region with the exception of one of the four Premières Crus (Château Haut-Brion), which was from the Graves region (Pessac). The white wine classification was limited to the sweet wines of Sauternes and Barsac, and comprised one Première Cru Supérieur (Château d'Yquem), with 11 Premières Crus and 15 Deuxièmes Crus. The classification has remained almost unchanged ever since, the only significant modifications being the addition of another Cinquième Cru in 1856, and the elevation of Château Mouton Rothschild from Deuxième to Première Cru in 1973. Not to be outdone by Bordeaux, Burgundy soon introduced its own cru classification, with the result that about 600 vineyards now qualify for the appellation Premier Cru, and 33 Grand Cru. The Champagne region also introduced its own cru classification in which each of the 300 villages in the region was given a percentage score indicating its grape growing potential. Grand Cru status is held by the 17 villages graded at 100%, and Premier Cru by the 38 villages graded at 90–99%. No village producing grapes for Champagne is graded at less than 80%. Some growers make their own champagne, but most sell their produce to the champagne houses or to cooperatives. Most champagne is therefore made from grapes from a variety of sources, but the famous champagne houses (the Grandes Marques) strive to use grapes with the highest possible average percentage score.

The story of whisky production in Scotland in this period is not so much one of changes in the methods of production, but one of an ongoing battle between the thousands of illicit distillers and the excise men. The Scottish Parliament had already started to tax the whisky in the preceding century, but after the Act of Union between England and Scotland in 1707, the taxes became ever more oppressive. Evasion of an unreasonable duty on the national beverage imposed by a government regarded as foreign was seen as acceptable behavior, and anecdotes abound of illicit stills in the countryside, of whisky being stored under pulpits or being transported in coffins, and there is one story of a 70 yard underground flue being constructed from an illicit still to a cottage chimney so that the smoke would not arouse suspicion. The situation was eventually brought under control when the Duke of Gordon, on whose land a number of illicit distillers operated, proposed in the House of Lords that all distilleries should be licensed, and that a fair duty should be paid on the whisky produced. This eminently sensible proposal was incorporated into the Excise Act of 1823,

and enabled whisky distillers to operate legally and to make a fair profit. Within 10 years almost all the illicit stills had disappeared.

A very similar situation existed in eighteenth century Ireland with the illicit production of their whiskey, but in this case one of the hated excise men was to perform an a valuable service for the industry. In 1821 Aeneas Coffey (*ca*.1780–1852) commenced experiments to devise a still, which made illicit abstraction of the product impossible. In 1824 Coffey resigned from the excise service to continue his experiments, and by 1830 his design was perfected. Aside from any possible benefits to the revenue, the Coffey still performed fractional distillation on a continuous basis and thereby produced spirit more quickly and more cheaply than the traditional pot still, which distils one batch at a time. The Coffey still (Section 3.1.3) continues to be widely employed in the production of spirits.

Licensed Scotch whisky distilleries increased in number and size as a result of the 1823 Excise Act, and this enabled modern whisky styles to evolve. The Coffey still was introduced for the manufacture of grain whisky, which is made by adding unmalted barley or other grains to malted barley prior to grinding. Malt whiskies are produced solely from malted barley, with two distillations being performed on the fermented wort using traditional pot stills. Blended whiskies contain 10–50% malt whisky mixed with grain whisky. In 1877 the Distillers Company was formed by the amalgamation of six grain distilleries, and it eventually grew to dominate the Scotch whisky industry. Whiskies are made in many countries, but to be called 'Scotch' a whisky must have been distilled at a licensed Scottish distillery and must have been matured in oak casks in Scotland for at least three years. Among the more famous whiskies produced outside Scotland is Bourbon, which gets its name from a former county of that name in the state of Kentucky. It was first produced there in the eighteenth century, and is made from a grain mixture containing mainly corn (maize), the remainder being wheat and/or rye, and malted barley (Section 3.3.4). It is aged in charred oak barrels. At around the same time the first whiskies were made in Canada by Scottish immigrants, and as a result the Canadian beverage is spelt the Scottish way (whisky) rather than in the Irish or American manner (whiskey).

Like the whiskies, other spirits acquired their modern characteristics during this period. Cognac (Section 3.6.2) emerged as one of the most famous brandies, and to be called a cognac the brandy must be distilled from wine made from certain specified white grape varieties grown in the Cognac region. The classification system was introduced by Hennessy, the firm founded in Cognac by the Irish immigrant Richard Hennessy in 1765, and indicates what period of aging in oak barrels the cognac has undergone, although the system is used on other brandies as well. The letters 'AC' indicate two years' ageing, 'VS' indicates three, 'VSOP' four and 'XO' six. In the nineteenth century, the Coffey still started to be used in the rum industry, and now the majority of rum is distilled by this technique (Section 3.5.3). The Coffey still produces a lighter rum than the pot still. After distillation the product is aged for at least a year, and is then usually blended to ensure consistent quality. In the nineteenth century the liqueur industry expanded considerably, especially in France. The most widely used technique is to add fruit or herbs to a spirit and then redistill (Sections 3.9.2 and 3.9.3). The distillate is then sweetened and possibly further flavoring may be added in the form of essential oils or clear vegetable extracts (Section 3.9.6)

1.2.4 Modern Times and Newer Processes (*ca*. 1900–Present Day)

In the early years of the twentieth century, the alcoholic beverages industry faced a threat that had been growing slowly for many years. This came from various temperance groups and societies opposed to the activities of the industry. They argued that alcohol consumption was a social evil, stressing that drunkenness resulted in family poverty, domestic violence, crime, insanity, immorality and suicide, to name but a few. Almost every country in the western world had its societies opposed to the industry, although their character varied considerably (Section 5.6.1). The movement was weakest in France, where the production of wine was

of huge economic importance and a matter of national pride, and its consumption was a national pastime. In as far as there was opposition to alcohol in France, it was mainly spirits which came in for criticism, for there was a reluctance to believe that the intoxicating principle in wine and spirits was the same substance in different concentrations. The temperance movement was stronger in Britain and the USA, although the many groups had different objectives. Some campaigned for an outright ban, while others wanted to ban just the sale of spirits or restrict the locations where alcohol could be sold and the times at which it could be purchased. In Britain it was the outbreak of World War I in 1914 that was the spur to the enactment of the first legislation (Section 5.6.1). There was a fear that inebriated personnel might disclose information to enemy spies, especially in the ports, and that drink might result in a reduction in efficiency of workers in factories producing goods for the war effort. Accordingly, restrictions were placed on the opening hours of public houses, brewers were forced to reduce the strength of their beer and excise duty was increased substantially. By the end of the war, beer consumption in Britain had fallen dramatically.

In the USA, individual States had on occasions banned alcohol at various times in the nineteenth century, but in 1920 the temperance movement achieved what it must have regarded as a triumph when prohibition was introduced. Although this had a serious effect on the alcoholic beverages industry, not all producers were forced to close. Products containing up to 0.5% alcohol by volume were permitted, and breweries produced drinks marketed as 'cereal beverages' which satisfied this criterion. They were known by the public as 'near beer.' An illegal practice was to add alcohol to near beer through the cork using a syringe, and the product was called 'spiked' or 'needle' beer. Some vineyards sold grape juice or bricks of dried grapes. These were often marketed with the warning that they could produce an alcoholic beverage if mixed with sugar, water and yeast – just what one needed to know for clandestine operations in the back kitchen. Not surprisingly alcohol continued to be available through illicit shops and bars ('speakeasies'). The ban undoubtedly encouraged criminality, and the Chicago gangsters Al Capone and his rival Bugs Malone made millions of dollars by selling illegal alcohol. In 1933 prohibition was rescinded. Prohibition was also in force in Canada around the same time, but the laws were enacted (and repealed) on a province-by-province basis and in a much looser and less well-defined manner. Nordic countries, with the exception of Denmark, also had periods of prohibition, and they still strictly control the sale of alcohol.

In Britain demand for beer picked up after World War I, and the industry revived in the USA after prohibition. However, in Britain, after the post World War I peak, there was a gradual decline in beer consumption. At the same time the number of common brewers decreased, as a result of closure, merger or takeover, while the size of the remaining companies increased. A typical example of this can be seen in Table 1.2.1, which traces the number of breweries in the (now) city of Cambridge from the late nineteenth century to the present day. This situation was partly due to the same factors that had started the process in the nineteenth century, especially the continuing improvement of the transport system, but also the rise of the advertising industry resulted in the promotion of nationally recognisable brands. Another reason was that many brewers owned a considerable number of pubs (tied houses), and as a result they were taken over by larger brewers who wished to increase the number of outlets for their products. The process of amalgamation has occurred the world over, with the result today that the largest breweries (along with their brands) in many countries are owned by national or international companies, most of which have diversified interests in other areas of the food, drink

Table 1.2.1 *Decline in number of breweries in the borough (now city) of Cambridge, UK*

Year	1888	1900	1925	1950	1975	1984	2008
Number of breweries	30	13	6	5	0	1	1*

*There are also two small breweries just outside the city boundary: one in Milton and one in Chittering.

or leisure industries. Most of the world-famous beers are owned by these companies: for example Beck's (Germany) is owned by Anheuser Busch InBev, Pilsner Urquell (Czech Republic) by SABMiller and Guinness (Ireland) by Diageo. By1999, the 10 largest groups controlled half the world beer market (see Section 1.3.2, Tables 1.3.1 and 1.3.2).

Scientific research and technical innovation continued to play a crucial role in the development of the industry. Beer was sold in bottles in England as early as the seventeenth century, but sealing the bottles was a problem. Holding down conventional corks with copper wire was not always successful. The crown cork (or crown cap) was invented in 1891, which not only provided a reliable seal, but meant that the bottling process could be automated. Another problem that technical developments were able to overcome around this time was that the bottled beers displayed cloudiness unless poured very carefully. This was due to a yeast suspension from secondary fermentation occurring in the bottle. Cloudiness was not really noticeable in the darker colored beers (porter and stout), but in the paler products that were gaining in popularity at this time it was undesirable. A variety of techniques were employed to render the beer completely clear and prevent further fermentation occurring. These included pasteurization, refrigeration and filtering (Section 2.6.9). Once the possibility of secondary fermentation had been removed there was no way that further carbon dioxide could be generated in the bottle to render the product sparkling, so the beer was artificially carbonated. These technologies were developed for bottled beer at the end of the nineteenth century, but were also applied when canned beer was introduced in 1935. Initially the cans were made of tinplate, but aluminium cans began to be used from 1959. At about this time breweries started to supply bars and public houses with beer treated in a similar manner and delivered in aluminium containers called kegs (Section 2.6.11). The barperson, rather than pump the beer up from a wooden barrel by a hand pump (Section 2.6.11), merely turned a tap and the beer was forced up by the pressure of carbon dioxide from a cylinder of the gas connected to the keg.

In the latter part of the twentieth century many breweries replaced their fermenting vessels with much larger cylindroconical tanks (Section 2.6.8). At the end of the fermentation, the yeast sinks into the conical lower portion of the vessel where it can be easily removed. An innovation for which there was initially much enthusiasm around 1960 is continuous fermentation (Section 2.6.8). In principle the idea is very attractive, for the yeast is immobilized and the wort passes through it on a continuous basis. This should result in a shorter production time being required to produce the beer, involve less time spent on cleaning operations than with the batch process, and hopefully give a more consistent product. However, although the process is used by some breweries, particularly for rapid maturation, it has proved to be difficult to operate and it has not been generally adopted.

Guinness's bottled stout was a famous drink throughout Britain during the first half of the twentieth century, advertized under the slogan 'Guinness is Good for You.' In 1964 draught Guinness was introduced, with the kegs containing nitrogen under high pressure, which resulted in a creamy head of fine bubbles forming on the drink when the liquid was forced through fine holes in a plate in the tap. So successful was this that the company embarked on research to replicate the effect on Guinness sold in cans. The result was the widget, a hollow plastic insert in the can that contains both the beer and nitrogen under pressure. Opening the can causes beer to squirt out of the widget, thus stirring the beer in the can and causing the release of further dissolved nitrogen as small bubbles. The idea has been adopted by a number of other brands, which are known as cream flow beers.

Towards the end of the twentieth century increasing health concerns created a demand for beers of reduced alcohol and/or carbohydrate content (Chapters 2.13 and 5.7, respectively). These are variously marketed as light (or lite), diet or low calorie beers. In the UK, for a beer to be described as alcohol-free it must contain no more than 0.05% alcohol, whereas up to 0.5% alcohol is permitted in the rest of the European Union. In the UK a beer designated as low alcohol must have no more than 1.2% alcohol. Thus consumer demand has led to the production of beers somewhat similar to those forced on an unwilling American public during prohibition.

A much more significant example of consumer pressure altering the practice of breweries in the UK is provided by the activities of CAMRA (Campaign for Real Ale) and similar groups. CAMRA was founded in 1971 to put pressure on breweries and public houses to provide traditionally produced cask-conditioned ales as an alternative to the keg beers, which by then had become almost universal (Section 1.3 4). Older styles of beers such as porter have been reintroduced, and the use of older flavorings such as coriander has been revived (Section 2.6.13). The campaign has been an undoubted success and cask-conditioned ales are now available in 65% of all public houses in the UK, and while overall beer consumption in the UK continues to fall slowly, the sale of cask-conditioned ales continues to rise. An allied change is that many public houses are once again brewing their own beer (real ale) in so-called microbreweries (Sections 1.3.3 and 1.3.4). This section of the market is growing strongly, aided by the introduction by the UK Government of a lower duty on beer produced by small breweries, and by increasing environmental concerns expressed by consumers (a locally produced beer has fewer 'beer-miles'). Among the many examples of microbreweries that can be cited is that owned by the Kirkstile Inn in the small village of Loweswater in the English Lake District. The landlord currently sells 30 000 l per annum of his own beer, 6000 l per annum of cask conditioned ales from other local breweries and 9000 l per annum of other beer, lager, Guinness and cider. If these figures seem large for a village with a total population of around 200, it must be remembered that a large part of the consumption is by visitors who come to this beautiful part of England and visit this lovely traditional hostelry. Another consumer demand that has grown recently is that for organic beer (Section 1.3.12). Although sales are still small, beers produced from organically grown barley and hops are increasing in popularity.

Looking again to the East, rice wine brewing technology advanced steadily during the twentieth century, particularly in Japan. The Japanese Government opened the Sake Brewing Research Institute in 1904, which was to become the focus of developments in sake brewing. Yeast and mould strains were isolated and those that were most beneficial to the brewing process were identified and cultured. Amongst other changes, enamel-coated steel tanks replaced wooden vats for the storage of sake. The tanks were not only considered more hygenic than wooden vats, but they also prevented the 30% loss due to evaporation in wood vats, thereby increasing the government's tax revenue from sake. Like its western counterparts, sake has long been taxed by the government. Again, like its western counterpart, the fortune of rice wine in general has been has been subjected to the vagaries of ecomomic and social changes, including wars.

During the Russo-Japanese War in 1904–1905, the government banned the home brewing of sake and toward the end of World War II, limitations were imposed on the use of rice for brewing, because of rice shortages. The World War II period also witnessed necessary production changes and innovations, some of which are still in use today. In the late seventeenth century, it had been discovered that small amounts of rice wine spirit could be added to sake before pressing to extract extra aromas and flavors from the rice solids, but during World War II, pure alcohol and glucose were added to relatively small quantities of rice mash, giving a considerably increased yield of sake. Today, much of Japan's sake is produced by a similar method, although more traditional methods are still in use (Section 2.7.1). The popularity of sake in Japan has more or less waned since 1945, in favor of shochu, domestic and imported whisky, wine and other drinks. However, sake has become a popular drink in many other countries, new sake breweries springing up in countries as far apart as Australia and the USA.

Prior to the annexation of Korea by Japan, rice wines were, along with soju, the everyday alcoholic drink of the Korean people, much of it home-brewed. However, during the period of Japanese occupation (1907–1945), the Government General of Choson collected a liquor tax from Korean people, which eventually led to the near disappearance of traditional brewing in Korea. Even as early as 1916, the severe control of alcohol liquor manufacture had reduced the variety of Korean alcoholic drinks to basic yackju, makkoli and soju and the production of high quality Korean traditional alcoholic beverages had diminished. By 1930, the recipes for brewing unique regional and provincial liquors had been put aside and these special drinks had disappeared.

After the liberation of Korea in 1945, a frequent shortage of rice and other grains led to a marked deterioration in the overall quality and consistency of Korean rice wines. Since the 1990s, however, the consistent use of rice for brewing, along with technical advances in the brewing process, has resulted in a steady increase in quality of all the different kinds of rice wine, so that today the consumer has a wide choice of high quality rice wines, including flavored versions (Section 2.7.1).

The grape wine industry in the twentieth century saw a series of dramatic changes, especially after the end of World War II. Shifting patterns of demand and supply, changing consumer preferences and continued improvement in transportation systems (especially containerized freight) all conspired to create opportunities for some wine producers and problems for others. Demand fell steadily in the traditional wine consuming countries of Europe, whereas production increased. In the late 1940s, per capita consumption of wine in France stood at 150 l, but 50 years later it had declined to 60 l. The social and cultural changes responsible for the decline have been much discussed, but one reason seems to be that as the standard of living has improved, the average French family, instead of consuming large quantities of *vin ordinaire* with every meal, now drinks smaller quantities of better quality wine. Another reason is the growing awareness of the dangers of consuming alcohol at the workplace or before driving a car. The reduced consumption in France, which was mirrored in other countries such as Italy, resulted in a vast European stockpile, called the 'wine lake.' In the early 1980s, the European Economic Community (as it was then called) sought to remedy the situation by forcing the distillation of some of the surplus to produce industrial alcohol, and introducing a compensation scheme to encourage farmers to pull up their vines and grow something else instead. This policy had the effect of reducing the total area of vineyards in Europe from 4.5 million hectares in 1976 to 3.4 million in 1997, and the huge surplus of European wine has almost disappeared.

As demand was declining in Europe, more efficient methods of production were being introduced around the world. Traditionally, caring for a vineyard had been a very labor intensive business, but the introduction of tall tractors with wheels that could straddle a row of vines meant that operations such as spraying could be carried out much more quickly and more thoroughly, and picking could be mechanized. Many changes have been introduced into the winemaking process itself, the aim being to improve efficiency or quality (or both). Refrigeration is often employed to preserve the quality of grapes or juice prior to fermentation, and some winemakers delay fermentation for long periods so that production can be continued in batches for much of the year. Although some winemakers still use traditional wooden vats and barrels for fermentation and maturation, vessels made out of stainless steel are now common (Sections 2.9.1 and 2.9.2). These can be very large compared to their wooden counterparts, and careful temperature control and cooling by refrigeration may be required to dissipate some of the heat produced in fermentation. This is especially important for white wines for which low temperature fermentation produces the best results. To press the juice from the grapes, the traditional wooden basket press has now largely been replaced by the pneumatic press, in which a slowly inflating airbag within a rotating perforated steel cylinder gently expresses the juice (Sections 2.9.1 and 2.9.2). Pumps and pipes are now universally employed to move liquids around. Sulfur dioxide is now added to wine in the form of potassium or sodium metabisulfite, which being solid is much easier to handle (Sections 2.5.2 and 2.5.3).

Traditional fermentation relies on yeasts existing on the skins of grapes or in the winery to enter the vat naturally. Usually inoculation by these so-called wild yeasts occurs satisfactorily, but sometimes a fermentation is slow to start, and a batch becomes stuck. Cultured yeast strains are now available to initiate fermentation in a reliable and consistent manner, but their use is still attended by considerable controversy. Another possibility available to the modern winemaker is to induce malolactic fermentation, the process by which malic acid is converted to lactic acid. The organisms responsible are bacteria (e.g. *Oenococcus oeni*) (Section 2.3.2) rather than yeasts. The process can occur naturally during primary alcoholic fermentation, but the winemaker usually suppresses this using sulfur dioxide to prevent undesirable strains of bacteria producing off flavors. Malolactic fermentation is then induced at a later stage by inoculation with a suitable

strain of bacteria. Most red wines and an increasing number of whites now undergo this process, the effect being to increase the stability of the wine, and to make it taste softer, but more complex. Research has been done to investigate the possibility of carrying out wine fermentation on a continuous basis. Experiments have been performed using yeast cultures immobilized on cuts of apple or quince, but so far this has not become a standard method in wine production.

While few would disagree that the traditional Champagne method (Section 2.9.3) produces the best sparkling wine, new (and less expensive) techniques were devised in the twentieth century. A simple method, which is only used for the cheaper wines, is to pump in carbon dioxide under pressure immediately before bottling. A more sophisticated process is one in which the carbon dioxide is produced by a second fermentation in a large tank (*cuvée close*), rather than in the individual bottles as in the true Champagne method.

Beaujolais is a wine that has received more attention in the second half of the twentieth century, mainly through the clever marketing of Beaujolais Nouveau. The fermentation technique employed is that of carbonic maceration (Section 2.9.2), in which whole bunches of grapes are allowed to ferment in an atmosphere of carbon dioxide. To a certain extent this used to occur naturally in barrels of grapes awaiting processing, when the grapes at the bottom became crushed, started to ferment and the carbon dioxide released displaced the air from around the grapes higher up. The process is now done on a controlled basis by pumping carbon dioxide into tanks containing the grapes. Fermentation takes place within the intact grapes, and after pressing and pasteurization to inhibit malolactic fermentation the wine is bottled. The carbonic maceration method produces a light fruity wine low in tannins that is best if drunk young. Sales have soared as a result of publicity surrounding the races to get the new wine to markets around the world immediately after the official release day a few weeks after harvest.

There is general agreement that good quality wines benefit from being aged in new oak barrels, but this is of course an expensive process and a modern alternative is to allow the wine to mature in a tank containing oak chips (Section 2.9.5). Traditionalists rightly point out that the effect is not quite the same, but their opposition to another innovation, namely plastic stoppers or screwcaps in place of corks, would appear to have little foundation. Prior to bottling, most wine is clarified to remove any traces of sediment or any remaining organisms. The new method of achieving this is by centrifugation or filtration, but the traditional method of fining is still much used (Section 2.9.4). Beaten egg whites are often added to wine matured in wooden barrels, while a type of clay called bentonite is used in larger tanks.

The alcohol content of wines varies considerably. Wines produced in cooler areas will, unless chaptalized prior to fermentation, be lower in alcohol (sometimes lower than 9%), but in general the alcohol content of wines has tended to increase in recent years, now typically being of the order of 12–14%, although fashions change from country to country and from year to year. In consequence a demand has grown for lower alcohol wines, and in the UK in 2007, the supermarket chain Sainsbury's launched their 'Ten%' range of wines. However in the same year the UK Food Standards Agency banned a company from selling wine reduced to 8% alcohol because the technology used to reduce the alcohol content (spinning cone column) (Section 2.13.3) was unauthorized. In California the Ariel company produces nonalcoholic wine (less than 0.5% alcohol) by the use of reverse osmosis (Section 2.13.3). Starting with wine of normal composition, the process removes both alcohol and water, so the resulting syrup is diluted with pure water. In the same way that a demand has arisen for organic beer, there is now likewise a market for organic wine, with the grapes being grown without synthetic fertilizers fungicide or pesticides, and without the use of sulfur dioxide.

While wine continues to be by far the most popular drink based on a fermented fruit juice, cider sales have been growing in recent years. In the UK one company, H.P. Bulmer, has more that 50% of the market (Sections 2.8.6 and 2.8.8). Perry experienced a surge in popularity in the UK after the launch of the Babycham brand in 1953, but its popularity declined in the 1970s. However, it was probably Babycham more than any other drink that introduced ladies in Britain to the pleasures of alcohol consumption. For many years concentrated grape juice has been imported into Britain to be diluted and fermented to produce so-called 'British' wines.

In the days when no grapes were grown in Britain for commercial production of wine, this labeling did not cause confusion. Nowadays, as a result of the efforts of an intrepid band of pioneers (such as Ray Barrington Brock, Edward Hyams, Gillian Pearkes, Sir Guy Salisbury-Jones and Jack Ward) and a warmer climate, the country boasts over 350 vineyards and in consequence there is now an element of misunderstanding. The wine from these British vineyards is labeled 'English,' 'Welsh' or 'United Kingdom.' Most of the vineyards are located in the southern parts of England and Wales, but a bold experiment is taking place in the north of England at High Cup Wines on the edge of the Pennines in Cumbria. The principal products are wines made from fruits such as elderberry and damson, but six varieties of grapevines are grown. The most successful at yielding wine so far has been the red *V. vinifera–V. amurensis* hybrid *Rondo*.

We have seen how international companies emerged in the brewing industry in the twentieth century. A similar process occurred in the wines and spirits industries. One such company is the French concern Pernod-Ricard, which was formed in 1975 by the merger of the rival Pernod and Ricard concerns. They both manufactured pastis, a drink similar to absinthe, but with a lower alcohol content and without wormwood, which was introduced after absinthe was banned in 1915. In recent years the company has acquired Irish Distillers, Orlando Wyndham (makers of Jacob's Creek wines), 38% of Seagram's wines and spirits business and Allied Domecq. Another large concern is the Japanese company Suntory, which produces beer and spirits in Japan, and has also branched out into the genetic engineering of plants with the Australian company Florigene, in which it now holds a 98.5% share. In 1987 a significant proportion of the Scotch whisky industry came under the ownership of United Distillers, which was formed from the merger of the Distillers Company Limited with Arthur Bell and Sons. It is now part of the Diageo group, which owns many world famous brands of beers, wines and spirits, producing one third of all Scotch whisky.

In the second half of the twentieth century, the pattern of whisky consumption changed. In the 1950s, the consumption of single malts was largely confined to Scotland, with the export trade consisting mainly of blended whisky (that is to say malt whiskies blended with grain whiskies). Somewhat confusingly, the term single malt refers to a blend of malt whiskies from a single distillery. In most cases, these will be malt whiskies from different casks and from different distillation batches (from different years) vatted together in a tank or vat for consistency, although the age statement on the bottle label (e.g. 15 years old) must reflect the wood-maturation age youngest component (Section 3.2.5). Gradually the rest of the world acquired a taste for single malts, with the result that they are now the flagship product of the industry worldwide. Demand for blended whiskies, including 'de luxe' blends, is still high, especially in some European countries (France and Italy) and in Asian countries such as India and South Korea.

Production of Korean soju, like rice wines, suffered under Japanese rule, but was revived after the liberation of the country in 1945. However, Korea at that time was a poor country, a situation not helped by the Korean War (1951–1953), and many of the traditional recipes had been obscured or even lost. On top of that, raw materials for the production of soju, especially rice, were periodically scarce. During the period 1965–1991, the Korean government amalgamated and reorganized the distilleries into regional and provincial conglomerates and forbade the direct production of soju from fermented rice mash. Instead, highly rectified spirit from any source was mixed with water and flavorings to create what is known as diluted soju. Although the restriction has now been lifted, this cheaper version of soju continues to be made this way and is presently the more popular form of the drink, and altogether, soju may well be the world's most highly consumed spirit (Section 3.4.4).

The Korean government regulates the alcohol content of diluted soju to less than 35% ethanol by volume; in practice it is usually 20–25% ABV. Since the 1990s, several regions have resumed the traditional production of soju, by the distillation of grain (especially rice) mashes or wines, resulting in what is known as distilled soju, the original version. The soju from the city and environs of Andong is the most famous of all, with an ABV around 45% and having a powerful, malty character. There is now (2009) a trend amongst distillers to produce lighter, less powerful traditional versions of soju with alcohol contents of around 20%

(v:v) in reply of the popular move towards milder, less alcoholic spirits (a trend also occuring in Japan) (Section 3.4.4).

1.2.5 The Development of Analytical Methods

We have seen how during the nineteenth and twentieth centuries there was an improving level of understanding concerning many of the processes involved in alcoholic beverage production. The scientific explanations of what had for many years been the work of craftsmen, and the introduction of new techniques, had resulted in many changes in the production of beers wines and spirits. But not surprisingly there was an increasing demand for the application of analytical methods to assist in achieving consistency by monitoring the various stages of production of beverages, and assessing the quality of the final products.

Aside from the antics of the ale conner, an early method of quantitative measurement was the application of the thermometer, used by brewers from around 1760 to measure the temperature of the hot water in the mash tun prior to the grist being added. Previously the brewer had judged the temperature to be appropriate if he could just tolerate immersing his hand in the water, or if the reflection of his face in the surface of the water was just not obscured by the steam. A few years later the saccharometer was introduced. This was a hydrometer that measured the specific gravity of the wort, and hence provided a measure of its sugar content. These two pieces of apparatus remained the sole monitoring devices in use until the nineteenth century, when breweries started to use the microscope. More recently an enormous number of analytical techniques have been developed, many of which have found application in the alcoholic beverages industries.

The application of the thermometer and saccharometer did not require specialist staff working in a laboratory for their application. When brewery laboratories were established from the mid nineteenth century, the trained chemists hired to work in them introduced methods such as gravimetric and volumetric analysis. Both found application in the analysis of water supplies used by breweries. Volumetric analysis was also used to measure the acidity at any stage of production (Section 4.6.3). The Kjeldahl method for nitrogen (mentioned previously) operates by converting the nitrogen to ammonia, which is then distilled off, absorbed into water and titrated with standard acid (Section 4.6.3).

The importance of close analytical control in beer production was demonstrated by the so-called Manchester beer epidemic of 1900, when about 7000 people became ill, of whom 70 died. It was discovered that all had been consuming beer from the same two breweries, who in turn had obtained their sugar from Bostock and Co. of Liverpool. The sugar contained arsenic, derived from the sulfuric acid used in its manufacture, which in turn had been made using iron pyrites containing arsenic compounds, common impurities of iron pyrites. Two years later arsenic was detected in beer from a Halifax brewery. On this occasion there were no fatalities, and the source of the contamination was found to be the coke over which the malt had been dried. In 1903 a Royal Commission recommended that liquid foods should contain no more than 0.14 parts per million of arsenic. The quantification of arsenic at such concentrations was achievable by the methods available at the time, but previously the tests had not been carried out.

Almost all the techniques used to analyze beers wines and spirits were developed in other contexts, and then found application in the alcoholic beverages industry. However, one very important method was developed by an industry insider. He was Joseph William Lovibond (1833–1918), the son of a London brewer. He worked with his father until 1869, when he went to be director of another brewery in Salisbury owned by the family. Once there, he set about devising an objective method of measuring the color of wort and of beer. The color of the wort provides an indication of its quality, and measuring the color of beer itself was important because consumers were now demanding that it should be of a consistent appearance. This demand had arisen partly because pewter tankards were being replaced by transparent beer glasses, and partly because the traditional dark and somewhat murky porter beers were being superseded by the newer pale ales. In so far as attempts

had previously been made to record the appearance of beer, it had been done solely by visual inspection, using terms such as 'very pale,' 'fairly dark,' etc. Lovibond devised an instrument called a tintometer in which a sample of beer or wort was placed in a small glass cell and viewed with one eye down a narrow tube at the base of which was a white screen. The other eye looked at the same screen down a parallel tube into which could be inserted up to three colored pieces of glass. Different glasses were inserted until the two shades perceived by the observer were identical. The instrument was supplied with 450 standard glasses of red yellow and blue colors of different intensities. Lovibond devised a notation system to describe the color of the sample based on the combination of glasses used. From 1863 it had been permissible to add colorants such as caramel to beer, and the tintometer also found application in the evaluation of colorant preparations. In 1885 Lovibond founded a company to manufacture the tintometer. The company is still in existence, and an updated version of the instrument continues to find application in breweries and in whisky distilleries, and it can also be used to measure the color of wine (Section 4.4.3).

The Lovibond tintometer is an example of a kind of colorimeter called a comparator, as the color being measured is compared to a previously prepared colored standard. Of much more widespread application today are instruments that measure the proportion of light absorbed over a narrow band of wavelengths (photoelectric colorimeters) or at an individual wavelength (spectrophotometers). A spectrophotometer can also record the absorption spectrum over the entire visible (and ultraviolet) range, and therefore provides an objective graphical representation of the light absorbing characteristics (and hence color) of a sample. Colorimeters and spectrophotometers find important application in the quantitative estimation of individual compounds in a mixture (Section 4.4.3). The usual procedure depends upon employing a chemical treatment to react the compound being sought to yield a colored substance, whose absorbance (which is directly related to concentration) is measured using a colorimeter or spectrophotometer. Two examples of this technique in use at the present time are the Folin–Ciocalteu method for measuring phenolic antioxidants in wines, and the Rebelein method for measuring the concentration of tartaric acid and tartrates in wine (Section 4.4.3).

The spectrophotometer provides an example of the entry into analytical chemistry of the kind of equipment that is now widespread and commonplace. Using instruments containing sophisticated electronics and linked to computers, the capabilities of analysts have been greatly extended. Alcoholic beverages, especially wine, contain an enormously complex mixture of organic compounds, and until the middle of the twentieth century the identification and quantification of most of these substances appeared to be a superhuman task. However, there was an overpowering need to obtain a better understanding of beverage composition, partly to assist in more rigorous quality control, and partly to quantify components that might convey health benefits or health risks to consumers (e.g. anthocyanins and *N*-nitroso compounds, respectively). Modern instrumentation has provided the means by which much of that need can be met.

Another benefit of the introduction of modern methods has been the increase in the speed at which analyses can be performed. This is well illustrated by the various methods that have been used to analyze metals in water and in drinks. The classical method of gravimetric analysis provides a very accurate measure of the concentration of a metal such as calcium, but it is a slow procedure. In the 1920s the method of flame photometry (Section 4.4.4) was developed, in which the solution under test is sprayed into a flame, with the consequence that some of the metal atoms are excited and then emit light of characteristic wavelengths as they return to the ground state. The light is passed through a suitable filter, and its intensity is measured, thus providing a measure of the concentration of the metal. The method is reasonably sensitive for metals such as sodium, potassium and calcium, but less so for metals such as copper (which could be in wine as a result of treating the vines with Bordeaux mixture), since the proportion of excited atoms is very low. In the 1950s the alternative technique of atomic absorption spectroscopy (Section 4.4.4) was developed, in which the much greater proportion of unexcited atoms is measured by passing an intense beam of light of a suitable wavelength through the flame and recording the resulting reduction in intensity. This technique can measure a much greater number of metals to an acceptable degree of accuracy. However, more recently still,

emission techniques have staged a comeback as a result of the development of plasma sources. In a plasma, a much higher proportion of metal atoms are excited and then emit light, and the intensity of the emission is measured. This technique (called inductively coupled plasma spectroscopy) is now the method of choice in many laboratories (Section 4.4.4).

Today, rather than using a titrimetric procedure, the acidity of a beverage is measured using a pH meter (Section 4.5.1). Although Sørensen introduced the pH concept in the early years of the twentieth century, direct reading pH meters were not available until soon after World War II, by which time the glass electrode, sensitive to hydrogen ion concentration, had been perfected, and suitable electronics had been developed for measuring the potential between the glass and reference electrodes. More recently a range of electrodes sensitive to other ions have been developed, and these ion-selective electrodes provide a rapid and nondestructive method of analysis (Section 4.5.1). One of the most important developments in this area has been the development of electrodes responsive to sulfites in wine. One recent publication described such an electrode that owed its sensitivity to sulfite by having the enzyme sulfite oxidase immobilized on its surface (Situmorang *et al.*, 1999).

Another electroanalytical method that has found increased application in recent years is that of conductivity measurement (Section 4.5.1), which is used to measure tartrate concentration in wine. Tartaric acid and tartrates are naturally present in grape juice, and may form crystals in the bottom of the wine bottle. Traditionally this presented no problem, but many contemporary consumers dislike seeing the crystalline deposit, and so tartrate removal, by cooling and filtering or by electrodialysis, is employed. Removal is continued until the conductivity has fallen to a level that indicates that tartrate crystals are unlikely to be deposited on storage.

The three techniques that have done most to revolutionize the identification of organic compounds are infrared spectroscopy (IR) (Section 4.4.2), nuclear magnetic resonance (NMR) (Section 4.4.1) and mass spectrometry (MS) (Section 4.4.5). An IR spectrophotometer records the various wavelength ranges at which a compound absorbs in the infrared, the pattern being due to the various types of chemical bond present in the molecule. In NMR the parameters under which a certain nucleus (e.g. ^{1}H or ^{13}C) resonates under the influence of radiofrequency radiation in an intense magnetic field are recorded; the exact conditions depend on the environment of that atom in the molecule. In MS a positive ion is generated from the molecule, which may then fragment, and electrical and magnetic fields are used to focus the various ions in turn on to a detector. The mass of the parent molecular ion and its fragmentation pattern are characteristic of the compound under investigation.

These methods are most frequently employed for qualitative analysis on individual compounds once they have been separated from the matrix in which they occur, but recently some quantitative applications have been developed for the beverage industry in which the entire sample is subjected to analysis. Thus an NMR spectrometer has been adapted in which an unopened bottle of wine can be placed in the machine to measure the ethanoic (acetic) acid concentration (Section 4.4.1) (Weekley *et al.*, 2003). This enables a judgement to be made as to whether an old bottle of wine has been spoiled by oxidation occurring as a result of a porous cork. Recent research has also been directed at the use of IR (Section 4.4.2) to monitor the progress of wine fermentation. Fructose, glucose, ethanol and a number of organic acids are measured on a continuous basis. Such methods are currently in their infancy, but they are likely to find wider application in the future.

However, more frequently, an individual compound is isolated from the matrix prior to analysis, and the most widely used separation technique is that of chromatography. This term covers a range of methods in which a sample of the mixture is injected into a mobile phase, which passes through a stationary phase. The compounds in the mixture will be attracted to both phases, and if different compounds experience differential relative degrees of attraction for the two phases, they will travel through the system at different rates. The technique was first demonstrated in 1903 by the Russian botanist Michel Semenovich Tswett (1872–1919), who separated a mixture of plant pigments using a vertical column containing calcium carbonate as the stationary phase, down

which was passed benzene as the mobile phase. Since that time a variety chromatographic methods have been developed, examples being thin layer (TLC) (where the stationary solid is coated on to a glass or plastic plate and the mobile phase rises up the plate by capillary action) (Section 4.3.1), paper (where the stationary phase is water held on paper and the water-immiscible mobile phase is soaked up by the paper) and gas-liquid chromatography (GC) (Section 4.3.2), where the mobile phase is gaseous and the stationary phase is a liquid held on solid beads). The technique most resembling Tswett's original method is high performance liquid chromatography (LC) (Section 4.3.3) where the mobile liquid is pumped through a column of stationary solid under pressure. All these methods have found application in the beverages industry.

The straightforward application of chromatography in qualitative analysis involves identifying the separated components by running known compounds under the same conditions. This is achieved in thin layer and paper chromatography by placing the sample and known compounds side by side on the same plate or paper, and then spraying the resulting dried chromatogram with a developing agent to reveal the final position of the compounds. In the case of GC and LC a detector is employed to signal a change in composition of the stream emerging from the column. The most widely used method of detection in GC is flame ionization, in which the gases emerging from the column are combusted in a stream of hydrogen in a burner. As they decompose, organic compounds produce ionic intermediates, which are detected by a pair of electrodes in the flame. Spectrophotometric detectors are most common in LC. These are set to a wavelength at which the analytes are known to absorb. Whatever the detection method, the retention time of a component in the mixture is noted, and this is compared with the retention times of known compounds run under identical conditions. The magnitude of the detector response (i.e. the area under the sample peak) provides a measure of the quantity of the analyte. Such techniques are well suited to detecting adulterants that have been added to alcoholic beverages. An infamous case occurred in 1985, when small quantities of Austrian wine were found to contain diethylene glycol, which had been added to make the wine smoother and sweeter (and able to command higher prices) (Prial, 1985). As a consequence, Austrian wine regulations are now some of the most stringent to be found anywhere, and the Austrian wine industry has recovered from the serious setback it received (Jarvis, 2005; Mariani, 2007).

Whilst chromatographic methods are by far the most important separation techniques employed in the beverages industry (and in chemical analysis generally), methods based on the phenomenon of electrophoresis are used as well (Section 4.6.1). The original method, introduced in the 1930s, used the application of an electric field to separate charged molecules applied to a horizontal support medium of paper or gel soaked in buffer. The rate of migration of an individual species depended on its size and charge. More recently the method has been miniaturized and automated and is known as capillary zone electrophoresis (CZE) (Section 4.6.1). The separation is achieved in a capillary tube up to 100 cm long and up to 100 μm internal diameter, and the emerging species are detected and quantified. An advantage of the method is that the bands of the various substances are much sharper than with LC. The method is applicable to the separation of uncharged molecules provided these can be converted to charged complexes (e.g. with borate), an example being its application to the quantitative determination of anthocyanins in wine. A technique that is to some extent a hybrid of CZE and LC is capillary electrochromatography (CEC). In CZE the buffer solution in the tube migrates under the influence of the electric field, a process known as electro-osmotic flow. CEC likewise employs as its mobile phase a buffer migrating under the influence of an applied electric field, but the capillary tube also contains a stationary phase similar to that used in LC. The high pressures of conventional LC are thereby avoided, and the method has the advantages of sample microvolume and sharp bands associated with CZE.

Most beverages are such complex mixtures that the analyst frequently requires a technique that will not only separate the individual components, but identify them as well. This can be achieved by linking a GC or LC apparatus to a mass spectrometer. Each compound yields a characteristic molecular ion and fragmentation pattern that are identified by reference to a computer database. The classical methods of ionization are

by electron impact (electron ionization or EI), which produces positive ions by electron loss, or by the gentler method of chemical ionization (CI), which produces positive ions by protonation. These methods are applicable to low mass thermally stable analytes. A more modern and even milder method of ionization for thermally sensitive compounds is atmospheric pressure chemical ionization (APCI), which, however, results in very little fragmentation of the positive ion. All these methods of ionization are applicable to small molecules, but it is also necessary to be able to detect and characterize large molecules such as proteins, as they affect the taste, clarity and stability of a beer or wine. For these samples a technique known as electrospray ionization (ESI) is used. The protein molecules are not fragmented, but emerge with a range of positive charges, typically in the range +10 to +22, and thus a spectrum of mass/charge ratios is produced.

It is frequently the case that the substances to be analyzed by GC-MS or LC-MS are present in such low concentrations in the matrix that some kind of prior concentration stage is necessary. Sometimes solvent extraction can be used, and on other occasions simultaneous distillation-extraction is appropriate (Sections 4.2.2 and 4.2.3). Other possibilities are the so-called sorptive methods, in which the solid phase sorbent and liquid containing the components (the matrix) of interest are allowed a certain contact time. During this time, components are selectively partitioned between sorbent phase and the matrix, usually according to their relative polarities (Section 4.2.4). The major techniques are known as solid phase extraction (SPE), solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE). The sorbent material is often silica-based, crosslinked polystyrene-divinylbenzene (e.g. for SPE) or polydimethylsiloxane (PDMS) (e.g. for SPME and SBSE), but other materials are used. The sorbent and matrix are then separated and subjected to either a thermal desorption process or solvent extraction to free the extracted components in a more concentrated form. If thermal desorption is used, the liberated compounds are usually subjected to GC-MS (Section 4.3.2), whereas if a solvent is used in the desorption stage, then LC-MS (Section 4.3.3) is employed. These methods find considerable application in the estimation of a wide variety of compounds occurring in beers, wines and spirits. Sometimes an analysis is required of the volatile compounds given off by a beverage, in which case the sample is confined until the air in the space above (the headspace) is saturated with the volatile compounds (Section 4.2.5). A sample from the headspace can then be injected into a GC-MS apparatus, or if a concentration stage is necessary, a fiber of an SPME material can be employed.

The mass spectrometer can also determine the ratios of isotopes of a particular element in a compound (Section 4.4.5). This is most useful, because the ratio of $^{13}C/^{12}C$ or $^{2}H/^{1}H$ in a sugar or in ethanol depends upon the plant from which it was derived and the climatic area in which it was grown. As a result, fraudulent practices such as the mislabeling of a wine to suggest it comes from another country or area, or the addition of more than the allowed quantity of sugar during chaptalization, can be detected.

An even more sophisticated isotopic ratio technique is to use NMR (Section 4.4.1) to deduce the relative abundance of a particular isotope (e.g. deuterium, ^{2}H or D) at two different sites within a molecule. The principal example has been to measure the ratio of methylene deuterium (CH_3CHDOH) to methyl deuterium (CH_2DCH_2OH) in ethanol by comparing the intensities of the deuterium NMR signals for the CHD and CH_2D peaks. The technique, known as site-specific natural isotopic fractionation NMR (SNIF-NMR) can be applied directly to spirits, when it is found that the ratio depends upon the plant from which the ethanol was derived (e.g. potato or sugar cane) and the area of the world in which the plant was grown. The ethanol in wines and beers is too dilute for direct application of the technique, so these beverages need to be concentrated by distillation beforehand.

The ultimate analysis of any beer, wine or spirit is that performed by the consumer. His or her opinion is what matters to the producer, and throughout the alcoholic beverage industry, expert tasters assess the quality and development of their company products and give valuable advice on blending and adjustments to be made before sale. Organoleptic assessment and sensory analysis are still of prime importance in the production of alcoholic drinks (Chapter 4.7). Analytical instruments will continue to improve in their sophistication, and

the individual components of the complex matrix that makes up any beer wine or spirit will be quantified with ever greater accuracy. But in the future a totally new kind of instrument, the so-called artificial nose, may play a significant role in assessing the quality of beverages. Such instruments are currently under development, and consist of an array of sensors (Section 4.5.3), which detect individual compounds or specific groups of compounds. In one design, the sensors are made of different types of electrically conducting plastic. The conductivity of the sensors changes when the volatiles in the air are absorbed by the plastics. The changes in conductivity of the various sensors is relayed to a computer, which is 'taught' to interpret the new pattern. Artificial noses will probably find application in areas such as medical diagnosis (patients with diseases such as cirrhosis exhale characteristic odors), airport security and in detecting spoilage in foods. In the beverages industry, artificial noses have already found application. In the brewing process, a buttery off flavor can develop owing to the presence of diacetyl, which is converted to tasteless butanediol if the batch is rested (Section 2.6.4). An artificial nose can detect if a resting period is required.

The long history of alcoholic beverages commenced with a wine being tasted by a lady from the harem of the king of Persepolis, and has now reached the stage when some degree of assessment can be achieved by an artificial nose or a portable artificial tongue (Moreno i Codinachs *et al.*, 2008). The enormous improvement in the quality and consistency of alcoholic drinks over a period of thousands of years has been due to the combined skills of craftsmen, technologists and scientists. There is no doubt that continuing changes, both in the various production processes and in the methods of performing analyses on the intermediate and final products, will yield further benefits for the industry and the consumer.

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1.3
Some Recent Trends and Developments

1.3.1 Overview

This section highlights some recent issues, developments and trends relating to alcoholic beverages and the drinks industry. Generally, it is hoped that the topics of this section give some pointers to the near future of alcoholic beverages. However, the authors feel disinclined to try to predict the detailed future of alcoholic beverages, even after a good session in the public bar of the Kirkstile Inn. The selected topics relate to what are perceived as major influences on the direction being followed by the world of alcoholic beverages and some of these are presently major points of discussion or even controversy. They are, allowing for a certain amount of overlap and connectivity between topics:

- Giant multinational companies and globalization of products
- Microbreweries, craft breweries and individualization of products
- Renaissance of cask-conditioned and top-fermented beers: revival of traditional styles
- Changes in beer drinking habits
- Global growth in wine drinking
- Growth of wine industries in countries with marginal climates
- Revival of cider and perry
- The rise of flavored alcoholic beverages ('alcopops')
- Health awareness, calorie counting and changes in social attitude towards alcoholic drinks
- Binge drinking
- Organic and biodynamic alcoholic drinks
- Use of genetically modified (GM) crops and microorganisms.

Some of the above issues have already been referred to briefly in Section 1.2.4, but will now be discussed in greater detail.

1.3.2 Big is Beautiful: Multinational Companies and the Globalization of Alcoholic Beverages

Like other industries, the alcoholic drinks industry has witnessed the growth of national conglomerates since the latter half of the nineteenth century and into the closing years of the twentieth century. The past 20 years

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

Table 1.3.1 *Brewery-based drinks companies (2006/2007)*

Company	Approx. Output/ hl $\times 10^6$ (2004)	Examples of beer brands	Approx. revenue	Approx. number of employees
Anhauser-Busch InBev	193.4	Beck's, Budweiser, Jupiler, Stella Artois	\$36 400 $\times 10^{6*}$ (2008)	> 100 000*
SABMiller	148.3	Castle Lager, Gambrinus, Miller Genuine Draft, Tyskie	\$18 620 $\times 10^6$ (2008)	69 116 (2008)
Heineken International	112.6	Amstel, Heineken, Starobrno, Zyweicz	€11 829 $\times 10^6$ (2006)	57 557 (2006)
Carlsberg	92.0	Baltika, Carlsberg, Holsten, Tetley, Tuborg	44 750 $\times 10^6$ DKK (2007)	33 420 (2007)
Molson-Coors	49	Molson Ice, Coors Blue Moon	\$6400 $\times 10^6$ (2008)	9700 (2008)

Data from http://www.europeanbeerguide.net (2008).
*Projected values on sale of Anheuser-Busch to InBev in July 2008.

or so have seen the rapid growth of multinational companies so that today national and international markets are dominated by a relatively small number of companies who own facilities (e.g. bottling plants, breweries, cider factories, distilleries, malting houses, orchards, vineyards, farms, wineries, etc.) and employ thousands of people in several countries. Although these companies tend to have diversified interests, many concentrate on the production of particular beverages, such as beer (Table 1.3.1) or distilled beverages, liqueurs and wine (Table 1.3.2). Several of these companies have interests in nonalcoholic beverages: for example, SABMiller is the main bottler and distributor of Coca Cola, and Anheuser-Busch InBev is a large producer of soft drinks and sports drinks. With the merger of Anheuser-Busch (USA) and InBev (Belgium/Brazil) in July 2008, the resulting company (Anheuser-Busch InBev) is the world's largest brewing organization, overtaking SABMiller. Anheuser-Busch held this accolade (calculated on revenue) for many years and was the last large American brewing company to be majority owned and operated in the USA (Miller merged with South African Breweries, and Coors merged with Molson earlier this century). It is estimated that the new company will have yearly sales of around \$36.4 billion (the sum of their individual annual sales before merger) and will control around 25% of the world beer market (Spain and Goldstein, 2008).

Diageo, formed in 1997 from the merger of Guinness Plc and Grand Metropolitan Plc, is the world's largest broad-based alcoholic drinks company, closely followed by Pernod-Ricard (Table 1.3.2). Their businesses are firmly based on aperitifs, liqueurs, spirits and (for Diageo) beer. Constellation Brands is the world's biggest producer of wine, with interests principally in the USA (especially California and New York State), Australia and New Zealand. The company also owns the UK firm of Matthew Clark, the world's second largest producer of cider and perry, noted for Blackthorn cider and Babycham.

Apart from providing work and economic prosperity, these companies provide valuable social benefits by sport sponsorships (e.g. of clubs or leagues), by organizing special events, by sponsorship of academic posts and research programmes, and by making charitable donations at local and international levels. At a more local level, the same can be said of many smaller alcoholic drinks companies.

Advancing technology and improved communications have helped the growth of these companies, and although they possess production plants in many countries (or have some of their products made under license by contract companies), there is a heavy reliance on transport, with its attendant rising costs and increasing

Table 1.3.2 *Spirits- and wine-based alcoholic drinks companies (2007)*

Company (main products in order of importance)	Revenue	Employees	Typical brands
Diageo (s, b, w)	7260 million GBP (2007) $16 142.8 million (2008)	24 373 (2008)	Bailey's Irish Cream, Captain Morgan, Guinness, Johnnie Walker, Piat (wine), Red Stripe (beer), Smirnoff, Crown Royal
Pernod[#] Ricard (s, w)	€6443 million (2007)	17 680 (2007)	Chivas Regal, Martell, Mumm, Pernod Anise, Ricard Pastis, Wild Turkey, Wyborowa
Baccardi (s, w)	$5500 million	–	Baccardi Breezer, Baccardi Oro, Benedictine, Bombay Sapphire, Dewar's, Drambuie, Martini & Rossi
Fortune Brands (s, w)*	$7300 million (2007)	34 000 (2007)	Fundador, Canadian Club, Cockburn, Courvoisier, Harveys, Jim Beam, Teachers
Constellation Brands (w, b, c, s)	$5200 million (2007)	7700 (2007)	Corona (beer), Hardy, Matthew Clark (cider/perry), Paul Masson, Robert Mondavi, Taylor, Tsingtao (beer)
Brown-Forman (s, w)	$2 444 million (2006)	3350 (2006)	Bolla, Casa Herradura, Fetzer, Finlandia, Jack Daniels, Southern Comfort

b = beer, c = cider/perry, s = spirits and liqueurs, w = wine (including fortified wine).
[#] After Pernod Ricard's acquisition of Vin & Sprit from the Swedish government in 2009, that company is now (2010) estimated to be the biggest.

pollution issues. These companies are also facing rising costs of basic materials, such as aluminium (for cans), barley and hops. Furthermore, some of the products of these large companies are not viewed altogether favorably by minor, but significant (and growing) consumer groups in a number of countries. Although the companies all have wide portfolios of products, the beers in particular are predominantly of a uniform type – pale lager. At least, this is the beer type that is promoted most heavily, through advertizing.

1.3.3 Small is Beautiful: The Growth in Microbreweries and Craft Breweries and the Localization of Products

Numerous consumer groups and commercial associations have appeared over the past few decades in many countries, notably Britain and the USA. Their aims vary, but are generally to protect and promote small-scale operations, traditional minority products and traditional methods. The activities of some of these groups and associations have fostered the growth in numbers of small-scale producers, the revival of dying minority products and a great increase in consumer choice, through a greater diversity of products. In the UK, the Society for Preservation of Beer from the Wood (SPBW) and the Campaign for Real Ale (CAMRA) have been fighting against the monopolizing and rationalizing tendencies of big breweries and cider companies since 1963 and 1972, respectively. CAMRA, in particular, has been successful in saving cask-conditioned ales and ciders from possible extinction. It has revived the fortunes of the once popular mild ale and its successes have inspired the setting up of many microbreweries and brewpubs in the UK and other countries, notably

the USA, from the late 1970s to the present day. Additionally, CAMRA has inspired the establishment of similar beer consumer groups in Europe, such as Objective Bierproevers (Belgium) and PINT (Netherlands). Indeed, CAMRA, along with the two aforementioned groups set up the European Beer Consumers Union (http://www.ebcu.org/) in 1990, which now has member groups in most European countries, with CAMRA having over 95 000 members, Danske Ølentusiaster (Denmark) over 10 000 and Zythos (Belgium) over 1000 members.

The interests of small-scale brewers in Britain are catered for by the Society of Independent Brewers (SIBA), and in America by the Brewers Association. SIBA, which set up in 1980 as the Small Independent Brewers Association, aims to provide high quality products and to protect and promote traditional styles, whilst encouraging creativity, invention and innovation. It acts as a general focus point (http://www.siba.co.uk) and helps its members promote, sell and distribute their beers via a 'direct delivery system.' Likewise in Belgium, the more specialized Hoge Raad voor Ambachtelijke Lambrikbieren promotes and protects lambic beers: many famous lambic brewers (such as Lindemans and Timmermans) are members of this council. In the USA, the Brewers Association (http://www.beertown.org/), formed from the Association of Brewers and the Brewers Association of America promotes and protects American craft beer, American craft brewers and the community of brewing enthusiasts.

1.3.4 Revival of Traditional Beer Styles

The late nineteenth and early twentieth centuries witnessed the growth of large regional and, eventually, national brewing companies and the disappearance of many local breweries in many countries (for an example, see Table 1.2.1 in Section 1.2.4). This was accompanied by the rapid domination of the brewing industry by pale, bottom-fermented beers and the decline or loss of traditional styles (both top- and bottom-fermented). An account of various styles of beer can be found in Section 2.6.13. Since the late 1970s, largely as a result of the activities and influence of consumer groups such as CAMRA, there have been revivals in both the numbers of small-scale brewers and of the brewing of traditional styles of beer, particularly top-fermented cask- and bottle-conditioned beers. Table 1.3.3 illustrates the growth in the number of breweries in many countries since the late 1970s. Previous to that, all countries witnessed dramatic declines. The growth trend is more or less worldwide, but is more pronounced in the USA and Britain, being most rapid in the 1980s and 1990s: it has leveled off in some countries, but growth continues in others, like the USA (Tables 1.3.4 and 1.3.5). A delayed growth rate in brewery numbers is apparent for some countries, notably Denmark (Table 1.3.3) and the other Scandinavian countries not listed in Table 1.3.3. Judging by the general level of interest in the revival of traditional beer styles (and cider), as indicated in the web pages of the Scandinavian members of

Table 1.3.3 *Numbers of breweries in various countries from 1956 to 2006*

Country	1956	1976	1986	1996	2006
Belgium	563			115	124 (2004)
Czech Republic	330*	80	71 (1989)	83	96 (2005)
Denmark	155	24 (1977)		15	55
Germany	2928	1626		1234	1284
UK	404	142	281	499	633
USA	305	85 (1977)			1437

Data from http://www.europeanbeerguide.net (2008), Jackson (1977) and the Brewers Association (2008).
*Data for Czechoslovakia.

Table 1.3.4 *Beer production in the USA, 2003–2007 (hl)*

Beer by brewery type	2004	2005	2007
Brewpub	740 395	762 399	844 652
Microbrewery	829 118	896 055	1 134 891
Regional craft	4 118 989	4 511 667	5 906 398
Contract*	991 258	1 143 053	1 513 840
Non-craft (industrial)	205 031 200	201 882 826	–

Data from the Brewers Association (2008).
*Beer brewed under contact for microbreweries and regional craft breweries.

the European Beer Consumers Union, the future promises some further growth of choice in these countries. Of course, a greater number of small breweries does not necessarily mean higher quality, but it does mean greater choice. With the level of education, assistance and expertise offered by both commercial/professional societies like SIBA, the Brewers Association and the Master Brewers Association of America, and consumer groups such as EBCU, it is likely that quality will be generally high.

Naturally, most of the breweries that have sprung up since the late 1970s were originally either brewpubs or microbreweries. Some have gone and some have expanded into altogether bigger concerns. Based on 2007 sales, two (originally) craft brewers (Boston Beer Co. and Sierra Nevada Brewing Co. are number 5 and number 7 (respectively) in the top 50 breweries of the USA. The Brewers Association in the USA defines craft brewers as those producing less than 2 million barrels per year, where at least 50% of the production is all malt beer and where less than 25% of the brewery is owned by an industry member who is not a craft brewer. A microbrewery is one that produces less than 17 600 hl of per annum, with 75% of sales being off site, whereas a brewpub is defined as brewing beer primarily for sale at a bar or restaurant: at least 25% of its beer must be sold on site.

A considerable growth in craft beer output in both Britain and America has been in cask-conditioned ales. In the UK, in 2008 (first half of year) although the volume of pub trade was down 8% on 2007 figures, sale of cask-conditioned beers of SIBA members was up 8% during the same period (http://www.siba.co.uk). Similarly in the USA, growth in craft beer production was up 12% during 2007 (Tables 1.3.4 and 1.3.5), whilst the growth rate for imported beers and domestic noncraft beer production was just 1.4%, and it is evident that cask-conditioned beers are gradually acquiring a wider audience (Garbee, 2008). However, to bring some perspective into the picture, the overall US beer market in 2007 was 211 489 982 (US) barrels. So, although craft beer production in the USA is only 4% or so of total production (Brewers Association, 2008), growth is steady (Tables 1.3.4 and 1.3.5), and it is worth remembering that just three decades ago craft beer production in the USA was almost nil. Bottle-conditioned beers (especially ales) have also been

Table 1.3.5 *Numbers of breweries according to type in the USA, 2005–2007*

Brewery type	2005	2006	2007
Large noncraft	18	20	20
Regional noncraft	23	23	23
Regional craft	48	49	53
Micro	380	359	392
Brewpub	979	977	975

Data from the Brewers Association (2008).

on the increase in many countries over the past decade – to such an extent that all the big UK supermarkets (e.g. Asda, Budgen, Co-op, Marks and Spencer, Sainsbury, Tesco, Waitrose and others) and smaller chains now (2009) normally stock several such beers. The past two or three decades have witnessed the rejuvenation of some declining and even extinct beer styles such as Dampfbier, mild ale, oatmeal stout and porter. New styles or extensions of already established styles have also appeared, especially in the area of flavored beers (Section 2.6.13). In America, the last few years have witnessed the large brewing corporations turning their hands to producing (relatively) small quantities of specialty beers with real character, such as Molson-Coors Blue Moon brands and Michelob chocolate beers (Section 2.6.13).

1.3.5 Changes in Beer Drinking Habits

Over the past two decades, there have been gradual changes in the beer drinking habits of consumers in certain European countries (Table 1.3.6). In particular, there has been a general shift away from drinking in bars, pubs or restaurants to drinking at home, in Belgium and Britain. Likewise, in the UK, drinkers have been gradually shifting away from draught beers to canned beers, indeed gradual shifts toward canned beers can be seen for all the countries listed in Table 1.3.6. This trend can be correlated with the trend towards greater beer consumption at home, but it should be noted that canned beers are available in some bars of certain countries. Denmark is remarkable for having virtually all its beer being sold in draught or returnable bottle form. From an ecological and environmental viewpoint, this is highly commendable, since these two forms of beer packaging use less energy and cause least pollution. Most nonreturnable bottles and cans are probably recycled, but these processes require considerable energy input, with the possibility of pollution. It can be seen from Table 1.3.6 that the beer drinkers of Denmark, Germany and Ireland are the most conservative. In Germany, since nonreturnable bottled beers are more likely to be bought in supermarkets and beers in

Table 1.3.6 *Changes in beer package type by country (Europe, 1998–2001) and beer consumed at home (1998–2001, with 2004 figure in square parentheses) (d = draught, rb = returnable bottle, nb = non-returnable bottle, c = can)*

	1998 (% home consumption)				1999 (% home consumption)				2000 (% home consumption)				2001 (% home consumption)			
Country	d	rb	nb	c	d	rb	nb	c	d	rb	nb	c	d	rb	nb	c
Belgium	39.7	49.6	7.5	8.2		–				–			39.0	46.0	2	13
		(39)				(39)				(41)				(42) [45]		
Denmark	9.9	90.1	0	0									11.0	89.0	0	0
		(74)				(73)				(75)				(75)		
Germany	19.9	59.9	4.7	15.4		–				–			19.5	55.5	5.1	19.9
		(65)				(65)				(65)				(65)		
Ireland	79.8	3.1	4.4	12.7		–				–			76.9	4.7	3	15.4
		(11)				(11)				(12)				(12)		
Sweden	12.5	26.7	0.5	60.3		–				–			12.5	22.6	1	63.9
		(79)				(79)				(79)				(79)		
UK	63.9	1.9	10.6	23.6		–				–			60.4	1.2	11.6	26.8
		(29)				(32)				(33)				(35)		

Data from http://www.europeanbeerguide.net (2008).

returnable bottles are more likely to be bought from breweries or in bars, it can be concluded that the average German beer drinker prefers bottled beers at home, but bottled beers are also popular in bars. The Irish are easily the top draught beer drinkers of Europe, which means that they are also the most enthusiastic pub goers. Britain and Belgium are not far behind, but shifts away from draught beer consumption in bars are more pronounced in these two countries.

1.3.6 Global Growth in Wine Drinking

As far as beer drinking with respect to the consumption of other alcoholic beverages is concerned, there is some evidence that the beer market in many countries has been unsteady or in decline for a number of years. As long ago as 2005, the large US brewing companies of Anheuser-Busch and Molson-Coors reported a 2–3% loss in net sales (Gross, 2005), which was blamed on younger consumers turning to wine and spirits. It was also suggested that the reason for the switch was the growing gap between East/West Coast professionals, who are more inclined to buy wine and spirits, and interior working people who are cutting back on six-packs of Bud or Miller. Another reason contributing to the diverging fortunes of beer on the one hand and wine and spirits on the other, is the general perception of wine (in particular) as a healthier drink than beer – this is discussed in Section 1.3.10. The USA is a major wine producer (28 750 000 hl in 2005 – see Chapter 1.1, Figure 1.1.4) and is also a keen wine importer. Growth in the number of Californian and total number of US bonded wineries since 1970 is indicative of the rapid growth of the US wine market (Table 1.3.7). California still provides the lion's share of production, but there are now large industries in New York State and other eastern and central states, as well Oregon and Washington States. In 50 years (1957–2007), wine consumption in America has risen from 0.89 to 2.47 gallons per head of population per year (Table 1.3.8 and Figure 1.3.1; 1 US gallon $\sim$ 3.8 l).

World wine consumption is also increasing (Figure 1.3.2): between 2001 and 2005 there was a 4.15% increase in consumption and it is estimated that between 2005 and 2010 there will be a further increase of 4.8%, giving a total of 238 825 million hl (30 384 billion bottles) of wine consumption (Wine News, 2007). Table 1.3.8 shows trends in per capita wine consumption for selected countries between 2001 and 2005. It can be seen that consumption is more or less level or slightly in decline in some major wine producing countries, notably Argentina, France, Italy, South Africa and Spain. On the other hand, there are marked upward trends in other wine producing countries, such as Australia, Chile, China, New Zealand, Portugal, Russia and the USA. It is not certain whether political events have any influence on the data in Table 1.3.8 (e.g. Russia/Moldova and Czech Republic/Slovakia).

There is evidence that traditional wine drinking countries (mostly also wine producers) outside the European Union are driving the growth in global wine drinking (Wine Business Insider, 2007). Between 1986 and 1990, the five leading exporters of the EU (France, Germany, Italy, Portugal and Spain) held an average of 78.8% of the world wine market, whilst the major producers of the southern hemisphere (Argentina, Australia, Chile,

Table 1.3.7 *Rise in the number of bonded wineries in California and the United States from 1970 to 2007*

Year	1970	1980	1990	2000	2007
Number of bonded Californian wineries	240	508	807	1450	2687
Number of bonded wineries in USA	441	920	1610	2904	5958

Data from the Wine Institute, 2008.

Table 1.3.8 *World per capita wine consumption 2001–2005 (liters per head of population per annum)*

	2001	2002	2003	2004	2005	% Change between 2001 and 2005
France	55.72	57.20	54.77	55.85	55.85	+0.24
Italy	51.86	47.66	50.48	48.16	48.16	–7.13
Portugal	44.29	43.84	49.88	46.67	46.67	+5.39
Spain	35.24	34.56	34.16	34.66	34.66	–1.67
Australia	19.62	19.77	20.71	23.93	24.67	+25.75
UK	16.16	16.36	17.53	18.97	18.97	+17.42
Argentina	30.15	30.03	29.09	28.81	28.81	–3.02
Chile	13.95	14.24	15.82	15.50	15.50	+11.11
New Zealand	14.87	16.09	16.19	16.44	16.68	+12.21
Russia	4.25	4.48	6.08	5.95	5.95	+40.09
Moldova	9.60	4.65	4.61	4.70	4.70	–51.05
Czech Republic	8.76	10.62	11.47	12.60	12.70	+44.93
Slovakia	11.77	11.18	10.90	11.03	11.03	–6.25
USA	7.90	7.92	8.16	8.41	8.69	+10.01
South Africa	8.99	8.79	7.89	8.37	8.37	–6.85
China	0.84	0.87	0.88	0.88	0.91	+8.56

Data from the Wine Institute, 2008.

New Zealand and South Africa) held only 3.1%. In 2006, these figures were 62.2% and 27.4%, respectively, if the output from the USA is included with that of the southern hemisphere. The countries with the largest populations (China, Russia and the USA) all show markedly increased per capita wine consumption between 2001 and 2005 (Table 1.3.8) and so will make major contributions to the world upward trend. It is estimated that by 2010, China and Russia will be in the world top 10 of both producers and consumers of wine (Wine News, 2007).

Increase in wine consumption is more pronounced for more expensive wines and is projected at 17.12% for wines selling for more than $10 per bottle, compared with 2.44% for wines that sell for under $5 per

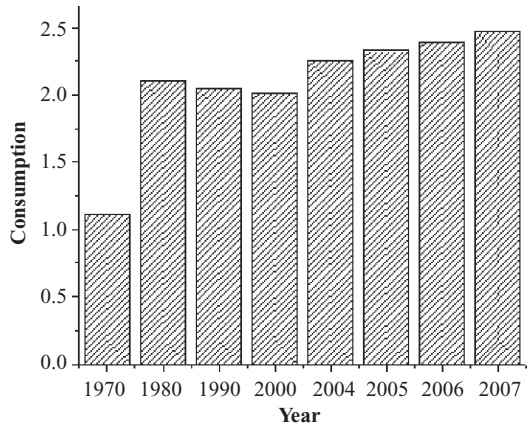

Figure 1.3.1 *Per capita wine consumption in the USA, 1970–2007 (gallons per head per annum). Data from the Wine Institute, 2008.*

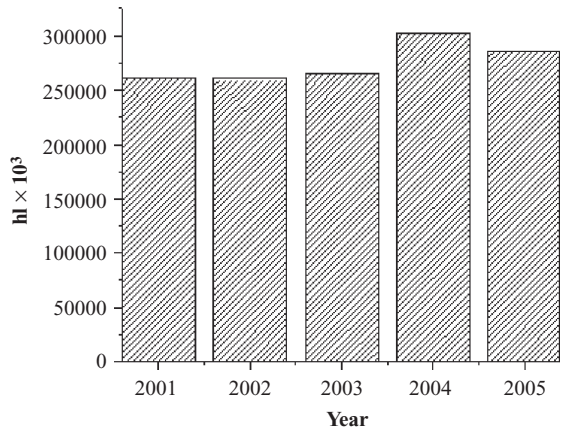

Figure 1.3.2 *World wine production 2001–2005 in hl × 103. Data from the Wine Institute, 2008*

bottle during 2005–2010 (Wine News, 2007). This supports the notion that it is the more affluent members of society who are either turning to wine or are increasing their consumption. Wine prices do not necessarily reflect wine quality and upward trends in wine consumption according to wine type or style may be largely driven by clever marketing and advertizing, perceived fashionability, status indication and perceived health benefits. Red wines show the biggest increase in consumption between 2001 and 2005 (7.48%) and there has been a renewed interest in rosé wines during this period (3.45% increase in consumption) (Wine News, 2007). It should be remembered that two decades ago, rosé wines were unfashionable. Sweet wines and sweet fortified wines are currently unfashionable, for obscure reasons, but possibly because they are considered to be 'high calorie' and 'old people's' drinks' by the young upwardly mobile consumers who spearhead the global growth in wine consumption. Domestic sales of Australia's fortified wines dropped from 27 million l per annum in 1993–1994 to 18.5 million l per annum in 2005–2006, although export trade remained fairly firm (Shiraz, 2007). Home sales of liqueur Muscat and Tokay styles have become so low that a project jointly financed by the government and the Muscat of Rutherglen group has been set up (2007) to revitalize this section of the industry. Many wine savants are of the opinion that these wines and many of the sweet wines of Europe constitute some of the greatest gems of the wine world. Many currently sell at prices that are far below their real value, so developments in this area are awaited with interest.

1.3.7 Development of New Wine Industries

Over the past three decades, wine industries have started (or have significantly increased in size) in a number of countries, many of which must be rated as marginal for the production of wine. For example, there are now small industries in the UK (England and Wales), Belgium, the Netherlands, Denmark and other Baltic/Scandinavian countries. Three of these countries, Belgium, England and Wales, have more or less revived their wine industries following a period of zero commercial production. In the case of England and Wales this was a centuries-long period (interrupted by just two or three localized short commercial eras) brought about by the abundance of cheap wine from Europe. In the case of Belgium, commercial production has been influenced by the proximity of France and by the devastation caused by two World Wars. In the UK, wine production peaked in the early 1990s, but has leveled off in recent years to around 100 wineries and about 700 hectares of vineyards (English Wine Producers, 2008). Average yield varies considerably from

year to year, from over 30 hl/hectare (1992, 1996, 2006) to 10 hl/hectare or less (1988, 1997). Likewise, in recent years total production varied widely from 1 280 000 l in 2005 to 2 526 700 l in 2006.

The small vineyard areas of the Netherlands are in the south of the country near the Belgian border, whilst the Belgian vineyards are mostly in the Ardennes area, not far from Luxembourg vineyards. *Vitis vinifera* crosses and hybrids have been bred in France and Germany (mostly) to perform well in marginal climates. *V. vinifera* crosses include Bacchus, Dornfelder, Faber, Huxelrebe, Kerner (and Kernling), Madeleine Angevine 7672, Müller Thurgau, Ortega, Reichensteiner and Schönburger, and hybrids include Orion, Phoenix, Regent, Rondo and Seyval Blanc. Classic *V. vinifera* varieties such as Chardonnay, Gamay Noir, Pinot Blanc, Pinot Gris, Pinot Meunier and Pinot Noir are also grown. Although contribution to world growth in wine production by these countries is and will remain very small, they do add to the overall variety, and sparkling wines made in England from Chardonnay, Pinot Meunier and Pinot Noir by the méthode champenoise have won many international awards. Most of the wines from these countries are dry or medium white, but rosé wines are also made and the proportion of red wines in UK wine production has risen from 7.1% in 1990 to 25.2% in 2006 and 28.3% in 2007 (English Wine Producers, 2008). It is more difficult to produce good red wines in marginal growing conditions, so increase in red wine production and general quality may be attributed to both increased expertise and generally warmer summers. Many established UK winemakers have reported longer growing seasons, indicated by earlier blossoming and fruit maturation, in recent years, compared with, say, the 1960s.

Wine industries are also growing in warmer climatic areas, which have been only recently globally recognized as winegrowing areas. An example is given by British Columbia, whose wine industry has grown rapidly since 1990, as shown in Table 1.3.9. The vineyards of BC range from the cool coastal areas around Vancouver, where typical cool-climate, mild-winter varieties like Huxelrebe, Müller Thurgau and Seyval Blanc are grown, to the vineyards of the much warmer inland area of the Okanagan Valley. It is here that most of the expansion described in Table 1.3.9 has occurred: French-American hybrids are grown for winter hardiness and classic *V. vinifera* varieties such as Chardonnay, Merlot, Pinot Noir and Riesling will generally ripen well in these vineyards.

India has a small but rapidly growing wine industry. Here, there is no worry about inclement summer weather, or hard winters, provided the vines are pruned in September and cropped in February or March. This southern hemisphere regime is required because of the searing heat and heavy rains in the middle months of the year. Wines are made from *V. vinifera* varieties, such as Cabernet Sauvignon, Chenin Blanc, Sauvignon Blanc and Syrah, but in some vineyards' harvest must be carried out in the relative cool of night, using refrigerated containers to convey the crop to the winery. Wineries such as Chateau d'Ori, Sula Vineyards (at Nasik, near Mumbai) and Chateau Indage (near Pune) can look forward to supplying the blossoming

Table 1.3.9 *Growth of the wine industry in British Columbia*

Year	Area under production (hectares)	Number of wineries	Wine production (l)
1990	597	17	–
1994	870	–	–
1999	1700	–	6 574 200
2004	2210	102	9 985 200
2006	2684	131	13 802 563
2008	3683	143	–

Data from BC Wine Institute (http://www.winebc.com/).

upwardly mobile Indian middle classes (with an estimated potential of 300 million!) over the next 10 years (Fabricant, 2007).

1.3.8 Revival of Cider and Perry: the Magic of Ice

The 1950s and 1960s saw renewed interest in cider and perry, as larger companies such as H.P. Bulmer, Gaymers, Merrydown, Showerings and others expanded, making their streamlined, industrialized and consistent products widely available (Section 2.8.8). Although many smaller cider firms were swallowed up by the larger companies, others survived and continue to produce distinctive and often traditional cider and perry. Brittany, Normandy and northern Spain did not experience such a marked expansion of industrial cider as the UK, and today most cider and perry from these areas is traditional.

In the UK, after a period of slower growth or equilibrium, revitalization has occurred in recent years in two quite different directions. Firstly, demand for craft (mostly traditional) cider and perry has increased, and this has been accompanied by a rise in the number of small-scale producers. No doubt this success is partly influenced by the successes of the big cider companies (see next paragraph), but greater public interest in health issues, natural or organic products and speciality or distinctive products must also be included as contributing to this success. Likewise, the support of the European Union and the establishment of cider production sites as major tourist attractions (like vineyards and wineries) have both helped boost sales. The EU has promoted cider and perry in an attempt to revitalize rural economies by providing profitable markets for apples and pears. New craft cider and perry producers have grown up alongside well-established companies in all of the specially designated cider and perry areas of the EU.

In the other direction, large-scale factory cider has received an enormous boost in the UK through advertizing that presents the drink to young consumers as chic and fashionable. In particular Magners, (C&C, Ireland), Bulmer's Original, Frosty Jack's and others have seen large increases in sales over just two or three years, largely because of their association (through advertizing) with an otherwise humble commodity – ice. The 'over ice' phenomenon, no doubt boosted by the warm summers of 2003–2007, has probably done more than anything else toward the 26% increase in 2007 UK cider sales over 2006 figures. Between March 2006 and March 2007, cider sales in the UK reached 1 003 050 401 pints (569 732 628 l): the first time that cider sales have exceeded 1 billion pints (Anon, 2007).

In America, the cider/perry (pear cider) market is dominated by factory products, such as those of E. & J. Gallo, HardCore, Woodchuck and Wyder's (Canada). In 2005, US cider production was estimated at 6 000 000 US gallons (~22 800 000 l) (Merwin, 2005): small compared with UK production and considering the size of the US population. Although annual cider production has increased substantially since 2000, growth in craft cider and perry production has not yet equaled growth in craft beer manufacture (Merwin, 2005) (Section 1.3.3). This may be because the American public is less familiar with cider (hard cider in the USA – cider is carbonated apple juice) and US bankers are reluctant to lend money for the expansion of an unfamiliar market. It appears as though craft cider and perry makers, like Fox Barrel (Colfax, California), are having trouble securing loans to finance expansion to cope with a threefold increase in demand over two years (Anderson, 2007). Indication that the production of craft (or 'high end') cider is gaining interest is shown by the establishment of many new companies, mainly in winegrowing areas, such as California, the Finger Lakes (New York), Oregon and Virginia (McNeill, 2008).

1.3.9 The Rise of Flavored Alcoholic Beverages ('Alcopops')

Alcopop is the common name for a flavored alcoholic beverage (FAB) that may be a pre-packed spirit (PPS), a wine cooler type drink or a flavored malt beverage (FMB). Another collective term for these drinks is ready

to drink (RTD), used in Australia and New Zealand. Essentially, FABs are distilled spirits, wine or malt beverages to which fruit juice or other flavorings have been added. They are often available in 330 ml or 355 ml bottles, with ethanol contents of between ~4% and ~18% (v:v). Although prepacked versions of sangria (red wine and orange juice), various wine coolers, gin and tonic and others have been around for some time, the current popularity of FABs began in the late 1980s and early 1990s with the introduction of Bacardi Breezer (rum-based), Two Dogs, Hooper's Hooch, Smirnoff Ice (vodka-based), Mike's Hard Lemonade, Zima (malt beverage-based) and other brands. Slick advertizing, aimed at younger consumers led to rapid success in the 1990s. Indeed, the falling sales of cider and perry in the UK during the mid to late 1990s (after a period of growth – see Section 1.3.8) have been blamed on the rise of FABs (Pratley, 1996).

The very success of FABs, along with the character of the advertizing led to criticisms and complaints in several countries that, these drinks were being consumed in significant quantities by underage drinkers (under 18 in Europe; under 21 in the USA). The popular term alcopops was coined by critics in order to link alcoholic and soft drinks ('pop') and to imply that FABs were aimed explicitly at underage consumers. Numerous complaints from newspaper columnists and various 'watchdog' groups have been issued since the 1990s, relating to advertizing and underage drinking. In the USA, the Federal Trade Commission (FDA), acting on complaints from the Center for Science in the Public Interest (CSPI) in 2001 and on a directive from the House and Senate Appropriation Committee in 2003, investigated the possibility that FABs were aimed specifically at underage drinkers (Evans *et al.*, 2003). In both cases, the FDA found no evidence for such assertions and concluded that the majority of FAB drinkers were over the age of 27.

In Europe, the most popular FABs are those of the premixed spirits type, but wine cooler type drinks and beer cocktails are also popular in some countries. In Canada, PPS type beverages tend to dominate FAB sales, whereas in the United States FMBs are more in evidence. Some of the spirits-based beverages have high alcohol contents, which has led to several countries imposing higher excise taxes on FABs, bringing the level of taxation up to that of spirits. This has happened in Ireland (2003), Germany (2004) and more recently in Australia (2008) (Herald Sun, 2008). Additionally labeling and other legislation has been imposed in several countries and certain states of the USA in order to remove any ambiguity regarding alcohol content. In EU countries, FAB bottles carry the warning that they are not meant for consumption by people under the legal drinking age. The state of California has recently (2008) passed legislation that compels manufacturers of FABs for sale in California to include the label warning 'Attention: this drink contains alcohol' (PRNewswire, 2008). Earlier the same year, the state of Illinois passed a similar law, whilst also restricting advertizing and other promotions away from the attention of children (Illinois Compiled Statutes, 2008). Labeling laws for FABs need not be regarded as draconian, since similar laws are already in force, or will soon be in force, for other alcoholic beverages in many countries. Despite other restrictions, many people (like the Australian Opposition leader Brendan Nelson) feel that consumption of FABs (RTDs in Australia) will increase despite extra taxation, but some people may turn to full-strength spirits (Herald Sun, 2008). The FDC report (Evans *et al.*, 2003) also mentioned that industry-based research suggested that most FMB drinkers regarded their drinks as substitutes for beer and were unlikely to consume more than two or three drinks in a session, thus indicating that this type of FAB at least is not associated with binge drinking (Section 1.3.11).

1.3.10 Calorie-Counting and Health Perception of Alcoholic Drinks

The past two decades have witnessed a surge in the general public's interest in health issues, particularly with regard to balanced, nutritional diets and exercise. This interest has naturally extended to alcoholic drinks, where health-conscious people, influenced by advertizements promoting healthy lifestyles and by dietary trends, generally perceive normal alcoholic beverages as being undesirable in a healthy, balanced diet. In general, alcoholic drinks, especially beer, have not been promoted as being healthy by modern diet

trendsetters, such as the South Beach diet (Agatston, 2003). The exception to this is red wine, which is frequently promoted for its nutritional and beneficial health values. On the other hand, with regard to health, beer is often regarded by people who are not primarily beer drinkers as the worst alcoholic beverage. In particular, it is considered to be fattening by people who like alcoholic drinks, but who are seeking to control their daily calorie intake ('calorie counters'). This perception is considered by some brewers in Japan to be a significant contributor to the decline in domestic beer sales over the past few years, with people either abstaining or turning to shochu, the traditional distilled spirit (Anon, 2008). It will be demonstrated in Section 5.7.1 that beer and red wine are nutritionally similar and the major health benefit of low alcohol intake (reduced risk of cardiovascular disease for certain individuals) is more or less the same for beer, spirits and wine (Section 5.6.3).

The first reaction of brewers to win over health-conscious potential beer drinkers was the launching, in the 1990s onwards, of many brands of low carbohydrate beers, variously known as 'low carb', 'light' or 'lite' beers (Sections 5.7.1 and 5.7.2), although some well-established low carbohydrate beers for diabetics already existed. The second reaction of brewers occurred this century with the launching of web-based educational campaigns by individual brewery companies (e.g. Coors Brewing Ltd, 2002; Anheuser-Busch Inc., 2006) and by other commercial organizations (e.g. the British Beer and Pub Association, 2005). The aim here was to focus on beer's natural, nutritional and health-giving values (assuming low to moderate intake) by providing a general educational background. Apart from these, many of the normal brewery websites also mention the naturalness or health-giving properties of beer.

Labeling legislation in many countries now requires the inclusion of nutritional information (per serving) somewhere on the product container. For example, in the USA, the regulations set by the Alcohol and Tobacco Tax and Trade Bureau (TBB) (2004) require average analysis values to include the number of calories, along with the number of grams of carbohydrate, protein and fat per serving size. Other information (on the main or on a subsidiary label) will include % alcohol (v:v) and in some countries, information regarding contribution (per serving) to daily requirements and information on specific components, such as additives, cholesterol or sodium content, also may be required. Legislation of this kind is really bringing alcoholic beverage labeling in line with general packaged foodstuff labeling, legislation for which has existed in many countries for a number of years.

How nutritional label information regarding alcoholic beverages influences the attitude and behavior of consumers is not well understood and there are conflicting conclusions in the literature (Wright *et al.*, 2008). In a recent survey of consumers' perceptions of alcoholic and nonalcoholic beverages, it was found that red wine was perceived to be the overall most healthful drink (Wright *et al.*, 2008). The consumers were visitors to a craft brewery and two wineries in northern California, who answered a verbal questionnaire on their perception of the health value of drinks before and after being given nutritional information on the beverages. The results showed that people at the wineries (mostly wine drinkers) and those at the brewery (mostly beer drinkers) viewed beverages differently and their perception altered after seeing the information. It was concluded that perceived health quality of a beverage is, in general, not the major driving force for choosing that beverage: the brewery visitors thought red wine the most healthy drink. Also, beer drinkers were more heavily influenced by the nutritional information, suggesting that inclusion of such information on product labels will increase beer drinkers' perceptions of the health quality of their beverage of choice.

1.3.11 Binge Drinking

Binge drinking can be defined in different ways, but it generally refers to the consumption of five or more standard drinks in a relatively short space of time (e.g. part of an evening), so that blood alcohol concentration reaches at least 0.08%. In Britain, binge drinking is sometimes defined as the consumption of more than 50%

of the maximum weekly number of alcohol units in one session (i.e. on one night out or at one party). Of course binge drinking, even amongst young people, is not a new phenomenon. However, the perceived scale and severity of binge drinking amongst young adults and underage drinkers is a more modern phenomenon, as is the strong drinking culture that accompanies it. Nowadays binge drinking often involves medium to large groups of youths drinking 'shots' while playing drinking games such as 'scrumpy hands,' 'Edward wineyhands' or 'Edward fortyhands.' Antisocial and even violent behavior is a frequent result of binge drinking, as are drink-induced accidents or illnesses, such as those caused by ruptured bladders (Atkins, 2007). The latter place strains upon the hospital accident and emergency services, especially on Friday and Saturday evenings, the main binge drinking times (BBC, 2003). There is little doubt that binge drinking is more damaging to the health than drinking the same number of alcohol units over a much longer period of time (Section 5.6.2).

Binge drinking takes various forms and varies in frequency and severity from country to country, but appears to be most prevalent in northern Europe, Australia, New Zealand and the United States. All kinds of alcoholic beverages can be used in binge drinking, but beer, cider, cocktails, FABs and spirits appear to prevail over wine and related drinks. The origins of binge drinking cultures in different countries with quite different sociological and political backgrounds are unknown, although many try to relate it to the legal age of drinking, the availability of alcohol or the degree of severity of drinking laws. It seems likely, however, that the desire to socialize and the potency of peer pressure (being seen to be fashionable or 'part of the crowd') play major roles. Remedies suggested by various governmental, health, drinks industry, educational and other organizations range from drastic curtailing of the availability of alcoholic beverages (e.g. by increasing the legal drinking age, increasing excise tax or deceasing the number of outlets) through further liberalization of drinking laws (e.g. decreasing the drinking age limit and making alcoholic drinks more freely available) to stricter enforcement of existing drinking laws, particularly with regard to antisocial or violent behavior. Naturally, these suggestions have led to controversy and even greater media coverage. For example, in the USA, the suggestion made by a group of college administrators that lowering the legal drinking age from 21 to 18 may help curtail the growth of binge drinking by university and college students (the main binge drinkers in America, it appears) has caused predictable reactions from the media (Editorial, New York Times, 2008). The growth of binge drinking this century has triggered specific governmental and other campaigns in a number of countries: in March 2008, the Australian government provided 53 million Australian dollars for an advertizing and educational campaign against binge drinking (Cooper, 2008) and the EU wine producers have launched a campaign designed to promote the cultural dimension of wine and to contribute to the reduction of alcohol abuse (Euractiv, 2008; CEVI (Confédération Europeénne des Vignerons Indépendants), 2008). Opposing remedial regimes suggested for application to the most modern form of alcohol abuse are nothing new: in the past there have always been organizations that argue for restriction (or even prohibition) and those that advocate liberalization to solve the same problem. Some argue that liberalization doesn't help, for example extension of UK bar opening times to 24 hours has not been seen as a cure for binge drinking (Johnston, 2006). However, history has shown that it is simply impossible to take care of every potential alcohol abuser and restriction or prohibition can lead to numerous other problems (as in prohibition America, 1919–1933). There is no historical precedent to modern binge drinking, as it involves most forms of alcoholic drinks, but it should be remembered that the British 2nd Gin Act of 1736, in which licence and excise duties were imposed on gin distillers and penalties were imposed for sale without licence, had the effect of doubling gin consumption in England. Ultimately, the 1751 Gin Act, preventing distillers selling by retail or to unlicensed owners of inns and public houses saw an end to the 'gin epidemic.' The act also raised the duty on spirits and prevented recovery of drinking debts through the courts. Additionally, the duty of £1 on a gallon of beer had been dropped in 1743 (Young, 1979). On this basis, raising taxes or decreasing the availability of all types of alcoholic beverages is unlikely to curb modern binge drinking.

1.3.12 Organic and Biodynamic Production of Alcoholic Beverages

The term 'organic' applied to foodstuffs and alcoholic beverages generally refers to the production of crops within a balanced farm ecosystem, without the use of artificial fertilizers or pesticides. Certain agricultural practices, such as the use of cover crops, green manuring, crop rotation and crop diversity (growing several crops together) are also features of organic farming. The last-mentioned is designed to provide a better ecosystem for the farm, so that pests and diseases are minimized. For example, it is not uncommon for viticulturalists in southern Europe to grow other fruit trees (e.g cherry trees) or walnut trees amongst the vines. Additionally, the processes that convert the crop into an alcoholic beverage should be as natural as possible and involve no or minimal additives. In practice, certain pesticides (e.g. sulfur, Bordeaux mixture and preparations made from natural sources) are allowed and sulfur dioxide is allowed in winemaking up to certain low levels (depending on the prevailing legislation – see Section 5.9.5). Organic beers are produced from organically grown barley (and other cereals) and hops, but these beers may well have been filtered and pasteurized. The same applies to organic cider, perry and wine.

Various nongovernmental organizations exist to oversee and help the production of organic crops, to promote organic products, to expand the organic marketplace, to liaise with governmental bodies, such as USDA (USA), DEFRA (UK) and the EU (most of Europe) and to issue certificates of guarantee for organic products. These organizations include the Soil Association (UK), California Certified Organic Farmers (CCOF) (USA), Stellar Certification Services (USA), Verein Deutscher Prädikatsweingüter (Germany) and the Soil and Health Association (New Zealand). The biodynamic organizations Demeter and Biodivin are mentioned later.

In parallel with the rise in health awareness, growth of organic beer, cider and wine production and consumption has been rapid during the past two decades: the California crop reports for 2005 showed the area of certified organic vineyards constituted 18% of total bearing vineyard area in Mendocino County, accounting for 1142 ha out of a total 6512 ha. Likewise, there are now (2008) over 2000 ha of organic vineyards in Germany. Less than 20 years ago, both these figures would have been much closer to zero. The CCOF reports that the vineyard area certified by itself has increased from 7761 acres in 2004 to 9240 acres in 2007. For a number of notable, but relatively small-scale winemakers, such as E. Dürrbach at Domaine de Trévallon (France), Frey (USA), Guerrieri-Rizzardi (Italy), Heller (USA) and J. Palacios (Spain), their output is entirely organic, but much larger producers such as Fetzer (California) and Penfold's (Australia) also make solely organic and biodynamic wines or make an organic series of wines alongside their conventional wines. Some breweries, such as Caledonian Brewery, Samuel Smith (both UK), Anheuser-Busch InBev (USA), Pinkus Müller and Riedenburger (Germany) brew organic beers along with conventional beers, whilst there are some purely organic breweries (e.g. Peak, Otter Creek Brewing and Santa Cruz Brewery – all from the USA). Organic beer sales are increasing, the 2005 sales being 40% up on 2004 sales (Associated Press, 2006).

Organic beverages tend to be more expensive than their conventional counterparts and although it is probably true that some of the produce is market orientated, it appears that many producers use organic techniques because they truly believe in them. Indeed, the organic (or biodynamic) aspect of some wines (e.g. those of Guerrieri-Rizzardi, Jasper Hill and Descendientes de Palacios) takes a low profile. Because of the meticulous care generally given to organically produced alcoholic beverages, their quality is usually high, but whether they are better than their conventional equivalents depends very much on the taster. What can be said is the presence of organic beverages greatly enriches the choice for the consumer.

From its genesis in the form of a series of lectures in 1924 on agriculture by the vitalist Austrian philosopher Rudolf Steiner, biodynamic (BD) viticulture has been making big ethereal waves in the world of wine over the last 10 years. BD wines are often mentioned in the same breath as organic wines, and indeed, under the auspices of the Demeter and Biodivin certification bodies, organic vineyards can convert to BD by adopting the special soil and plant preparations with reference to astrology, as originally advocated by Steiner. This

involves making mixtures of animal, mineral and vegetable origin for soil improvement and highly diluted plant preparations for crop spraying to improve plant health. It also involves keeping animals for biodiversity and ecological balance. These, and the various winery operations (such as racking and bottling) are carried out with strict reference to cycles of the moon and relative positions of constellations of the zodiac (Zacharkiw, 2008). The whole purpose of these procedures is to balance lunar and zodiacal influences in favor of the vineyard, its workers and its products: in this way, the wine expresses its 'terroir' to maximum degree.

Biodynamic viticulture has become popular and much more high profile over the past few years. It now includes some the world's finest wine producers as adherents, including Castagna (Australia), Domaine Chapoutier (France), Coulée de Serrant (France), Descendientes de J. Palacios (Spain), Grgich Hills (USA), Millton Vineyard (New Zealand), Domaine Leroy (France) and Domaine Zind Humbrecht (France) (Crosariol, 2008). Critics often argue that improvement in vineyard health and wine quality may have occurred anyway if standard organic farming had been used, without the mysticism and considerable extra effort of biodynamics (Smith and Barquín, 2007; Chalker-Scott, 2004; Treue, 2002). Other critics say that the success of BD viticulture owes more to the consummate skill of the winemakers: they would make superlative wine whether or not BD practices were used.

Evidence for the efficacy of BD farming is mixed: in Switzerland, a 21-year project found that soil fertility and biodiversity were improved by BD methods over organic or conventional culture (Mäder *et al.*, 2002). On the other hand, the only detailed vineyard study (conducted between 1996 and 2003) concluded that BD farming gave improved grape sugars and tannins in one year, but otherwise gave no further improvement on those obtained using standard organic practices (Reeve *et al.*, 2005). Wines of unquestionably high standard are produced using the biodynamic method, although for the onlooker the controversy regarding exactly how much the method itself contributes to high quality remains unsolved.

1.3.13 Use of Genetically Modified (GM) Crops and Microorganisms

Genetic modification of any kind remains a highly controversial issue and the first introductions of GM foods has met with a good deal of consumer resistance in many countries. However, GM foods are now approved in many countries, including those of the European Union (since 2004), subject to fulfillment of labeling requirements. Genetic modification is carried out on crop plants in order to give them an additional genetic characteristic (trait) that is beneficial to producer and consumer alike. Otherwise the genetic characteristics of the original and modified plant remain identical. Alien genes, often from bacteria or fungi, are incorporated into the plant genome, often by microparticle bombardment of plant cells or tissue. For example, an additional gene may be added to confer disease resistance, insecticidal properties, resistance to herbicides or improved food value. Before being released for general agricultural use, GM plants undergo extensive laboratory and field trials that look for unfavorable influences on the environment (affects on beneficial soil bacteria, fungi, beneficial insects, etc.), as well as examining the possibility of cross-pollinated propagation.

The first beer to be brewed using GM produce as part of the grain bill was Kenth Beer of the Oesterienbryggarna Brewery (Sweden) in 2005. This beer was brewed using maize that has a specific genetic modification that makes it resistant to attack by *Ostrinia nubilatis* – the European corn borer. The variety, *Zea mays* L. Yieldguard, has an added gene that expresses a truncated version of the insecticidal protein Cry1Ab, derived from *Bacillus thuringiensis* (Bt) (Agrobios, 2008). Cry1Ab is lethal only to the juveniles (caterpillars) of *Lepidoptera* species (butterfies and moths) and only then if the insects attempt to feed on the plant. Other insects and animals are unaffected by this protein, their gut linings not having the required receptors. An added advantage arises from the fact that minimization of insect feeding damage also minimizes attack by pathogenic fungi, so the GM plants are effectively protected from fungal diseases.

More recently, two GM barley lines have been investigated at trial field sites by researchers at the University of Giessen and the Friederich-Alexander University of Erlangen-Nürnberg (Kogel, 2006; GMO Compass, 2006). One barley line has an additional gene that expresses endochitinase (derived from *Trichoderma herzianum*), an enzyme that hydrolyzes fungal (but not insect) chitins. The plants are resistant to attack by the soil fungi *Rhizoctonia solani* and *R. oryzae*, which cause barley root rot, a disease that is widespread in many countries, especially the United States. The field trials are being conducted partly to determine whether the presence of this GM barley line affects beneficial soil fungi: laboratory trials have already shown that there is no effect. The second GM barley line has an extra gene that expresses a glucanase enzyme in the kernels. Glucanases (Section 2.6.2) hydrolyze β-glucans in the cell walls of cereal kernels during malting, hence they contribute much to the production of a haze-free beer. The German field trials are also testing whether this GM barley line also affects beneficial soil fungi (supposing that some kernels drop to the soil and decompose), since β-glucans occur in the cell walls of almost all fungi. If these and other trials are successful it is expected that GM barley may be commercially available in the near future, depending on the extent of consumer resistance, which still tends to be high in some countries, especially in Europe. Here, it is not unknown for GM opponent groups to exhibit rather more than passive resistance, as witnessed by the persecution of Kenth beer (Moore, 2005) and the destruction of part of the University of Giessen's GM barley field (Kogel, 2006). A survey of consumer attitudes in Western Australia toward hypothetical GM beer (brewed with either GM barley or GM yeast) indicated a general aversion to GM products, but significant numbers of the survey subjects were willing to choose a GM beer at lower cost or even at premium cost if the product could demonstrate additional health benefits (Burton and Pearse, 2002).

The use of GM yeasts in winemaking has been a battleground since the early days of the century (Lawton, 2002) and has continued for several years (Goode, 2005). At the time of writing (2008), two GM yeasts, ML01 (developed in South Africa by Volschenk and coworkers in 1997)) and ECMo01 (developed in the USA) have been approved by the Food and Drugs Administration in the United States, in 2005 and 2006, respectively. The FDA treats the organisms as substances and has granted 'generally recognized as safe' (GRAS) status (see Section 5.9.5) to both. These yeasts are now available to winemakers in Canada and the USA, and commercial wines (which at present do not need to be identified as being made using GM yeast) have been produced. In many other countries, there are restrictions on the use of GM organisms, such as yeasts, in foodstuffs, although in some of these countries research is taking place on producing genetically engineered yeast for research or analysis purposes (Chambers, 2007; Cummins, 2007). There is a certain natural genetic variation in wine yeasts, giving rise to a wide range of fermentation characteristics that result in differences (though often subtle ones) in finished wines (Chapter 2.2). Non-GM mutagenic techniques have the potential for producing tailor-made novel yeasts, but GM techniques are generally recognized as offering greater scope (Chambers, 2007).

Genetic engineering of crops differs considerably from yeast genetic engineering. The former is based on illegitimate recombinations resulting in unpredictable and uncontrollable sites of gene insertions, whereas the latter uses legitimate recombination, which allows gene insertion at specific sites. Genetic engineering of yeast falls into two major categories: transgenic and self-cloning. The former involves insertion of heterologous genes (derived from different species or organisms) into *S. cerevisiae*, whereas the latter involves insertion of genes from a different strain of the same species. The GM yeast ML01, is *S. cerevisiae* with two extra genes, one of which is a malate transporter gene from *Schizosaccharomyces pombe* (another fungus) and the second is the malolactic enzyme gene from *Oenococcus oeni* (a bacterium) (Section 2.3.2). The advantage of this yeast is that it performs alcoholic and malolactic fermentation at the same time, thus cutting back wine processing time and producing wines with lower levels of biogenic amines, which are sometimes produced during conventional MLF with certain lactic acid bacteria (Sections 2.3.14 and 5.11.3). The second commercially available GM yeast ECMo01, derived from Davis (California) *S. cerevisiae* strain 522, contains three genes derived from other strains of *S. cerevisiae*. These, DUR1,2 gene, a promoter gene and a terminator gene, increase the

expression of urea amidolyase (urease), which catalyzes the hydrolysis of urea, a byproduct of alcoholic fermentation (Sections 2.2.3 and 2.2.8). Urea reacts with ethanol during winemaking (and distillation) processes to give the carcinogenic ethyl carbamate (urethane) (Sections 2.2.3, 2.2.8 and 5.11.4), although other biochemical routes to this compound exist (Section 2.3.6). Fermentation trials have revealed that ECMo01 produces red wines with levels of ethyl carbamate reduced by up to 89%, but with similar qualities and characteristics of red wines produced by the parent strain (Heller, 2007). Other, as yet noncommercial, GM yeasts include those possessing genes that facilitate glycoside hydrolysis to liberate aroma compounds, those that allow production of more glycerol and those that endow antimicrobial power. There are many others (see Cummings, 2005).

Scientific objection to the widespread commercial use of GM yeasts are centered on the possibility of mutations caused by unknown chromosome rearrangements, with the possible formation of toxic end-products (Cummings, 2005). Wine yeast cells have been shown to be hyperactive in mitotic recombination and this is considered to be a contributor to the well-known genetic instability of wine yeasts (Sections 2.2.3 and 2.2.4). There is also concern about GM yeast cells (and their degradation products, such as RNAs) found in bottles of wine being consumed and also there is fear of GM cells persisting in wine and in wineries (and their environs) (Cummings, 2005). More generally, there is fear of contamination of native or traditional specialized yeasts in wineries (Martenson, 2006). It is also felt by some that not enough health and safety groundwork has been done regarding already sanctioned GM yeasts, and a rush of newly approved GM yeasts would indeed exacerbate this situation (Cummings, 2005).

There have been field trials with GM grapevines since the late 1990s in many countries, including Australia, France, Germany, Italy and the United States (Information Systems for Biotechnology, 2008). The aim is mostly to breed plants that are resistant to attack by pathogenic fungi (e.g. *Uncinula necator* or *Oidium Tuckeri*), bacteria or even viruses (e.g. *nepovirus*). The inserted genes include synthetic genes that express antimicrobial peptides, or genes from *Trichoderma harzianum* that express fungal chitinase. Other aims of transgenic vine breeding include modification of grape color or quality and regulation of the plant hormone auxin, thus altering the vine's life cycle. The majority of field test release applications are from university departments, such as California, Cornell and the State University of New York, but field trials are also conducted by private companies and wine research institutes (Cummings, 2005). There is concern over what happens to GM-expressed compounds (e.g. antimicrobial agents) or to the inserted genes themselves during winemaking (Cummings, 2005). Vines are rather prone to mutations (e.g. bud mutations) that produce different strains of the same variety (a recent example is Kernling, derived from Kerner), which may differ, for example, in grape skin color, vine life cycle, disease resistance or grape quality. What would happen to donor genes in such a situation is unknown at the present time and may need longer trials to establish.

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Part 2

Fermented Beverages: Beers, Ciders, Wines and Related Drinks

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Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

2.1

Introduction: Overview of Fermentation and Microorganisms

Natural fermentations have played a vital role in man's development. Some of these fermentations made changes in food materials that were quickly recognized as desirable.

—N. Potter, 1986

Consumers have a considerable portion of their nutritional needs met through fermented foods and beverages. Fermentation is of great significance because it provides mankind with a way to preserve foods and beverages in a wide diversity of flavors, aromas and textures. The affluent Western world has the technology to preserve food through canning and freezing, but the developing world still relies upon fermentation and dehydration (Steinkraus, 1997). The microorganisms associated with fermentation constitute a diverse group, including both prokaryotes (bacteria) and eukaryotes (yeasts and molds). The manufacture of alcoholic beverages and cultured dairy products represent the leading fermentation industries worldwide. The word 'fermentation' can have a variety of meanings, from informal to more scientific definitions. Any definition needs to include activities that produce alcoholic beverages or acidic dairy products, large-scale microbial processes occurring with or without air, or any energy-releasing reaction that occurs under anaerobic conditions. It also covers the spoilage of food by microbes, like the transformation of wine to vinegar by acetic acid bacteria.

In its broadest definition, fermentation is the anaerobic catabolism in which an organic compound serves as both electron acceptor and donor, and in which ATP is produced by substrate level phosphorylation. In catabolism (the breakdown of molecules), the electron donor, or more accurately, the chemical reaction in which the electron donor is oxidized, is the source of energy. For chemotrophs (organisms that rely on a chemical electron donor), two mechanisms of energy conservation are known, respiration and fermentation. The final result is the production of ATP for the organism, which is then used in further reactions. Fermentation and respiration differ in that respiration requires oxygen, or some other externally supplied terminal electron acceptor, whereas in fermentation, the electron acceptor is generated from the initial substrate. In addition, ATP is produced by different mechanisms during the two processes. In fermentation, as previously stated, it is generated by catabolism of an organic compound (substrate-level phosphorylation), while in respiration, ATP

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

is produced through coupling to the proton motive force in oxidative phosphorylation. Glycolysis (also called the glycolytic pathway) is a series of 10 reactions that take place in the cytoplasm of all cells, beginning with the metabolic process of chemical breakdown of glucose, and producing a three-carbon product, pyruvate. These reactions are accompanied by the production of two molecules of ATP, and the reduction of two molecules of NAD^+ to NADH and H^+.

Under anaerobic conditions, the total useful energy yield per glucose molecule is only two ATPs. When oxygen is involved, and pyruvate can be completely oxidized, about 38 ATPs are produced. Looking at the processes from a different perspective, cellular respiration occurs when the pyruvate is converted aerobically to energy-poor carbon dioxide. Fermentation produces relatively energy-rich molecules such as lactic acid or ethanol in anaerobic conditions, so the energy extracted from the original glucose molecule is far less (Purves *et al.*, 2001). Fermentation is therefore an incomplete oxidation, and organisms that are unable to carry out aerobic respiration are energetically disadvantaged. However, some organisms are confined to totally anaerobic environments, and their survival will be dependent on their ability to ferment, so it is a ubiquitous process in nature. Catabolic targets can be as diverse as cellulose in the rumen of terrestrial herbivores, or the simple monosaccharides found in the juice of various fruit. A medium such as grape juice, with a high concentration of monosaccharides represents a nutrient-rich environment in which a host of organisms could respire or ferment, if permitted. Management of the conditions in which grapes and their juice are protected from, or exposed to, microorganisms is initially the job of the viticulturalist, and then the winemaker or oenologist.

It could be argued the management of microorganisms is the most important task in the quest for excellence in wine quality, but opinions on this vary. Robinson (2003), for example, writes, 'It is a healthy sign that more and more winemakers are admitting wine is made in the vineyard and not in the cellar,' whereas Fleet (2003) states that '. . .although grape cultivar and cultivation provide the foundations of wine flavour, microorganisms, especially yeasts, impact on the subtlety and individuality of the flavour response.' Goode (2005) argues, '. . .yeasts don't get enough credit. When it comes to wine, grapes get all the glory.' The fact is that no matter how good your grapes, without yeast to transform them, they would not become wine. Indeed, even in the most modern production facilities, incorrect microbial choices and poor fermentation practices will produce a bad product from good starting material.

In addition to yeast needing management, other organisms including fungi, acetic acid bacteria and lactic bacteria also produce substances that can add to the organoleptic (sensory) qualities of the a product (positively or negatively), and may affect the progress of fermentation. It is important to understand the impact of microorganisms, and identify as many of the ecological interactions that occur between the different microbial groups, species and strains involved in a single fermentation as possible. These interactions encompass yeast–yeast, yeast–filamentous fungi and yeast–bacteria responses (Fleet, 2003).

The general processes involved in fermentation by yeast are discussed in more detail in Chapter 2.2, as well as in the winemaking sections 2.9.1, 2.9.2 and 2.9.3. There is also discussion of alcoholic and other fermentations relating to the brewing of beer (Sections 2.6.4, 2.6.5, 2.6.6 and 2.6.7), other cereal-based beverages (Chapter 2.7) and to cider making (Section 2.8.4).

Malolactic fermentation is discussed in Chapter 2.3, as well as with respect to various drinks (Sections 2.8.4, 2.9.1, 2.9.2 and 2.9.3). Additionally, Section 2.10.3 deals with some of the special characteristics of flor yeasts and the fermentations performed by a broad spectrum of microorganisms are discussed in Sections 2.6.7, 2.7.1 and 2.7.2.

Finally, the influence of microbiological activity (especially lactic acid bacteria, LAB) on the organoleptic character of certain spirits is discussed in Sections 3.2.3, 3.5.4, 3.5.5 and 3.7.2, and the activities of spoilage organisms are discussed in Chapter 2.4.

2.1.1 Yeasts

Yeasts are ubiquitous in soils and on plant surfaces, but the microbial population found on fruit will vary according to a number of factors, including the type of fruit, climatic influences and agricultural practices. The developmental stage at which fruit is examined, and any physical damage will also play an important role in which organisms are present. Although rich in sugars and other nutrients, the majority of microorganisms will not thrive in grape juice and must, due to high acidity and low pH, high osmotic pressure of the concentrated sugar in solution and (in a wine-making context) added sulfur dioxide.

Recent ecological evidence shows that the main yeast involved in most industrial alcoholic fermentations, *Saccharomyces cerevisiae,* is isolated with extreme difficulty from conventional habitats, such as soil or the surface of ripe fruit, but it is almost the only species colonizing the surfaces of the winery equipment (Martini, 1993). In fact, for all the importance of this yeast to mankind in brewing, baking, research and winemaking, the origins and natural habitat of the species are still uncertain (Jackson, 2000). *Saccharomyces cerevisiae* is unable to utilize the starch of barley, so when beer is produced, the grain is first germinated and the resulting malt is extracted with hot water to yield wort containing fermentable mono-, di- and trisaccharides and other yeast nutrients (Campbell, 2000) (Section 2.6.4).

The most frequently isolated species from grapes is the apiculate yeast, *Kloeckera apiculata* (and its sexual counterpart *Hanseniaspora uvarum*), identifiable by its lemon-shaped cells, which normally accounts for 50% or more of the yeast population present (Pretorius, 2000). The other significant but smaller populations identified on the surface of healthy grapes are the so-called 'wild' species of *Candida, Cryptococcus, Pichia, Klyveromyces, Brettanomyces, Hansenula, Aureobasidium pullulans, Metschnikowia* and *Rhodotorula*, depending on the stage of grape maturity (Fleet, 2003). In enology, a distinction is made between wild and wine yeast by Boulton *et al.* (1996), who define those genera which are found on the grape and can carry out a limited alcoholic fermentation with production of volatile esters, as 'wild yeast,' and those that will carry out a complete fermentation of grape juice without producing any atypical sensory effects, as 'wine yeast.'

The 'bloom' on the cuticle of the grape, previously thought to consist of wine yeast in their millions, has been proved to be a relatively dry and sterile environment of overlapping waxy plates where any yeast cells will be in a dormant state (Jackson, 2000). Yeasts require moisture in order to be metabolically active and unless the berry is damaged, the right conditions only occur on the cuticle when there is precipitation of some sort in the vineyard. Actively metabolizing yeast are most frequently found around stomata or next to cracks in the cuticle, where they form small colonies on nutrients seeping out of openings in the fruit (Martini *et al.*, 1996). Few yeasts grow on grape leaves or stems, but they may be found on the receptacle and pedicel.

Despite being present in very low numbers in the vineyard, crushing grapes under aseptic conditions, and sampling during active fermentation will provide evidence that *Saccharomyces cerevisiae*, with its combination of an efficient fermentative catabolism and relatively good alcohol tolerance, is the dominant, and sometimes the only species that survives in a wine fermentation, with most others declining after a few days (Pretorius, 2000). See also Sections 2.8.3 and 2.8.4 for details of microorganisms found on the skins of apples and pears and their involvement in the production of traditional cider and perry.

The choice and treatment of the yeast by the producer can have a huge influence on the quality of the beverage. Goode (2005) observes that of the 1000 or so volatile and flavor compounds that have been isolated in wine, yeasts are responsible for the production of about 400 of these. This figure is also the one used by Campbell (2000) to enumerate the metabolites that contribute to beer flavor. The effects of fermentation on aroma are outlined in more detail in Section 2.2.9 and more specific discussions can be found in Sections 2.6.4 (beer), 2.8.4 (cider and perry) and 2.10.3 (flor Sherry).

It is fairly routine in wineries these days for the inoculum of *S. cerevisiae* to be rehydrated from a freeze-dried form (known as active dry (wine) yeast or ADY), chosen for a particular set of characteristics

by the winemaker, and added to achieve a population of 10^5–10^6 cells/ml of juice or must. Although the inoculation of juice with selected strains of *Saccharomyces cerevisiae* is routine in many wineries, there are really only a few circumstances in which it is essential. If the grapes are in poor condition and harbor a large population of wild yeasts, fungi and bacteria, an inoculation with a commercial killer yeast strain will give the yeast a competitive advantage, and hopefully lead to a clean fermentation. After thermovinification, microbial populations on the grapes have been killed off by high temperatures, and trying to conduct a fermentation with natural yeast will be difficult, if not impossible. In the event that fermentation slows and sticks, inoculation with a large population of actively fermenting yeast cells can kick-start the process, and ferment any residual sugar. In order to initiate the secondary fermentation in sparkling wine production, an inoculum of an appropriate strain is necessary, because conditions in the bottle or tank (fairly low pH, presence of sulfur dioxide and reasonably high concentrations of alcohol) are not normally conducive to yeast growth (Section 2.9.3).

The alternative to inoculation is to use the natural yeast populations on the grapes, in which case the alcoholic fermentation will be characterized by the successional growth of various yeast species and strains, where yeast–yeast interactions determine the ecology, (Fleet, 2003). The advantages of this practice are that it costs nothing and very often the yeasts found naturally in an area are particularly suited to the type of wine produced. The wild yeast may be responsible for the initiation of fermentations, but by mid fermentation, *S. cerevisiae* represents almost all of the yeast isolatable, due to its tolerance of low temperature and pH, and higher alcohol concentrations. It is widely acknowledged that non-*Saccharomyces* species are not as ethanol tolerant as *Saccharomyces* species, resulting in S. *cerevisiae* dominating the fermentation at around 4% (v:v) alcohol, reducing the impact of non-*Saccharomyces* species on the aroma and quality of the final product. *K. apiculata, C. stellata* and *C. krusei* produced maximum ethanol concentrations in the range 2.7–6.6% when grown as single cultures in grape juice (Heard and Fleet, 1988). These authors also noted that an increase in pH from 3.0 to 3.5 increased the tolerance of apiculate yeasts to alcohol. Although the addition of SO_2 is thought to inhibit or kill indigenous yeasts it is unlikely to kill the *S. cerevisiae* present (Jackson, 2000). The presence of 'killer' yeast strains in either the inoculated or indigenous yeast can have an effect on the make up of the populations of yeast present. Killer strains produce exocellular toxins that have a lethal effect on more sensitive strains. Dominance of the inoculated strain if it does not possess a killer factor, is not always assured. Killer activity has been observed in the genera *Saccharomyces*, *Kluyveromyces* and *Hansenula* (Palpacelli *et al*., 1991). Killer factors are now deliberately included in many commercial dried yeast strains in order to ensure their domination in solution.

Wild yeasts in low concentrations, at the beginning of fermentation, are known to provide depth and complexity to the flavor and aroma of wine. A similar situation is believed to exist in the production of traditional cider and perry (Section 2.8.4). However, if the species is able to withstand conditions that allow it to completely ferment grape sugars (e.g. *Schizosaccharomyces* and *Zygosaccharomyces* species), the product is far less appealing. It has long been advocated that the distinct nature of wines from long-established vineyards and famous wineries come from the various yeast genera in residence, and even *Brettanomyces* ('brett') character is now thought to be part of the 'terroir' character of a particular wine. The decision to inoculate with a reliable, 'designer' *S. cerevisiae* strain, or go with a natural fermentation and its associated complexity is a stylistic choice or it is made according to the philosophy of the winemaker and brand. There are certainly many winemakers who shy away from New-World techniques, and advocate a natural fermentation from juice with a high solids content (rather than cultivated yeast-inoculated fermentation of sterile juice), with the aim of producing a more characterful, individual product.

Traditional brewing yeasts are unlikely to be pure cultures, so there is also the requirement to maintain a constant composition of the mixture (Campbell, 2000). One strain of the yeast population may predominate in the early stages of the fermentation, another strain later and a third strain later still. The yeast 'head' must be skimmed off several times during the fermentation to prevent it collapsing back into the beer and creating

off flavors, but only part of the recovered yeast is selected for reuse in subsequent fermentations (see Sections 2.6.4 and 2.6.5). Flocculation of yeast in beer production is also an important characteristic, as too early flocculation brings the fermentation to a premature end. Nonflocculent yeasts, however, remain in suspension and have to be removed by centrifugation or filtration. Pioneer yeast taxonomist E.C. Hansen first recognized pure brewing strains from beers in Belgium, Britain and Germany as *S. cerevisiae*, and differentiated them from strains isolated from Czech and Bavarian beers (*S. carlsbergensis*, now known as *S. ovarum*). Pure cultures of the latter yeast are now used worldwide for production of the Pilsener style of beer (Campbell, 2000) (Section 2.6.6).

Although yeasts are the organisms primarily associated with alcoholic fermentation, bacteria (and body cells) perform innumerable anaerobic conversions that are as useful and important. Different fermentations are distinguished by the final product of the process. In lactic acid fermentation, pyruvate is reduced to lactate. This fermentation takes place in a number of different environments, as well as in muscle cells in the human body. Lactic acid will accumulate in muscles that undergo anaerobic metabolism, leading to stiffness and pain. Nerve cells, however, are unable to reduce pyruvate to lactic acid, as they lack the enzyme, and therefore, without adequate oxygen, these cells (including those in the brain) rapidly cease functioning (Purves *et al.*, 2001).

2.1.2 Lactic Acid Bacteria

The fermentation carried out by lactic acid bacteria (LAB) is crucial in the production of other foodstuffs like yogurt, cheese, sauerkraut and pickles. Traditionally, bacteria do not play a role in winemaking until after the alcoholic fermentation. Nearly all red wines, and certain styles of white wine undergo the second or malolactic fermentation (MLF), during which malic acid, and occasionally other substrates, are converted to lactic acid (and other products) by lactic acid bacteria. This fermentation tends to happen naturally in the barrel during red wine maturation, but can also be deliberately induced through inoculation with a bacterial starter culture. The species most commonly used for this purpose in winemaking is *Oenococcus oeni* (previously known as *Leuconostoc oenos*). As well as making wine 'softer,' by converting malic to lactic acid, this fermentation adds complexity to the wine and increases microbial stability by removing an important metabolic substrate from solution. Lactic acid bacteria, like *Saccharomyces cerevisiae*, are unusual in their preference for fermentation over respiration, and their ability to survive in the relatively harsh conditions in wine. They occur in grape juice at low concentrations (10^3 cfu/ml) and usually decrease to even lower numbers during fermentation unless there is a delay in the onset of fermentation, in which case they grow and utilize substances otherwise available for the yeast. They can also produce acetic acid, which can limit yeast growth. Some authors therefore consider inoculation with bacteria for malolactic fermentation before alcoholic fermentation to be unwise. Swiegers *et al.*, (2005) noted that during malolactic fermentation, bacteria do not only provide deacidification, but they are also able to enhance the flavor profile of the wine. Malolactic fermentation is considered in more detail in Chapter 2.3.

2.1.3 Molds and Spoilage Organisms

The influence of molds (filamentous fungi) on the modification of the composition of the grape juice is not a subject of much investigation in the current literature. Their main influence occurs during grape cultivation, when they cause grape spoilage. Damaged and diseased fruit may also have populations of filamentous fungi such as *Aspergillus*, *Botrytis*, *Penicillium*, *Plasmopara* and *Uncinula* species. Fungal growth on grapes can produce various metabolites and conditions that may disturb the ecology and growth of yeasts during

alcoholic fermentation. There are reports that *Botrytis cinerea*, *Aspergillus* spp. and *Penicillium* spp. produce metabolites that retard the growth of yeasts during fermentation (Fleet, 2003). The activities of *Botrytis cinerea* in the vineyard may lead to high quality noble wines, or spoilage and taints, depending on climatic conditions at the time of their involvement with the grapes. If wet conditions persist in the vineyard during a period of infection, other organisms will become involved, leading to the formation of the notorious 'grey rot.' There is little that can be done with grapes in this condition. If weather conditions are conducive to the formation of noble rot (i.e. short damp periods, followed by dry, warm conditions, when the grapes are ripe) the effect of the mold will be to concentrate the sugar and flavor compounds in the berries through desiccation. Good quality noble rot wines can fetch high prices, which will offset the loss of income normally achieved through volume.

The role of specific species of molds in the notorious cork-taint problem and the microbiota associated with various cork manufacturing processes are still not fully understood (Álvarez-Rodríguez *et al.*, 2002), but *Trichoderma* and *Fusarium* were able to carry out the conversion of 2,4,6-trichlorophenol to 2,4,6-trichloroanisole when grown on cork. The role of *Penicillium* and *Aspergillus* in the production of corky and moldy taints was also noted (Jackson, 2000).

Damaged grapes have increased populations of lactic and acetic acid bacteria that impact on yeasts during alcoholic fermentation. Acetic acid bacteria have the ability to oxidize ethanol to acetic acid (vinegar) in the presence of oxygen. Under anaerobic conditions, they are able to use quinones as hydrogen acceptors and maintain a limited metabolism (Jackson, 2000). Their survival in tanks and bottles of wine without oxygen present, while being unexpected, is therefore still possible. If there are traces of oxygen present, such as those absorbed by wine during transfer operations or while in barrel, the survival of a population of acetic bacteria is almost guaranteed. The bacteria grow on damaged fruit and the population may reach 10^6 cfu/ml, in affected juice with the species *Gluconobacter oxydans* dominating. During alcoholic fermentation, populations of the bacteria tend to decrease, but given the right opportunities, *Acetobacter aceti* may grow. The most well known consequence of acetic acid bacterial activity is the production of volatile acidity, comprising mainly acetic acid. Ethyl acetate, also an important component of volatile acidity, is produced through nonenzymic esterification, as well as by other organisms and is not necessarily associated with acetic bacteria (Swiegers, *et al.*, 2005). The significance of acetic acid bacteria is greater than the production of 'vinegar' characters, however. Acetic acid is known to be a growth inhibitor for yeasts, and the presence of a large population of acetic or lactic acid bacteria can be antagonistic to yeast growth and may assist in causing stuck fermentations. Spoilage organisms are discussed in more detail in Chapter 2.4.

Alcoholic fermentation and associated topics, covering all alcoholic beverages, are the subjects of a very large number of research papers and have also been reviewed and summarized in many book chapters, including Zoecklein *et al.* (1995), Boulton *et al.* (1996), Jackson (2000), Ribéreau-Gayon *et al.* (2000), Boulton and Quain (2001) and Berry and Slaughter (2003).

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2.2
Alcoholic Fermentation

2.2.1 Introduction

Mankind has harnessed the abilities of yeast in brewing and bread making since the beginning of recorded history, and yeasts are probably one of the earliest organisms to be domesticated. However, it was only clearly established in the mid nineteenth century that conversion of glucose and fructose to ethanol and carbon dioxide (fermentation) is a microbial process. Pasteur, in around 1876, showed that fermentation did not arise spontaneously and isolated the yeasts responsible for it (Madigan *et al.*, 2003). He also demonstrated the effect of oxygen on the assimilation of sugar as well as the production of secondary products by fermentation, i.e. glycerol and carbon dioxide. As noted in Chapter 2.1, the most significant species involved in winemaking and brewing are *Saccharomyces cerevisiae.* According to Ribereau-Gayon and coworkers (2000a), after investigating the delimitation of winemaking species, all the strains of '*bayanus*' isolated in their study were found to belong to the species *S. cerevisiae*. On the other hand, yeast called '*uvarum*' principally belong to the species *S. bayanus*. In short, yeast species involved in commercial fermentations for wine and beer comprise a very large number of genetically similar strains, with varied technological properties. Yeast strain can affect the rate of fermentation, the efficiency and success of conversion of sugar to ethanol, and the nature and quantity of by-products.

2.2.2 Physiology and Morphology of Yeast

There are over 100 genera of yeast, representing around 700 species (Swiegers *et al.*, 2005), but a limited number are used in the production of beverages. In a winemaking context, for example, there may be up to 16 species involved, of which only one (*S. cerevisiae*) is of any real significance. Despite the considerable phenotypic differences among the yeast strains used for commercial brewing and winemaking purposes, most of them are now considered to be physiological strains of *S. cerevisiae.* The assignment of most of the traditional wine yeast strains to a single species does not, however, imply that all strains of *S. cerevisiae* are equally suitable for the various wine fermentations; they differ significantly in their fermentation performance and their contribution to the final bouquet and quality of wine and distillates (Pretorius, 2000).

Yeasts used in brewing and winemaking (e.g. *S. cerevisiae)* are unicellular fungi that mainly belong to the *Ascomycetes* (sac fungi) and *Deuteromycetes* (imperfect fungi) classes. They do not possess chlorophyll, cannot photosynthesize (Madigan *et al.*, 2003), and therefore utilize complex foods for their nutrients,

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

which they can metabolize with or without oxygen present. Each yeast cell possesses one membrane bound nucleus enclosing the chromosomes (in contrast to other fungi which may be multinuclear). The genome of *S. cerevisiae* has been under scrutiny for decades, with no less than 12 genetic maps of the organism published between 1960 and 1997 (Boulton and Quain, 2001a). It is probably reasonable to claim that *S. cerevisiae* are genetically the best understood of all organisms, and this intimate knowledge of the genome has many applications in fermentation technology. As fungi, *S. cerevisiae* are classified as ascomycetes because they produce ascospores through meiosis (sexual reproduction). This is triggered when yeast are nutritionally stressed, for example by deprivation of a carbon or nitrogen source. The diploid yeast in the population produces four haploid nuclei, which are incorporated into four stress-resistant ascospores, encapsulated in the ascus. Another characteristic of *S. cerevisiae* is that they are also able to grow vegetatively, i.e. they can reproduce asexually by budding multilaterally or extruding a bud from a point on the mother cell (Jackson, 2000a). When this extrusion reaches about half the size of the parent cell it is pinched off and becomes a daughter cell, leaving a 'budscar' on the mother cell. Buds may arise at any point on the mother cell surface, but never again at the same site. Branched chaining (pseudohyphae) may occasionally follow multilateral budding when buds fail to separate. Under optimal nutritional and cultural conditions *S. cerevisiae* double their mass every 90 minutes (Pretorius, 2000). Vegetative reproduction is normal under the conditions found in must and wine, as ascal development is suppressed by high concentrations of glucose, ethanol or carbon dioxide.

Like all plant cells, yeast have two cellular envelopes, the cell wall and cell membrane – the properties of which distinguish them from other eukaryotes. The cell envelope, comprising a cell wall, periplasm and plasma membrane, surrounds and encases the yeast cytoplasm. It makes up 15 to 20% of the dry weight of the cell, and incorporates chitin, a major constituent of the cell walls of other fungi, but only as a component of bud scars. The cell wall is fairly rigid, but a little elastic, and consists of mannoproteins and β-glucans (Feuillat, 2003). The β-glucans are of three types: fibrous (β-1,3), amorphous (β-1,3) or ramified β-1,6 glucans. The mannoproteins can be extracted and are important in the stability and clarity of the product. The cell membrane is a selective barrier controlling exchanges between the living cell and environment. Like the membranes of most eukaryotes, it is stable and mainly hydrophobic in nature. The principle components of the membrane are lipids and proteins, as well as a number of other factors important in selectivity and efficiency as a transport organ and barrier. The concentration of sterols in the cell membrane, for example, affects the penetration of glucose into the cell, as well as yeast survival during fermentation (Larue *et al.*, 1980). Ribereau-Gayon and coworkers (2000b) stated that ergosterols are indispensable to yeast in complete anaerobiosis. Membrane ATPase has a role in yeast resistance to alcohol, and the presence of ethanol will slow the penetration of glucose and arginine into the cell and limit the output of protons from the membrane. The membrane also permits the yeast to react to external stimuli like sex hormones. Optimum functioning of the cell wall and membrane are essential for a successful fermentation, flocculation of the cells after fermentation, the release metabolic products from the cell that add to flavor of the product, and autolytic characters during *sur lie* (on lees) maturation after fermentation (Ribereau-Gayon *et al.*, 2000c).

The structural organization of the cytoplasm, containing organelles such as the nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria and vacuoles, is maintained by a cytoskeleton. Several of these organelles derive from an extended intramembranous system and are not completely independent of each other (Pretorius, 2000).

2.2.3 Nutritional Requirements of Yeast

Yeasts have relatively simple nutritional needs. Unable to carry out photosynthesis, they require a reduced carbon source, which can be as complicated a compound as a disaccharide or as simple as acetate. In addition, they need the vitamin biotin and an organic nitrogen source such as ammonium, urea or the amino acids.

Like most eukaryotic organisms, they also require nitrogen in an assimilable form, and a variety of salts and trace elements for metabolic purposes. In brewing and winemaking terms, however, the most important nutritional ability of yeast (specifically *S. cerevisiae*) is that in the absence of oxygen, they are able to transform mono- and disaccharides to alcohol through the process of fermentation. As will be discussed subsequently, they are also capable of respiration (using oxygen as a final electron acceptor during metabolism) under specific conditions.

Of all nutrients assimilated by yeast during wine fermentations, nitrogen is quantitatively second only to carbon. Research has found that the total nitrogen utilized during the catabolism of 200 g/l glucose when all amino acids are in excess is around 400 mg/l, and nitrogen utilization is influenced by the presence of air in the fermentation headspace, ammonium supplementation and initial glucose concentration (Jiranek *et al.*, 1995). *S. cerevisiae* is incapable of adequately hydrolyzing grape proteins to supplement nitrogen deficiency, and relies therefore on the ammonium and amino acids present in the juice. Wine yeasts can distinguish between readily and poorly used nitrogen sources. Ammonium is the preferred nitrogen source and, as it is consumed, the amino acids are taken up in a pattern determined by their concentration relative to the yeast's requirements for biosynthesis and to total nitrogen availability. Deficiencies in the supply of assimilable nitrogenous compounds remain the most common causes of poor fermentative performance and sluggish or stuck fermentations, as nitrogen depletion irreversibly arrests hexose transport (Pretorius, 2000). Other problems related to the nitrogen composition of grape must include the formation of reduced-sulfur compounds, in particular hydrogen sulfide, and the potential formation of ethyl carbamate from metabolically produced urea. The ability to utilize a specific nitrogen-containing compound as a carbon source depends on a variety of factors, e.g. the degradation of the amino acid proline for use as a nitrogen source requires molecular oxygen. Addition of ammonium delays metabolism of amino acids, but increases the total nitrogen consumed (Jiranek *et al.*, 1995). The metabolism of individual amino acids varies between strains of yeast, but arginine, serine, glutamate, threonine, aspartate and lysine typically comprise the bulk of nitrogen used by yeast. Proline and arginine account for 30–65% of the total amino acid content of grape juices, with high proline accumulation in grape must associated with grapevine stress, in particular with low moisture (Pretorius, 2000). When a readily used nitrogen source (such as ammonium, glutamine or asparagine) is present, genes involved in the uptake and catabolism of poorly utilized nitrogen sources (including proline) are repressed. This repression impairs the assimilation of proline, as well as arginine, since both amino acids depend on the proline utilization pathway. Since the proline content of wine is generally not less than grape juice, it appears that proline is not taken up by wine yeast under anaerobic fermentative conditions.

Bell and Henschke (2005) state that both the form and amount of yeast-assimilable nitrogen (YAN) have implications for quality. Low must YAN leads to low yeast populations and poor fermentation vigor, increased risk of sluggish fermentations and production of thiols and higher alcohols, and low production of esters and long chain fatty acids. High must YAN increases biomass production, greater fermentation vigor, increased production of volatile acidity, the possibility of protein hazes due to higher levels of protein and microbial instability. Injudicious use of diammonium phosphate supplements often results in excessive levels of residual nitrogen, leading to microbial instability and ethyl carbamate (and phosphate in the case of DAP) accumulation in wine (Pretorius, 2000). The degree of supplementation of inorganic nitrogen in grape juice is usually regulated. This implies that knowledge of the nitrogen content of grape juice and the requirement for nitrogen by yeast are important considerations for optimal fermentation performance and the production of wines that comply with the demands of regulatory authorities and consumers. Urea, short peptides and nitrogenous bases (excluding thymine) can also be degraded by *Saccharomyces*. Nitrates, nitrite and most of the other organic amines cannot be utilized (Jiranek *et al.*, 1995). For more discussion on metabolism of amino acids by brewers' yeast in beer production, see Section 2.6.4.

The only vitamin that is required by *Saccharomyces* is biotin, although other vitamins can stimulate growth. *Saccharomyces* quickly depletes the supply of vitamins in the medium. A number of metals, including

iron, copper and manganese are also required at trace levels (Jackson, 2000b). *Saccharomyces* species are somewhat limited in the compounds that can be used as carbon and energy sources, when compared to other microorganisms. The monosaccharides glucose, fructose, mannose and galactose support growth and metabolism, and the disaccharides sucrose, maltose and melibiose can be utilized by most strains of *S. cerevisiae*, depending on their genetic background. Raffinose, a trisaccharide can also serve as substrate. Substrates that support oxidative or respiratory growth are also limited: pyruvate, lactate, ethanol, acetate and glycerol. Glycerol provides weak support when it is the only carbon source and energy source in a minimal, defined medium (Walker, 1998a). Other organic acids or alcohols cannot be utilized as carbon and energy sources by *Saccharomyces*, although some, such as malic acid, are metabolized to other compounds by some strains. Pentoses are not utilized by the strains of *S. cerevisiae* used in winemaking.

2.2.4 The Use of Naturally Occurring ('Wild') Yeasts in Fermentations

Formerly, producers relied on making yeast starter cultures from juice (sulfited to eliminate wild yeast and promote *S. cerevisiae*), which were inoculated into fermentors at 2–5% after several days of spontaneous fermentation. Spontaneous fermentation can be regarded as a heterogeneous microbiological process involving the sequential development of various yeasts and other microbiological species, affected by the prevailing fermentation conditions in a particular vat or tank (Pretorius, 2000). When grape must is used as a culture medium, selective pressures favor the yeasts with the most efficient fermentative catabolism, particularly strains of *S. cerevisiae* and perhaps strains of closely related species such as *S. bayanus*. For this reason, *S. cerevisiae* is almost universally preferred for initiating alcoholic fermentation, and has earned itself the title of 'the wine yeast.' For winemaking purposes, yeasts are frequently categorized as 'wild' yeasts or 'wine' yeasts. Generally speaking, these terms differentiate between yeast that are present naturally in the fermentation medium as a result of contamination of surfaces of substrate and equipment with organisms or spores, and yeast that is inoculated via a commercial starter culture (usually a single strain). All yeasts are wild in the sense that they may occur naturally in the environment, however the majority are not well suited to the conditions of alcoholic fermentation. Most wild yeasts are aerobic (Ribereau-Gayon *et al.*, 2000d) and they stop metabolizing when alcohol levels reach around 4%, or must density drops to 1.070–1.060. Although they can play a positive role in the character of the product through ester formation, some wild yeast may produce unwanted characters like volatile acidity, acetaldehyde and hydrogen sulfide. The practice of spontaneous fermentations remained prevalent in 'Old World' wine-producing areas until the 1980s because of the popular belief that superior yeast strains associated with a particular 'terroir' gave a distinctive style and quality to wine. The final product of spontaneous grape must fermentation is a result of the combined action of different yeast species, which contribute in different ways to the organoleptic properties of wine, (Shimazu and Watanabe, 1981). Conversion of grape sugars to alcohol and other end products by mixed populations of yeast may undoubtedly yield wine with distinct sensorial quality, often described as wine with a fuller, rounder palate structure. This may well be the consequence of higher concentrations of glycerol and other polyols produced by indigenous yeasts. Producers may utilize the various fermentative characteristics of wild yeast in the early phase of fermentation to potentially improve the product, and achieve stylistic distinction and vintage variability, as long as the process is managed carefully. Using natural flora also reduces costs to the producer, if all goes as planned (quite the opposite if not). The outcome of spontaneous fermentation depends not only on the numbers and diversity of yeasts present in must, but also upon grape chemistry and processing protocol, and is difficult to predict.

The characteristics and metabolism of non-*Saccharomyces* yeasts are discussed in more detail in Chapter 2.4. Also, further discussions on spontaneous fermentations can be found in Sections 2.6.7 (lambic beers), 2.7.2 (indigenous African beverages and Mexican pulque) and 2.8.4 (cider and perry). Also, the influences

of various non-*Saccharomyces* yeasts and other microorganisms on the organoleptic qualities of spirits are discussed in Sections 3.2.3 (Scotch whisky) and 3.7.3 (grape pomace spirits).

2.2.5 The Killer Factor

Certain yeast species, known as killer (zymocidal) strains, secrete exocellular toxins into their environment, which are lethal to other, sensitive strains of yeast and therefore provide a competitive advantage in fermentations (Ribereau-Gayon *et al.*, 2000e). The killer phenomenon in *S. cerevisiae* is associated with the presence of a proteinaceous toxin (zymocin), which is secreted by the zymocidal strains and is lethal to sensitive strains of the same species. Some yeast strains are immune to these zymocins, but do not produce active toxin. These are the so-called 'neutral' strains (Pretorius, 2000). The killer factor exists in most strains of *S. cerevisiae,* as well as other genera including *Hansenula, Kluyveromyces* and *Candida.* Killer yeasts offer both control (when inoculated to achieve a desired result) and a risk (when working as contaminating agents). When used as inoculants they offer certain benefits, including domination over wild yeasts which may cause delayed fermentation or production of off flavors, and protection in the wine from subsequent infection by spoilage yeasts. Shimizu and coworkers (1985) studied the distribution of killer yeast strains by geographical location. Australian wild yeast analysis showed killer strains only in *S. cerevisiae*. In Japan similar analysis showed killer properties in *S. cerevisiae* and *Hansenula* strains whilst Beaujolais (France) showed very high incidences (83%) of killer strains amongst a greater range of wild yeasts, including *Saccharomyces*, *Kloeckera*, *Candida*, *Hansenula and Torulaspora.* Most Mediterranean vineyards showed higher incidences of killer properties (65–90%), probably due to a combination of viticultural practices, climate and the extent of continual cultivation. In red wines, the killer function appears to be inactivated after about three days, possibly due to complexing of the toxin proteins with tannins. As the killer factors are proteins, their stability will be affected by ethanol levels, SO_2 concentration and by interactions with tannins and fining agents. The killer function is also inactivated by high temperature and high pH (Shimizu, 1993), but is favored by the acidic conditions of grape must (pH 2.8–3.8). Apart from sporadic reports, which imply that the presence of killer yeast could contribute towards stuck (incomplete) or sluggish fermentations, zymocidal *S. cerevisiae* strains have no negative effect on the wholesomeness and sensorial quality of wines that are produced (Pretorius, 2000).

2.2.6 The Use of Selected (Cultured) Yeast Strains

In 1890, Müller-Thurgau from Geisenheim introduced the concept of inoculating wine fermentations with pure yeast starter cultures (Pretorius, 2000). Currently, yeast manufacturing companies market a wide variety of dehydrated cultures of various *S. cerevisiae* strains. Most commercial freeze-dried cultures (or active dry yeast, ADY) contain yeast that was originally selected by various manufacturers from successful natural fermentations. In large-scale wine production, however, where rapid and reliable fermentations are essential for consistent wine flavor and predictable quality, the use of selected pure yeast inocula of known ability is preferred. These large wineries will be the main beneficiaries of programs aimed at producing new yeast strains with even more reliable performance, reducing processing inputs and facilitating the production of affordable high quality wines. The brewer or winemaker now has the option of selecting a commercial strain for a specific attribute (for example, the ability to hydrolyze monoterpenes from glycolysated precursors). There is a strong incentive to develop wine yeast strains with an ability to accumulate trehalose and glycogen as starter cultures, because trehalose appears to stabilize cell membranes of lyophilized yeast, as well as their cellular proteins by replacing water and forming a hydration shell around proteins. Glycerol accumulation

seems to control the concentration of solutes inside the cell relative to that of the culture medium, thereby counteracting the negative effects of dehydration.

The choice of yeast will be based on previous experience, the style of beverage required and the technical specifications of the yeast culture. Selected ADY is sold freeze-dried in packets which should be stored in a cool place and resealed if opened and not fully used. Yeast will still be viable if a packet is left unopened until the next season (or the use by date). Rehydration according to the manufacturer's recommendations is important for the survival of the yeast, as it affects the cell membranes and if the wrong method is used, viability will be reduced, for example if the recommended rehydration temperature is 35–40 °C, 100% of the yeast should be viable in this range (Morgan *et al.*, 2006). In inoculated fermentations, the actively growing starter culture dominates the native yeast species present in grape must, as long as the manufacturer's rehydration protocols are followed. There should be sufficient yeast to ferment the required juice if the dose rate recommended by the manufacturer is used, (usually 10–15 g/hl or 10^6 cells/ml in a winemaking context (Ribereau-Gayon *et al*., 2000f), giving a cell density upon inoculation of 1–3 million colony forming units (cfu)/ml (Pretorius, 2000). Walker (1998b) noted the absolute requirement of oxygen by *S. cerevisiae* for build up of cell numbers in the yeast starter, and ensuring adequate synthesis of sterols that aid yeast growth and survival during fermentation. Hence the starter should be aerated before addition to must to ensure a high count of active cells. If oxygen is deficient in the must, the lag phase between yeast addition and fermentation onset will be prolonged, thereby increasing the chances of infection by spoilage organisms.

2.2.7 Fermentation Vessels

Fermentation systems will reflect the type of beverage being made, the tradition of the country of production and the throughput of the producer. Vessel design, the method of operation and the variety of yeast used are related. Traditional vessels continue to be used and replaced, even in newer concerns, as brewing and winemaking tend to be conservative industries, and shortcomings of the systems are accepted as the price to be paid for continuity of style and the perceived quality of the product (Boulton and Quain, 2001b). In many countries, however, there is a trend towards fewer and larger producers with ever increasing batch sizes. Larger fermentation vessels represent a considerable risk if the product in them is in any way atypical. Material of construction, ease of cleaning, capacity, geometry, and monitoring and control facilities are all important when a system is being considered for use.

2.2.8 The Growth of a Yeast Population

A typical fermentation is almost completely anaerobic. The molecular oxygen that is available in the processed must or juice is quickly utilized by any microorganisms present in the medium. Yeasts require oxygen to synthesize and assimilate substances vital to their reproduction. If the must is not aerated, oxygen will become the limiting factor, yeast numbers will be too low to ferment effectively and the fermentation may end prematurely. In these circumstances it is not unusual for the fermentation medium to become reductive and hydrogen sulfide may be formed (Morgan *et al.*, 2006). The modern trend in winemaking is to use large doses of selected yeasts that have been grown in hyperaerated media and are rich in the necessary growth factors. If aeration is required in order to enhance aromatic complexity, exposing the must to oxygen at the beginning of the fermentation is much less risky and more effective then at the end, when the yeast growth factors are depleted. Musts from rotted vintages should be aerated as little as possible due to the presence of oxidative enzymes in solution. Red musts can be aerated to a greater extent to increase the rate of fermentation (Morgan *et al.*, 2006).

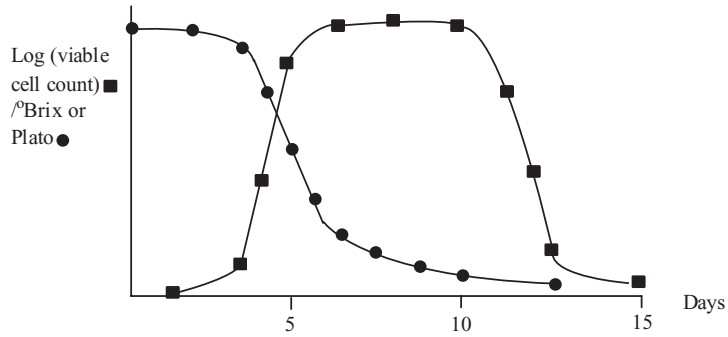

Figure 2.2.1 *Fermentation profile. This is a general profile; actual profiles depend on temperature and other factors. See also Figure 2.6.17, Section 2.6.5*

As stated previously (see Chapter 2.1), although *Saccharomyces* is virtually untraceable in the microbial flora of the grape, it very quickly dominates the fermentation so that at completion a nearly pure culture of this yeast is observed. Inoculation of the fermentation with *Saccharomyces* causes a faster domination of the fermentation by this organism. The reason for this domination can be found in the specific adaptation to high sugar, virtually anaerobic environments. Indeed, such environments appear to be the specific ecological niche in which this organism can flourish and dominate all other microorganisms. Yeast cells multiply during grape juice fermentation and their growth kinetics follow a typical microbial growth curve (Figure 2.2.1) which reflects the growth of cell number and decrease in sugar concentration (° Brix).

Margalit (1996) identified four key stages in the growth pattern of yeasts, a 'lag' period during which the yeast cells become acclimatized to conditions in the must and start multiplying; a period of rapid, exponential growth to a final population of around 2×10^8 cells/ml; a stationary phase during which the number of cells undergoing fission equals those dying, and a decline or death phase in which the number of cells dying exceeds the number undergoing fission. Sugars are metabolized through the first three phases.

Factors affecting the growth of a yeast population, and therefore the progress and efficiency of a typical fermentation are as follows: The sugar concentration can cause problems if it is too high, or too low. If it is too low, the yeast will respire if there is any oxygen present, producing carbon dioxide and water instead of ethanol. If it is too high, the osmotic pressure exerted on the yeast can delay the onset of fermentation. Approximately 50% of the total available sugar is used during the exponential growth phase, while the rest is fermented by the cells during the stationary phase. Sugar concentration may also influence the composition and concentration of aroma compounds produced by the yeast (see Section 2.2.2).

Apart from the inhibitory effect of excessive sugar content on yeast growth and fermentation, the production of excessive amounts of ethanol, coming from harvest of over-ripe grapes, is known to inhibit the uptake of solutes (e.g. sugars and amino acids) and to inhibit yeast growth rate, viability and fermentation capacity. The concentration of ethanol, as stated, can have an effect on yeast as it is a physiological toxin. The physiological basis of ethanol toxicity is complex and not well understood, but it appears that ethanol mainly impacts upon membrane structural integrity and membrane permeability (Pretorius, 2000). Resistance to ethanol toxicity is strain dependent, and factors increasing resistance are linked to decreased membrane permeability, for example the uptake of palmitic acid into the cell. Several factors are known to enhance the inhibitory effects of ethanol. These factors include high fermentation temperatures, nutrient limitation (especially oxygen, nitrogen, lipids and magnesium ions) and metabolic by products, such as other alcohols, aldehydes, esters, organic acids (especially octanoic and decanoic acids), certain fatty acids, and carbonyl and phenolic compounds. The effect of ethanol on suppression of sugar uptake by cells has been documented,

and this effect is increased with increasing temperature. Higher alcohols are even more toxic than ethanol, but normally do not occur in high enough concentrations during a normal fermentation to cause problems (Pretorius, 2000). Cell viability and sensitivity to alcohol toxicity are increased at higher sugar levels, and above 250 g/l, the likelihood of a stuck fermentation increases considerably (Jackson, 2000c), with ethanol production declining significantly. The producer is confronted with the dilemma that, while ethanol is the major desired metabolic product of grape juice fermentation, it is also a toxin that can lead to sluggish or stuck fermentations. A sluggish fermentation is more likely in small volume fermentations and occurs when the yeast ferment slowly and sometimes stop before the medium is dry.

Most so-called survival factors (e.g. certain saturated long chain fatty acids and sterols) are formed only in the presence of molecular oxygen, which in part explains the great success in the use of commercial starter cultures that are cultivated under highly aerobic conditions and in low glucose concentrations. These starter yeast cells contain high levels of the survival factors that can be passed onto the progeny cells during the six or seven generations of growth in a typical fermentation. Successful yeast cellular adaptation to changes in chemical or physical environmental parameters during fermentation requires the timely perception (sensing) of these parameters (e.g. fermentable sugars, assimilable nitrogen, oxygen, vitamins, minerals, ergosterol, ethanol, acetic acid, fatty acids, sulfite, agrochemical residues, killer toxins and unsaturated fatty acids), followed by accurate transmission of the information to the relevant compartments of the cell (Pretorius, 2000). Signals of a physical nature include factors such as temperature, pH, agitation and osmotic pressure in a complex, interconnected network.

Nutritional shortages can also give rise to a lagging or incomplete fermentation, naturally depending on the degree of the limitation. As well as causing fermentation to slow down, a shortage of nutritive substances, particularly nitrogen, can give rise to the formation of undesirable end products of metabolism, such as hydrogen sulfide and acetic acid. Under most winemaking conditions, a minimum of 150–250 mg/l assimilable nitrogen is recommended, and some authors have suggested levels as high as 500 mg/l (Jackson, 2000c). Nitrogen utilization usually occurs early during fermentation, before 5% ethanol has accumulated in the medium. Excess nitrogen has been linked to the formation of urea, which has been implicated in the formation of ethyl carbamate, a carcinogen (Bell and Henschke, 2005) (see Section 5.11.5). Mid chain length (C_8–C_{10}) saturated fatty acids and phenols are also known to affect yeast if present in high enough concentrations, affecting the progress of fermentations. Most manufactured lyophilized yeast has been grown in the presence of sufficient sulfur dioxide to provide some sort of resistance, but this is strain variable, and the preservative in high enough concentrations will impede membrane function in yeast, but at moderate concentrations (50 mg/l), most fermentations will progress unimpeded. Sulfur dioxide does have some other effects, for example, it inhibits acetic acid production, but favors glycerol synthesis. Elemental sulfur, if present in the medium as a result of antifungal treatment of fruit, can be assimilated, and in the worst case, reduced to hydrogen sulfide, thiols and mercaptans, thus adversely affecting the aroma of the resulting product. It is usually a complex problem. Other factors affecting yeast growth include build up of toxic by products, oxygen depletion, and carbon dioxide and osmotic pressure.

The lag phase usually lasts for 1–2 days, but may be longer if SO_2 concentrations are high, there is insufficient yeast present or there are other inhibitory substances such as spray residues in solution. The temperature should be controlled during the exponential growth phase to give the desired rate of fermentation. During the exponential and stationary phase, the bulk of the sugar is converted into alcohol. Towards the end of the stationary phase the population decreases, but significant sugar metabolism still takes place in the absence of growth of the population. The sugar is utilized exclusively as an energy source during this stage, and not as a carbon source in the production of biomass. The conversion to alcohol slows down towards the end of the stationary phase as the yeasts run out of food and die. In the death phase, the total number of cells remains constant but viability decreases. After this, there is little activity and fermentation finishes. Yeast cells will precipitate, and form a dense layer at the bottom of the fermentation vessel. Depending on

the strain, and temperature during this '*sur lie*' period, autolysis will set in and cell contents will be released back into the medium.

2.2.9 An Overview of Alcoholic Fermentation

Under anaerobic conditions, deprived of oxygen, yeast can only convert sugars to carbon dioxide and ethanol, recovering less of the energy stored in the substrate molecules:

$$C_6H_{12}O_6 + \text{Yeast} \rightarrow 2C_2H_5OH + 2CO_2 + \text{ATP(Energy)} + \text{Heat}$$

A carbon compound (acetaldehyde) acts as terminal acceptor of electrons, which are generated during the conversion of sugar metabolites to energy in the form of ATP, and ethanol is formed. The final concentration of ethanol depends on the initial concentration of sugars (or other substrate) in the must or juice, as well as the fermentation temperature, since some ethanol is lost during warmer, faster fermentations. In winemaking, the sugar content of the grapes is therefore a very important factor when the time of harvesting is chosen. The amount of residual sugar in solution after fermentation is also an important stylistic consideration and the final sugar, acid and alcohol levels must be balanced in the finished product. Residual sugar is desirable in dessert or sweet wine, but not in most table or dry wines. A better understanding of the factors that influence sugar utilization and fermentation rate will allow for greater control over the fermentation parameters and therefore allow better diagnosis of problem fermentations.

The main sugars present in grape must, glucose and fructose, are metabolized to pyruvate via the glycolytic pathway in *S. cerevisiae* (as well as the majority of other organisms). In yeast, under fermentation conditions, the pyruvate is then decarboxylated to acetaldehyde, and further reduced to ethanol. The rate of fermentation and the amount of alcohol produced per unit of sugar during the transformation of grape must into wine is of considerable commercial importance. During wine yeast glycolysis, one molecule of glucose or fructose yields two molecules each of ethanol and carbon dioxide. However, the theoretical conversion of 180 g sugar into 92 g ethanol (51.1%) and 88 g carbon dioxide (48.9%) could only be expected in the absence of any yeast growth, production of other metabolites and loss of ethanol as vapor. In a model fermentation, about 95% of the sugar is converted into ethanol and carbon dioxide, 1% into cellular material and 4% into other products such as glycerol (Pretorius, 2000).

All fermentation pathways are anaerobic. Fermentation is a complex process, involving around 30 different steps, each with its own enzyme. Each 100 g of glucose theoretically produces 51.1 g of ethanol and 48.9 g of carbon dioxide. As discussed previously (Chapter 2.1), yeasts draw their energy from degradation of organic nutrients and the useful ('free') energy obtained is transported within cells as ATP. Depending on aerobic conditions (with or without oxygen), yeast can degrade sugars using two metabolic pathways: alcoholic fermentation and respiration. These two processes begin in the same way, glycolysis, where one sugar molecule produces pyruvate and a net gain of two ATP molecules. The process of fermentation requires two enzymes to metabolize pyruvate, the first is involved in the decarboxylation of pyruvate to acetaldehyde; the second reduces the acetaldehyde to NAD^+ and H^+ as well as ethyl alcohol (Purves *et al.*, 2001). It does not need oxygen or an electron transport system, and uses an organic molecule as the final electron acceptor. When phosphate groups are removed from ATP to produce ADP or adenosine diphosphate, 30.5 kJ (7.3 kcal) worth of energy per mole of compound is released, and some of this energy is coupled to useful activities within the cell like transport, movement or synthesis. The rest is dissipated as heat.

In a liquid environment like grape juice, yeasts will settle to the bottom of the container and grow anaerobically, consuming any dissolved oxygen as they oxidize simple carbon sources, such as monosaccharides, ethanol, acetate or glycerol, to carbon dioxide and water. On a nutrient surface or in a ventilated container,

they grow aerobically, with each cell (or colony forming unit) forming visible colonies within a few days. The pathway that operates is determined by the available substrate and the growth conditions.

A complete fermentation pathway begins with a substrate, includes glycolysis and results in various end products. The different fermentation pathways typically are named for the end products that are formed. As far as energy is concerned, fermentation itself does not generate ATP directly, but recycles a limited amount of NAD^+ back into glycolysis to keep the process going, and each pass through glycolysis generates two ATP molecules by substrate level phosphorylation. The change in free energy degrading a mole of glucose, via pyruvate to acetaldehyde and ethanol (and CO_2) is 167 kJ (40 kcal). Two ATP molecules are produced yielding about 63 kJ/mol glucose of useful energy, the rest (around 104 kJ) is dissipated in the form of heat (Jackson, 2000b). In respiration, the pyruvate is used with oxygen to produce nearly 15 times more biologically usable energy, as described later in this section. The availability of oxygen plays an important role in the metabolism, as molecular oxygen is the terminal electron acceptor during respiration, but it is needed for other reasons during high sugar, anaerobic fermentations. Despite the gains to be made from respiration, it is only in low sugar environments that yeast will respire pyruvate in preference to fermenting. In high sugar environments (e.g. musts) yeasts can only metabolize sugars by the fermentative pathway. Even in the presence of oxygen, respiration is impossible. This is known as the 'Pasteur effect' (Ribéreau-Gayon *et al*., 2000g). The aeration (oxygenation) of musts can still help alcoholic fermentation, but only because it enhances the production of fatty acids and sterols required by yeast cell membranes for proper function. According to Larue and coworkers (1980), aeration conditions of the medium determined the sterol content of yeasts which act as growth factors, fermentation inhibitors and survival factors for the yeast, particularly important if cells are to survive adverse conditions later in the fermentation. Ergosterol is the predominant sterol in yeast cells, but zymosterol, lanosterol and others are also present. In the cells, sterols account for 6% by weight of the protoplasmic membrane dry matter. The sterol content of the cells also differs with the strain of yeast and the conditions of culture. Molecular oxygen is also needed for the synthesis of the vitamin nicotinic acid (Jackson, 2000b).

The first step in alcoholic fermentation is the transport of sugars into the cell. This can be done in one of three ways: simple diffusion, facilitated (or carrier-mediated) diffusion or active transportation. Glucose, fructose and mannose are transported via facilitated diffusion, a process that does require energy and shares the same transportation system. Sucrose is sometimes used for enrichment, or chaptalization, in cool climate wine areas. Yeast cannot metabolize this disaccharide directly and it is hydrolyzed outside the cell by an excreted enzyme, invertase. The monosaccharides that result (glucose and fructose) are then transported into the cell (Jackson, 2000c). The disaccharide maltose is transported into the cell via an active transport mechanism, which requires energy. In other words, the rate of alcohol production by wine yeast is primarily limited by the rate of glucose and fructose uptake, and the loss of hexose transport towards the end of fermentation may result in reduced alcohol yields. Based on the spectacular increase in the amount of information on sugar sensing and their entry into yeast cells that has come to the fore over the last few years, several laboratories have identified this main point of control of glycolytic flux as one of the key targets for the improvement of wine yeasts (Pretorius, 2000).

The most common pathway for glucose (as well as fructose and mannose) catabolism is glycolysis, and the main purpose of this pathway under anaerobic conditions is energy production. It is noteworthy that when cells capable of fermentation like *S. cerevisiae* become anaerobic, the rate of glycolysis speeds up 10 times or more (Purves *et al.*, 2001), so a growth-sustaining level of energy generation is achieved despite the low yield of ATP generated by the metabolism of glucose through this pathway. During glycolysis, parts of the original glucose carbon skeleton will also be used for biomass formation or anabolism (the synthesis of biological molecules, amino acids, nucleic acids, lipids etc., according to the need of the growing and multiplying cells).

The glycolytic pathway (or glycolysis) (Figure 2.2.2), the conversion of glucose to pyruvate, is a universal pathway for glucose catabolism that is encountered throughout the eukaryotic kingdom, as well as in many

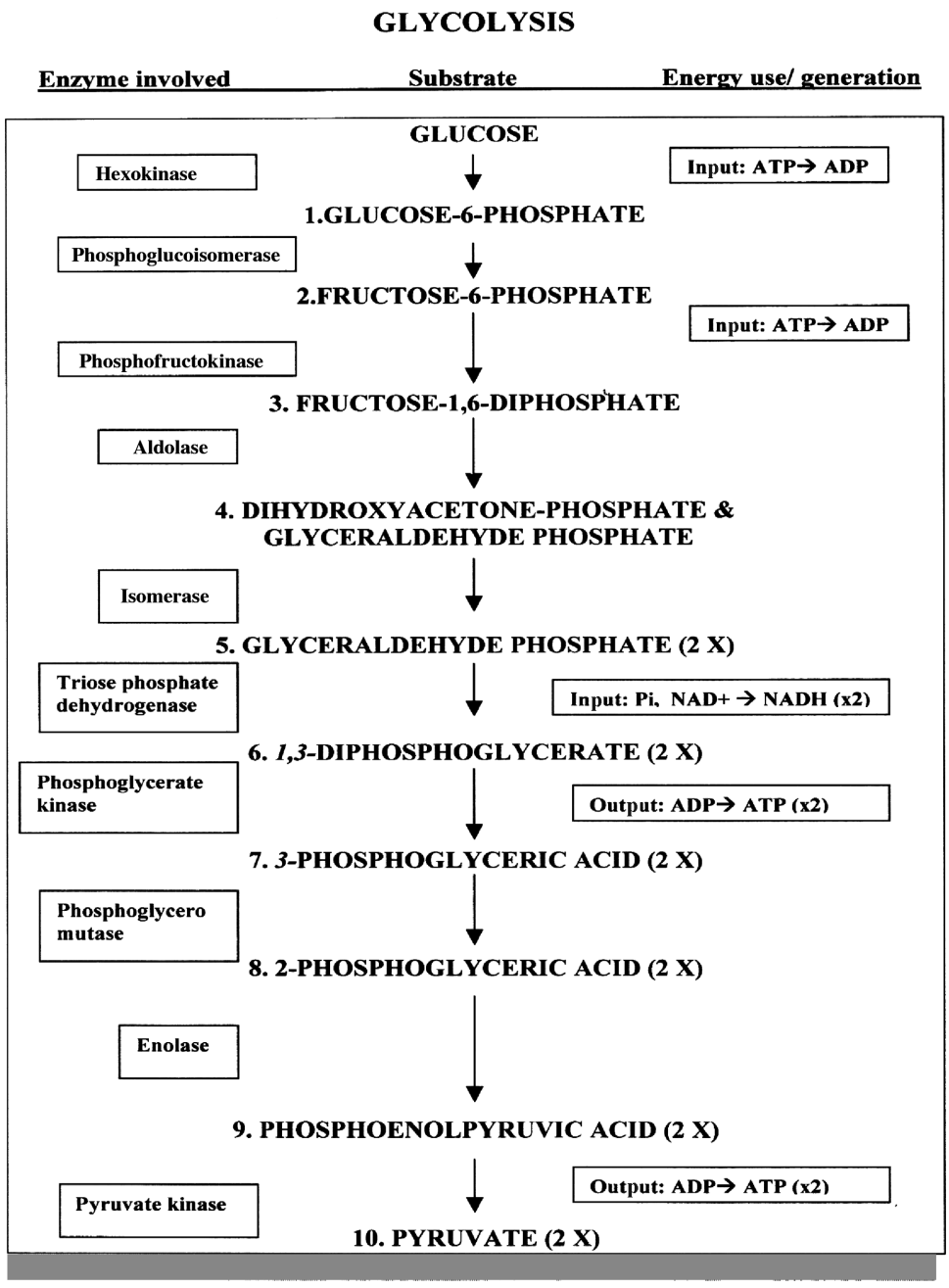

Figure 2.2.2 *An overview of the glycolytic pathway*

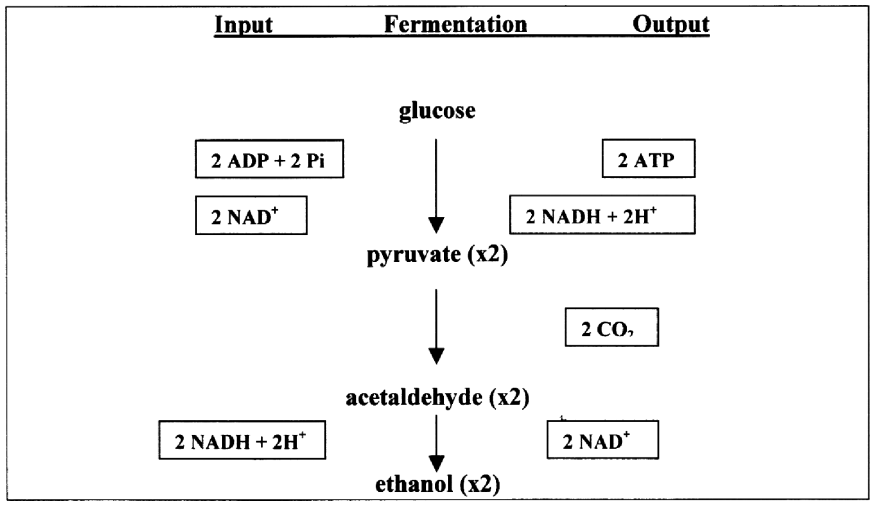

Figure 2.2.3 *An overview of alcoholic fermentation*

prokaryotes. This pathway is active under both fermentative and respiratory metabolism. Glycolysis consists of 10 steps, each catalyzed by a specific enzyme. The carbon skeleton of the sugar molecule is gradually dismembered during this process, and energy from the bond breaking is harvested in the form of ATP from ADP, and NADH from NAD^+. The first steps in glycolysis require an input of energy in the form of ATP, and serve to reorganize the sugar molecule. In fact, energy is invested in the first five reactions of glycolysis, and harvested in reactions 6, 7 and 10 (Jackson, 2000c). In step 4, the six-carbon sugar is split into two three-carbon moieties (glyceraldehydes and dihydroxyacetone) by the enzyme aldolase, and the preparatory reactions are complete. Thereafter, dihydroxyacetone is converted to glyceraldehydes and the two glyceraldehydes molecules are oxidized (hydrogen atoms with their electrons are removed), in the process reducing NAD^+ is to NADH. High energy bonds formed during glycolysis between phosphate groups and the substrate molecules are gradually broken, and the phosphate groups transferred to ADP molecules to form ATP.

During alcoholic fermentation (Figure 2.2.3), the pyruvate generated during glycolysis is converted to acetaldehyde, releasing carbon dioxide as a by-product. In *Saccharomyces*, the NADH from glycolysis acts as a reducing agent, and reduces acetaldehyde, which acts as the terminal electron acceptor, to ethanol. This process is necessary to maintain an acceptable redox balance within the cell (Jackson, 2000a). In the absence of oxygen, yeast cells are unable to transfer the energy stored in NADH to ADP and thus generate ATP other than through the reduction of acetaldehyde. Without this step, and the regeneration of NAD^+, the fermentation of glucose would cease altogether. In the early phases of fermentation, growth and cell division require considerable quantitities of reducing power (in short, the ability to get rid of excess electrons from within the cell). This is achieved through glycolysis, the diversion of sugar through the pentose phosphate pathway (to generate NADPH and precursors for nucleic acid synthesis), and the oxidation of pyruvic to acetic acid. The incorporation and reduction of compounds such as acetaldehyde and acetic acid that are initially released into the fermentation later on (Jackson, 2000c) helps to maintain the redox balance, and permit continuing fermentation. In contrast, during the declining phase of the fermentation, NADH and NADPH tend to accumulate.

Other organisms use other electron acceptors during fermentation and form, for example, reduced acids, such as the lactic acid bacteria. These organisms utilize pyruvate as a hydrogen acceptor during the reduction

to lactic acid, as do the cells of muscles in the human body during periods of strenuous activity (Purves *et al.*, 2001).

During respiration, which can be important in the earliest phases of the alcoholic fermentation and in all phases of commercial yeast production, most of the energy of the catabolism of carbon compounds is captured in the form of ATP. This is the result of the functioning of two metabolic pathways – the tricarboxylic or citric acid cycle and the electron transport chain. A further substrate level phosphorylation occurs during the succinyl-CoA-synthase reaction.

Fermentation and respiration are not mutually exclusive processes. If oxygen is available and glucose concentrations are low, *Saccharomyces* will switch to respiration for ATP production if fermentation cannot supply the ATP required for continued activity. This is not an instantaneous process, however, and yeasts need a period of adaptation to different growth conditions.

The next stage in respiratory metabolism is the citric acid cycle, also known as Kreb's cycle. Enzymes of this cycle and the electron transportation chain are situated in an endogenous organelle, the mitochondrion. Under anaerobic conditions, mature, fully functional mitochondria are not present in *Saccharomyces.* Since respiration is entirely dependent on oxygen, the enzymes required are only synthesized when needed, so in this respect, the availability of oxygen is a control mechanism for gene expression. In yeast, the expression of these enzymes is also controlled by the concentrations of glucose and other fermentable sugars in the medium. The expression is also suppressed by high concentrations of glucose and this regulatory phenomenon is known as glucose repression or the Crabtree effect (Boulton and Quain, 2001c). When the substrate is not limiting (i.e. present in high concentrations), yeasts depend entirely on fermentation or substrate level phosphorylation for the production of ATP. If the sugar concentration becomes limiting, the yeast need to convert to the respiratory metabolism in order to produce sufficient energy for continued growth and metabolism.

In the absence of oxygen, this metabolic shift will not take place. The citric acid cycle also provides the intermediaries that are required for amino acid and nucleotide biosynthesis. The enzymes, which are required for biosynthesis, are present in the cytoplasm in *Saccharomyces* under anaerobic conditions, as mitochondria are not functional. The mitochondrial and cytoplasmic forms are isozymes, i.e. similar in structure, but the product of different genes. The complete citric acid cycle does not exist in the cytoplasm under anaerobic conditions, but a form of the pathway serves to generate biosynthetic intermediates or end products of amino acid metabolism. Reverse flow along the pathway uses acetyl-CoA and helps form acetate esters. If the conversion of acetaldehyde to ethanol regenerates only limited NAD^+ from NADH, the reductive arm of the citric acid cycle can be used for this purpose with the associated production of malic and succinic acids. Otherwise, if reducing power in the form of nicotinamide adenine dinucleotide (NADH) is required, it can be generated via the oxidative arm of the citric acid cycle. Yeasts can utilize another metabolic process, the glyoxylate cycle (Walker 1998b), for the synthesis of sugars that are needed for cell wall structural components, glucans and mannans, and for growth substrates such as ethanol and acetate, but this pathway is only used if the cells grow in the absence of sugars. In the heterolactic malolactic acid bacteria, the pentose phosphate pathway is used for the metabolism of pentoses (five-carbon sugars) as carbon and energy sources, but *Saccharomyces* is not able to do this as the yeast requires this pathway for the formation of ribose-5-phosphate, a precursor of nucleotide biosynthesis (Jackson, 2000c). *Saccharomyces* also uses the pentose phosphate pathway for the formation of NADPH for biosynthetic purposes and erithrose-4-phosphate, which is required for the synthesis of aromatic amino acids. The pentose phosphate pathway therefore functions during both fermentative and respiratory growth.

In the cycle, pyruvate is decarboxylated and reacts with coenzyme A to form acetyl-CoA. The enzyme that catalyzes this reaction is pyruvate-dehydrogenase. Acetyl-CoA condenses with a four-carbon acid, oxalic acetate, to form citrate, a six-carbon acid, as well as a variety of other organic acid-intermediaries (Walker, 1998c). Under fermentative conditions, decarboxylation of pyruvate to acetyl CoA is inactive. One completed citric acid cycle generates two molecules of carbon dioxide and also regenerates oxalic acetate.

Three molecules of reduced (NADH) and one molecule of flavin adenine dinucleotide ($FADH_2$) are also generated.

Respiratory chain-linked NADH yields three molecules of ATP and two molecules of ATP from $FADH_2$, for a total of up to 14 molecules of ATP for each pyruvate that is metabolized. The generation of ATP during respiration is called oxidative phosphorylation; that which is formed during glycolysis is known as substrate level phosphorylation (Madigan *et al.*, 2003).

A further substrate level phosphorylation occurs at the succinyl-CoA-synthase reaction. Thus two molecules of pyruvate (equivalent to one glucose) moving through the citric acid cycle generate 30 molecules of ATP. Two molecules of ATP are produced during glycolysis giving a total of 32. If the pyruvate is completely oxidized to carbon dioxide, the two molecules of NADH that are produced during glycolysis can also be a source of ATP. This NADH molecule is cytoplasmic and not mitochondrial, and in order to be able to generate ATP the electrons must be transported to the mitochondria. Depending on the transportation mechanism that is utilized, two or three ATP molecules can be obtained for each NADH molecule that is oxidized. So, a single glucose molecule, when it is fully respired, will generate between 35 and 37 molecules ATP in total, of which 33 to 35 are from the oxidative phosphorylation and two from substrate level phosphorylation (Ribéreau-Gayon *et al.,* 2000h). The energy yield from the yeast metabolism is therefore around 15 times higher for aerobic sugar metabolism in comparison with anaerobic sugar metabolism.

To summarize then, under anaerobic conditions, or in a medium containing high levels of substrate, yeast will metabolize through fermentative pathways, rather than through respiration. Fermentation, despite producing so much less energy than respiration, still achieves two important metabolic goals. It uses NADH to reduce the pyruvate (or one of its metabolites) generated by glycolysis, and consequently NAD^+ is regenerated. This ion is used once more in glycolysis, so the cell can carry on metabolizing glucose. Also, fermentation allows the cell to produce a small amount of ATP through substrate level phosphorylation. Metabolic intermediates needed for cell growth will be supplied by glycolysis, the pentose phosphate pathway and the citric acid cycle.

Monitoring a fermentation allows the producer to ensure the fermentation is performing as required and allows prompt remedial action if any problems develop. The normal methods used are measurement of sugar (or, more accurately, dissolved solids) by hydrometry, measurement of temperature and sensory evaluation. One mole (180 g) of glucose yields about 103 kJ (24.5 kcal) as heat, and each percent of sugar (1° Brix) in the must generates enough heat during fermentation to raise its temperature by 1.3 °C/l, if no heat is lost. Hence, a wine must of 22° Brix would generate enough heat to raise its temperature by nearly 30 °C during the fermentation process, although some of this heat will be lost with the evolved CO_2 (Jackson, 2000b).

Important 'fermentation problem indicators' are high or low fermentation rate, temperature variations, turbulence and production of off odors such as H_2S. In difficult fermentations one of the first signs of a problem is the yeast settling as the fermentation slows down. It is thought that yeast cells die very quickly at the bottom of the tank due to sugar and nutrient depletion in that area, and that yeast that are in suspension are more likely to survive. Certainly, brewers know that suspended yeast cells provide the most efficient fermentation of beer wort (Section 2.6.4). Measures to rescue such 'sluggish' or 'stuck' fermentations include the increase of fermentation temperature, addition of vitamin supplements, limited aeration by pumping over and reinoculation (Pretorius, 2000).

Although alcohol is a major component of wine, there are many by-products of fermentation which will have a major impact on quality. Glycerol is not only produced by yeasts, but can also serve as carbon source in aerobically grown cultures. During wine fermentations, the main role of glycerol synthesis is to supply the yeast cell with an osmotic stress-responsive solute and to equilibrate the intracellular redox balance by converting the excess NADH generated during biomass formation to NAD^+ (Pretorius, 2000). Glycerol-3-phosphate, the precursor of glycerol, is an essential intermediate in the biosynthesis of membrane lipids.

Glycerol is viscous and has a slightly sweet taste (threshold 5.2 g/l in wine), giving smoothness, consistency and overall body to the product, so if it is produced abundantly during fermentation, it is usually seen as a positive contribution. The amount of glycerol produced by yeast during fermentation will vary according to nitrogen composition, sugar levels, sulfur levels and pH of the grape must, as well as fermentation temperature, aeration, choice of starter yeast strain and inoculation level. Typically, under controlled conditions, glycerol concentrations are higher in red wines than in white wines, varying from 1 to 15 g/l. About 4–10% of the carbon source is usually converted to glycerol, resulting in glycerol levels of 7–10% of that of ethanol. The overproduction of glycerol by yeast at the expense of ethanol could fulfil a growing need for table wine with lower levels of ethanol. Conversely, wine yeasts in which the glycerol pathway has been minimized would yield more alcohol, which would be of great value for the production of brandy and other distilled products (Pretorius, 2000) (Chapter 3.6).

The characteristic fruit flavors of wine are primarily due to a mixture of hexyl acetate, ethyl caproate, ethyl caprylate, isoamyl acetate and 2-phenylethyl acetate. Some of these aroma compounds have specific functions in the yeast cell, while others are still speculative (Pretorius and Lambrechts, 2000). Higher alcohols are produced during alcoholic fermentation through the conversion of the branched chain amino acids present in the medium (valine, leucine, isoleucine, threonine and phenylalanine), and are important precursors for the formation of esters, which are associated with pleasant aromas. They can also be produced *de novo* from a sugar substrate (Clemente-Jiminez *et al.,* 2005). Their exact function is unknown, but they may serve to detoxify any aldehydes produced during amino acid catabolism or be involved in the regulation of amino acid anabolism. Moreover, oxidative deamination provides the yeast with a mechanism for obtaining nitrogen. For this reason, amino acid metabolism to higher alcohols is restricted to the exponential growth phase. Tropical fruit notes are linked to acetates of higher alcohols, i.e. alcohols with carbon numbers greater than that of ethanol, such as isobutyl, isoamyl and active amyl alcohol. These compounds are produced by wine yeasts during alcoholic fermentation from intermediates in the branched chain amino acids pathway leading to production of isoleucine, leucine and valine by decarboxylation, transamination and reduction (Pretorius, 2000). At high concentrations, these higher alcohols have undesirable flavor and odor characteristics, but in wine they are usually present at levels below their threshold values and therefore they contribute favorably. Since higher alcohols are concentrated by the distilling process, they are not desired in wines that are to be distilled for brandy production (Chapter 3.6).

The effect of yeast on aroma and flavor development of the product is further discussed in Section 2.2.11.

The amount of alcohol produced by the fermentation depends, as previously stated, upon the initial sugar level and the strain of yeast used. Approximations can be made from the soluble solids level of the grapes, but the final alcohol level of the wine still needs to be measured for labeling purposes. As alcohol is a toxin as well as an intoxicant, there are strict regulations surrounding its production. Terminology for alcohol in winemaking is therefore complex, and can be confusing. Alcohol that could be made from the natural sugar in the wine is termed 'potential alcohol,' and needs to be determined before the fermentation process starts. Once this sugar has been converted to alcohol, it is termed the 'natural' alcohol of the wine. When alcohol is derived from added sugar (chaptalization), the wine is termed 'enriched' and this amount (according to UK legislation) has to be less than 3.5% (4.5% in poor years) (Morgan *et al.,* 2006). 'Actual' alcohol is that which is present in the wine after fermentation and chaptalization, and 'total' alcohol is determined by adding the contribution of potential alcohol from residual sugar in wine to the actual alcohol concentration. The way in which the alcohol content is displayed on the label is also subject to regulation, for example, the alcohol figure on a wine label must be accurate to within $\pm 0.5\%$ and prominently displayed. Duty is usually payable according to the alcoholic content of the wine. Alcohol is measured mainly by ebulliometry or distillation followed by hydrometry or pycnometry (Iland *et al.*, 2004), but there are many other methods (see Sections 4.3.2 and 4.6.3).

2.2.10 Flocculation

Yeast flocculation is a physical process and is of fundamental importance in most brewing and winemaking applications. During flocculation, yeast cells aggregate to form multicellular 'flocs' (or clumps) which sediment rapidly from the medium through precipitation, or are entrained by carbon dioxide and taken to the surface of the medium. The aggregation is brought on by interaction between lectin-like proteins on flocculent cell surfaces and mannose receptors on other cells. This interaction starts in early stationary phase, and it has been suggested that flocs form as a response of yeast cells to stress (Boulton and Quain, 2001d). Flocculation is critical to yeast recovery in a brewing context (Section 2.6.4) and to postfermentation clarification during winemaking. Yeasts can be removed through skimming the surface of the medium in open fermentors, or racking off (cropping) sediments in closed vessels. In-process changes in yeast flocculance are reflected in head formation during brewing. Although not usually quantified or monitored, yeast flocculation is a high profile fermentation parameter and is as important to a successful fermentation process as the rate and efficiency of conversion of sugar to alcohol.

If the fermentation is at the point where the producer wishes to finish it, a combination of refrigeration and/or accelerated clarification is normally used. The time taken to cool the ferment sufficiently should be taken into account and the ferment stopped earlier than the actual sugar level required. Clarification gets rid of the active yeast and allows other methods to work. The addition of SO_2 by itself to stop fermentation does not work in all cases, as the products formed during the fermentation (predominantly acetaldehyde) may bind and inactivate the SO_2 before it has had a chance to act against the yeast. If the number of suspended yeast cells has been lowered by clarification and activity reduced by cooling there is more chance it will be successful. It may still be necessary to add a larger than usual amount of SO_2 to ensure cessation of activity by the yeast. It is vital to keep a close check on the product towards the end of fermentation to see whether it is developing detectable levels of hydrogen sulfide. This is particularly true in the case of wines undergoing lees contact, which should be stirred through and, once the lees have settled, tasted. If there are high levels of hydrogen sulfide, the wine should be immediately racked and aerated or a copper treatment may be necessary, either by passing the wine over copper chips or by adding 0.2–0.4 mg/l copper sulfate (Morgan *et al*., 2006).

A wine chemist (Mulder, *ca.* 1857), working over a century ago, stated:

> 'A wine ferment ... is not essentially different from a beer ferment, for both consist of membranous cells which are formed out of gum or vegetable mucilage. Both possess albuminous contents, which exude through the walls of the cells, and when brought into contact with sugar, cause it to ferment. These contents are soluble in water, more so in vegetable acids, and peculiarly so in tartaric acid. So much of the albuminous substance as is soluble is decomposed during fermentation, and more or less of its products must be contained in the liquid.'

This quote is provided (with great respect to the author) to show how our knowledge of such a fundamental process has increased in detail, accuracy and magnitude over time. *S. cerevisiae* has enjoyed a long and distinguished history in the fermented food and beverage industries; it is without doubt the most important commercial microorganism with GRAS ('generally regarded as safe') status. With the emergence of modern molecular genetics, *S. cerevisiae* has again been harnessed to shift the frontiers of mankind's newest revolution, genetic engineering. *S. cerevisiae* was the first genetically modified organism (GMO), as distinguished from a genetically modified product (GMP), to be cleared for food use, as a baking and brewing strain (Pretorius, 2000).

2.2.11 Aroma Compounds and Fermentation

The aromatic potential inherent in grapes can either be realized or destroyed during the alcoholic fermentation. The yeast *S. cerevisiae* synthesizes a variety of volatile aroma compounds that form a very important part of the 'character' of the final wine and the task of choosing a yeast strain to produce desirable attributes in the final wine is significant. The preferable flavors of wine depend on a balance of volatile constituents such as acids, alcohols, aldehydes, ketones and esters, with yeast bouquet resulting largely from higher alcohols and esters (Swiegers *et al.,* 2005). Relative concentrations of the many by-products of fermentation can be influenced by the choice of yeast, the nutritional factors and the environmental conditions of the fermentation. Several studies suggest that the formation of varietal aroma is an integral part of yeast metabolism and not a simple cleavage of aromatic moieties from nonvolatile precursors (Loscos *et al.,* 2007; Koslitz *et al.*, 2008). The yeast chosen, given conditions in which it will ferment optimally, should produce the desired by-products, and few off odors such as hydrogen sulfide, mercaptans or acetic acid. It is generally accepted that these positive fermentation characters are easily lost during aging through oxidation and other chemical reactions in the bottle, so a wine that is dependent on yeast bouquet and/or primary aroma will be designed to be consumed early.

Some of the by-products of alcoholic fermentation are listed in Table 2.2.1, above. Glycerol provides sweetness and viscosity to the wine, affecting mouthfeel (body), but not aroma. The normal content in wine is 3–14 g/l but there is a higher concentration in 'noble rot' wines, as the fungus *Botrytis cinerea* produces glycerol in significant quantities. Specific yeast strains and high temperatures will also cause higher glycerol levels (Romano *et al.*, 1997). Levels of acetaldehyde, the major volatile carbonyl compound found in wine, vary between 10 and 75 mg/l. Wines exposed to air or to aerobic film-forming yeast will develop high levels of acetaldehyde as the oxidation of ethanol, through chemical or metabolic means, will produce it. It is characteristic of the aroma of oxidized or 'madeirized' wines like Madeira (Section 2.10.5) and also of flor wines like fino Sherry (Section 2.10.3), and can be detected sensorially at concentrations as low as 0.5 mg/l in aqueous ethanol (Francis and Newton, 2005), although the detection threshold would be significantly higher in wine (see Chapter 4.7 for discussion of sensory analysis). Acetaldehyde is frequently confused with ethyl acetate, which is a component of volatile acidity. The latter has the aroma of pear drops at lower concentrations, and is sharp and vinegary at higher concentrations. Ethyl acetate adds to complexity at lower concentrations, and is not considered to spoil a wine until levels become detectable (around 100–125 mg/l) (Francis and Newton, 2005). It is produced by yeast and bacterial metabolism, as well as through the chemical esterification of ethanol and acetic acid during wine aging. Acetic acid is formed by most yeast species during fermentation, but some strains produce more than others (Swiegers *et al.*, 2005). It has a sour, vinegary taste and aroma and is the major component of volatile acidity. It is produced by yeast as an intermediate in the pyruvate dehydrogenase pathway, but excessive concentrations are likely to be the result of metabolism of ethanol by acetic acid bacteria. It can also been seen as an indicator that the yeast is struggling to survive, as even *S. cerevisiae* strains that normally produce little acetic acid will show increases acetic acid production in musts with high sugar concentrations, low temperatures and marginal nutrition. Under normal circumstances, 0.25–0.35 g/l of acetic acid are produced during fermentation and concentrations of around 200 mg/l are detectable in aqueous ethanol (Francis and Newton, 2005). However, concentrations of acetic acid up to about 0.5 g/l do not have a detrimental effect on wine. The species *Hanseniaspora/ Kloeckera* is known to produce large amounts of acetic acid and ethyl acetate during fermentation. (Henick-Kling *et al.*, 1998). Research has also shown that it is a high producer of glycerol, esters and acetoin (Cadez *et al.*, 2002). Acetoin (3-hydroxybutanone) can be detected (organoleptically) at around 150 mg/l in aqueous ethanol and has a buttery or creamy aroma (Francis and Newton, 2005). Diacetyl and 2,3-butanediol, which are known to produce off flavors in higher concentrations, can be derived from acetoin, the former by chemical oxidation and the latter by yeast reduction. Generally, for wines fermented with pure cultures of apiculate yeasts,

Table 2.2.1 *Some odorous byproducts of fermentation*

Fermentation byproduct	Characteristic odor description
Acetic acid	Sour, vinegary
Acetone	Nail varnish
Acetoin	Buttery, creamy
Aldehydes (various)	Buttery, fruity, nutty
Butanediol	Sweet, sickly
Butyric acid	Rancid butter
Carbonyl sulfide	Ether, chemical
Diacetyl	Buttery, cloying
Dimethyl sulfide	Quince, truffle, stagnant water
Diethyl sulfide	Ether, pungent
Dimethyl disulfide	Quince, asparagus, garlic
Diethyl disulfide	Garlic, rubber
Ethanal (acetaldehyde)	Sherry-like, bruised apple, oxidized, unpleasant
Esters (various)	Fruity, floral
Ethyl mercaptan, ethanethiol	Onion, garlic
Formic acid	Tart, pungent
Fumaric acid	Smoky, pungent
Glycerol	Mouthfeel, sweetness
Higher alcohols (various)	Pleasant floral→ sickly fusel aromas
Hydrogen sulfide	Rotten egg
2-Mercaptoethanol	Burnt rubber
Methionol	Cooked cabbage, potato
Methyl mercaptan, methanethiol	Stagnant water, canned vegetable
Propionic acid	Cabbage, rancid

Source: Adapted from Swiegers *et al.* (2005)

high levels of acetoin are found in the final product, in contrast to mixed culture fermentations and pure *S. cerevisiae* fermentations, which have low levels (Romano *et al.*, 1993). This suggests acetoin may be utilized by *S. cerevisiae* to form 2,3-butanediol. Control of the production of diacetyl during fermentation is particularly important in the brewing of beer (Section 2.6.4). Higher alcohols (fusel oils) have agreeable odors at low concentrations and contribute to wine complexity, but at higher concentrations they are cloying and sickly. They are associated with fast, hot fermentations (Molina *et al.*, 2007) and are derived from amino acids via the Ehrlich pathway or synthesized as intermediates during sugar metabolism. Moreira and coworkers (1999) examined aroma production in *Hanseniaspora uvarum*, *H. guilliermondii* and *S. cerevisiae* in commercial culture media and grape musts, and found that media and wines fermented with *H. uvarum* present a low content of higher alcohols and sulfur compounds. As previously mentioned, esters are the products of reactions between alcohols and acids. They are very aromatic and provide aromas of banana, apple, peach and tastes of hazelnut or butter. *H. guilliermondii* produces relatively large quantities of esters (2-phenethyl acetate and isoamyl acetate in particular) and 2-phenylethanol, when inoculated as pure or mixed cultures in commercial media and grape must (Moreira *et al.*, 1999).

The effect on the aroma properties and chemical composition of coinoculating *S. cerevisiae* and *Candida stellata* in Chardonnay wine was studied by Soden and coworkers (2000). The fermentation did not progress to dryness and there was an increase in the concentration of glycerol and acetic acid. When the two species were fermented separately, as expected, sensory descriptive analysis showed a substantial difference in aroma

between the wines. The *C. stellata* produced significantly more intense 'honey,' 'apricot' and 'sauerkraut' aromas, and diminished the 'lime,' 'banana,' 'tropical fruit' and 'floral' aromas ascribed to *S. cerevisiae*. The authors suggested that a reliable mixed culture fermentation strategy may be a way of exploiting unconventional, fermentation-impaired yeasts for producing greater flavor diversity in wine.

Loscos and coworkers (2007) added an odorless flavor precursor fraction extracted from different nonfloral grape varietals to grape must and fermented it with three different yeast strains. The addition of the precursor fraction brought about a significant increase of the wine floral notes, irrespective of the yeast used. The levels of 51 wine aroma chemicals were found to depend on the precursor fraction addition and, in most cases, also on the yeast strain. Only β-damascenone, α-ionone and vinylphenols were produced at concentrations well above threshold. However, the concerted addition of groups of compounds has shown that lactones, cinnamates, vanillins and terpenes are together active contributors to the floral note.

Oxygen can affect the onset, progression and duration of the fermentation, favoring the growth of yeast cells and their fermentative activity (Ribćreau-Gayon *et al.*, 2000i). Yeasts require oxygen to synthesize and assimilate substances vital to their reproduction and if the must is not aerated sufficiently at the start of fermentation, oxygen will become the limiting factor for yeast numbers. The aeration of grape must prior to fermentation is usual in the winemaking process as a result of its transfers during the various processing stages. In the absence of oxygen, cell metabolism can be altered at the start of the fermentative process to favor the production of some aroma compounds, particularly esters and higher alcohols (Pretorius and Lambrechts, 2000). Exposure to oxygen in the initial stages of fermentation seems to increase survival for yeast during the declining phase of fermentation, leading to less residual sugar in the final wine. This may be explained by the partial removal of toxic medium length (C_8–C_{12}) fatty acid chains and accelerated synthesis of long chain (C_{16}–C_{18}) fatty acids and sterols, both factors contributing to a better sugar uptake through the cell membrane. The amino acid proline, which is not assimilable under anaerobic conditions, can be used as a supplementary nitrogen source in the presence of molecular oxygen. These effects seem to be more pronounced upon indigenous yeast than on active dry yeast multiplied under aerobic conditions (Ribereau-Gayon *et al.*, 2000i). The modern trend is towards the aeration in white wines to preserve greater aromatic complexity. This can be done using large doses of selected yeasts, which have been grown in hyperaerated media and so are rich in growth factors. Nevertheless, a slow onset of fermentation at normal temperatures indicates a lack of aeration (Zoecklein, 2006). If the fermentation medium is too reductive, yeast numbers may be too low, the fermentation will be slow and may end prematurely. Under these circumstances, there is a far greater chance that hydrogen sulfide will be formed. Therefore, early aeration of the must is recommended, particularly when using indigenous yeasts. Musts from rotted vintages, however, should be aerated as little as possible due to the risk of enzymic oxidation. Red musts can be aerated to a greater extent as the higher levels of phenolics present protect the must from oxidation to a certain extent. Rojas and coworkers (2001) found that highly aerobic conditions encouraged ester production by non-*Saccharomyces* yeast, whereas *S. cerevisiae* produced higher levels in minimally aerobic conditions. In an experiment regarding the effect of oxygen in the fermentative rate of different yeast strains, Valero *et al.* (2002) reported that the production of all higher alcohols monitored were dependant on the oxygenation conditions. The yeast strains studied (*S. cerevisiae* and *S. capensis*) produced lower concentrations of higher alcohols in musts in the absence of oxygen, which was attributed to the decreased growth of both yeast races under anaerobic conditions. The major esters studied showed a similar pattern, being produced in lesser quantities in the absence of aeration in both yeast races. Wines with higher ester/higher alcohol ratios possess an enhanced fruity flavor and better aroma quality, and there was a decrease in the ratio observed for the oxygenated wine compared to the nonoxygenated wine (Valero *et al.*, 2002). From these reports, it is therefore suggested that oxygen contact with the fermenting must should be kept to a minimum, in order for the resulting secondary compounds produced to have a positive impact on the sensory properties of the wine.

Sulfides in wine have distinctive aromas such as rubber, onion, garlic, cabbage, kerosene etc. Hydrogen sulfide in particular has a 'bad egg' aroma, and is a fault in wines that have had a stuck fermentation, or have been made and stored reductively. Hydrogen sulfide is a normal by-product of yeast metabolism, and the small quantities produced will escape with the carbon dioxide generated during fermentation. There can be several causes for abnormally high sulfur levels in wine, which include: spray residues, fermentation conditions such as low aeration, high temperatures and high solid levels, wild yeast strains, high concentrations of sulfur dioxide and long lees contact (Zoecklein, 2006). Yeast metabolism and chemical breakdown may lead to the production of volatile sulfur compounds from these sulfur sources, which can be very detrimental to the aroma. Any excess of hydrogen sulfide, if not removed immediately, will react with the alcohols in the wine to form other sulfides and mercaptans which have 'oniony,' 'rubbery,' 'cabbagey' or 'gasworks' aromas. Even exposing wines to a source of heat or to light can initiate chemical reactions involving methionine, which eventually lead to the production of methanethiol, which, on oxidation, forms dimethyl disulfide. Methionol (olfactory perception threshold of 1200 μg/l) is an unpleasant compound which is associated with atypical (premature) aging aroma in white wines. It is formed from an amino acid (methionine) in the grape juice, which undergoes successive deamination and decarboxylation reactions during yeast metabolism to produce methional and the alcohol.

Low levels of inorganic nitrogen may also cause hydrogen sulfide formation during fermentation. The optimal concentration of fermentable nitrogen depends on several factors, including yeast species and strain, and determining it is time consuming and expensive. Off odor formation in some yeast strains appears to be relatively independent of total fermentable nitrogen status over a fairly wide range, and may be more closely related to the ratio of certain amino acids in the must. However, yeast variability, with regard to reductive odor defect, is greatest under conditions of low levels of nitrogen or other stress factors. It is for this reason that most winemakers will automatically add diammonium phosphate (usually around 50 mg/l) to the must as a failsafe.

During fermentation, high levels of sulfur dioxide bind acetaldehyde, but if not enough of this compound is present, other juice components, such as sulfate, may be reduced instead, resulting in H_2S formation. One of the ways of avoiding this is to keep the concentration of sulfur dioxide before alcoholic fermentation below 8 g/hl (Zoecklein, 2006), or delaying sulfiting until after fermentation. It is important to act quickly if H_2S is detected during fermentation – wine should be aerated (briefly, so as to avoid oxidation of other components) and diammonium phosphate added, in case the production of H_2S is due to nutritional deficiencies. Hydrogen sulfide is oxidized by oxygen under these conditions ($2\ H_2S + O_2 \rightarrow 2\ H_2O + 2\ S$). The wine can then be racked, and the precipitated sulfur will be removed. One of the potential problems of using aeration to lower the concentration of some low boiling point volatiles, like H_2S and mercaptans, lies in the potential oxidation of mercaptans to disulfides (see above). To help avoid unwanted oxidation, especially in white wines, H_2S may be removed by sparging the must or wine with inert gas, such as nitrogen. However, this may take a significant quantity of gas, involve expense and manual labor, and may strip out positive aroma characteristics as well as the hydrogen sulfide.

The sulfur odor (reductive) defect may be an indication that a wine has a low oxidation–reduction (redox) potential, which can loosely be regarded as a measure of availability of oxygen (Zoecklein, 2006). The presence of off odor sulfur-containing compounds in a wine, and the corresponding smell, requires low oxygen concentrations (around –220 mV, compared to wine values of +220 mV to +450 mV, when wine is exposed to air). Reductive odor problems are not as great in barreled wines, due to the higher oxygen (redox potential) of barrel maturation. At the end of alcoholic fermentation, the addition of sulfur dioxide can represent a major cause of the formation of volatile sulfur compounds, mainly H_2S and methanethiol, in wines that have not been racked off the lees. Frequent barrel stirring to put the lees in suspension, and limited oxidation across the staves, inhibits the formation of postfermentation sulfur volatiles by increasing

the oxidation–reduction values. This occurs more rapidly in new barrels, as the oak contains more oxygen, and pores in the wood have not yet been blocked by phenolic and tartrates deposits. During barrel aging, volatile sulfur compounds generally decrease. All wines should undergo an odor screen for reductive-odor defect before bottling (Zoecklein *et al.*, 1995).

The temperature of fermentation has a considerable effect on the aromatic character of the wine. Low temperatures and slower fermentations are well known to help retain the fruit character of the juice. These aromatic compounds are lost at high temperatures. White wines should be fermented at low temperatures to preserve volatile aroma constituents that can be 'boiled off' with fast release of carbon dioxide. There is also a significant reduction in the levels of ethanol and glycerol produced at higher temperatures. Most authors agree that the optimum fermentation temperature for dry white and rosé wine production is about 15–20 °C and the maximum 23 °C, though the current trend is to ferment at temperatures below 15 °C. 'Noble rot' wines are usually fermented a couple of degrees higher than this. Red wines should be fermented at higher temperatures to maximize the extraction of color: the minimum recommended temperature for reds is 20 °C, the optimum 25–30 °C and the maximum 32 °C. In a study conducted by Molina *et al.* (2007), the influence of fermentation temperature on the production of yeast-derived aroma compounds at 15 °C and 28 °C was investigated. Higher concentrations of compounds related to fresh and fruity aromas were found at 15 °C, while higher concentrations of flowery related aroma compounds were found at 28 °C. The formation rates of volatile aroma compounds varied according to growth stage. In addition, linear correlations between the increases in concentration of higher alcohol and their corresponding acetates were obtained. These results demonstrate that the fermentation temperature plays an important role in the wine final aroma profile, and is therefore an important control parameter to fine tune wine quality during winemaking. In red wine fermentations the temperature also has an effect on the extraction of the phenolics and color. If the rate of fermentation is high, yeast growth and metabolism are encouraged, which then raises the temperature even further, leading to an even faster rate of fermentation. If this process is allowed to continue the heat produced may be sufficient to kill the yeast. This type of problem is more likely to occur in large volume fermentations with insufficient cooling at high ambient temperatures. In smaller fermentations it can occur if the cooling fails. Early recognition allows early intervention, which increases the chances of successfully increasing the fermentation rate so the ferment does not stop. On the other hand, fermentations that are too slow are inconvenient and give an increased probability of high acetic acid production. Lower temperature fermentations also favor estery characters in the bouquet that may not be desired in more full-bodied styles. The rate of fermentation and temperature should be chosen with the style of wine in mind, and both should be monitored constantly.

The formation of volatile compounds during alcoholic fermentation depends not only on the yeast species, but also on the particular strain (Pretorius and Lambrechts, 2000). It is important to know the potential differences in volatile production by yeast in order to select the best species, strain or mixture thereof, to produce a wine of a certain style and quality. Most cultured wine yeasts are not likely to produce off odors in large quantities. If a wild fermentation is carried out it must be watched carefully so that action can be taken if these faults develop. Some yeasts produce high ester concentrations (for example: strain QA 23) and these are used for neutral grapes as the yeast characters can add to the wine. Other yeasts such as VL3 are used with Sauvignon Blanc as they intensify the varietal aroma. In some cases yeasts such as CY 3079 are chosen for their influence on the body of the wine. In red winemaking, yeasts such as F10 are chosen for color and flavor extraction for early drinking reds. If fermentation difficulties are encountered, then EC1118 is a good strain to use. It can be used for restarting stuck ferments. In a study conducted by Patell and Shibamoto (2003), Symphony grapes were fermented with 20 different strains of the yeast *Saccharomyces*. Among 53 volatile compounds identified in the wines using gas chromatography/mass spectrometry, the major volatile compounds found were seven alcohols, seven esters and four acids. Isoamyl alcohol was the compound found in highest amounts with 19 yeast strains of Symphony wines. The amounts of isoamyl alcohol

ranged from 6.04 mg/l (A350/VL1/Fermiblanc) to 14.33 mg/l (Fermirouge). The other major compounds were 2-phenylethanol, ethyl-2-hydroxy propionate, monoethyl succinate, octanoic acid and hexanoic acid. Several esters, including ethyl-2-hydroxy propionate, may contribute to the fruity flavor of Symphony wines. Overall, 18 *S. cerevisiae* yeast strains (except A350/VL1/Fermiblanc and T73) used to ferment Symphony grapes produced the same major components, with certain variations in formation levels. The formation and composition of the volatiles produced by A350/VL1/Fermiblanc and T73 yeast strains were significantly different from the other strains.

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2.3
Malolactic Fermentation

2.3.1 Introduction

For centuries, winemakers observed that wines released small bubbles of gas and formed a haze, sometimes months after the alcoholic fermentation had been completed, and even in the bottle. This often occurred in the spring, when temperatures in the winery began to increase. Louis Pasteur and Hermann Müller-Thurgau recognized the bacterial causes of malolactic fermentation over a century ago, but it was not until the mid 1960s that the organism responsible was isolated, characterized and named *Leuconostoc oenos* by Ellen Garvie (Bartowsky, 2005). This organism is now known as *Oenococcus oeni*.

Malolactic fermentation (MLF) is the bacterial-driven decarboxylation of diprotic (S)-(-)-malic acid (sharp green apple character) to monoprotic (S)-(+)- and/or (R)-(-)-lactic acid (softer, yoghurt character) and carbon dioxide. It usually occurs after alcoholic fermentation, but studies investigating advantages of cofermentation (inoculating yeast and lactic acid bacteria simultaneously) are ongoing (Jussier *et al.*, 2006) and in the production of traditional Spanish cider, alcoholic and malolactic fermentations proceed more or less concurrently (Section 2.8.5).

In winemaking, it is one of the most difficult processes to control and can affect the final aroma and taste balance of the product by modifying fruit derived aromas and producing aroma active compounds (Nielsen and Richelieu, 1999). One of the main effects of the malolactic fermentation is deacidification, which is particularly desirable for high acid wine produced in cool climate regions, such as New Zealand and the United Kingdom. Malolactic fermentation is often spontaneous and difficult to stop in red winemaking, possibly due to the higher temperatures involved and/or through the activities of indigenous lactic bacteria found in cooperage. Producers will allow it to proceed or actively encourage it early in the life of a wine in order to prevent it happening in the bottle. After undergoing a malolactic fermentation, and as long as residual sugar levels are low, the wine is relatively microbiologically stable.

In addition to its occurrence in wine, this fermentation occurs in other fermented beverages, such as cider (Lea and Drilleau, 2003) (Section 2.8.4). *Lactobacillus* and *Pediococcus* are amongst the important spoilage organisms isolated from beer, and Van Vuuren (1976) noted that there is no doubt that the presence of lactobacilli during brewing greatly influenced the quality of beer produced. *Streptoccoccus* and *Leuconostoc* were also observed by this author to produce diacetyl in beer. Certain beers, such as the 'spontaneous' lambic beers of Belgium (Section 2.6.7) and certain native beers and fermented drinks of Africa, Asia and South America (Chapter 2.7) rely on the activities of LAB to contribute special character to the product. Likewise,

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

certain LAB can contribute positive characteristics to the fermented malted barley wort used for distillation into malt whisky, giving a product with greater complexity (Section 3.2.3). Nevertheless, in the production of both standard bottom-fermented (lager) beers and top-fermented ales, LAB are spoilage organisms, their presence being avoided at great lengths.

Lactic acid bacteria may also be present in significant numbers in base wine destined for brandy production (Chapter 3.6). In a study conducted by du Plessis in 2002, spontaneous malolactic fermentation was found to occur in around half the base wines studied, caused by populations of *Lactobacillus paracasei*, *Lb. plantarum* and *Oenococcus* spp. Concentrations of isoamyl acetate, ethyl acetate and 2-phenyl acetate were found to have decreased after the malolactic fermentation, but acetic acid, lactic acid and diethyl succinate increased. These changes had a significant effect on the quality of distillates from these wines, and most panellists preferred the distillates from wines that had not undergone MLF.

Many studies on the occurrence of malolactic fermentation in wine have indicated that lactic acid bacteria originate from grapes and winery equipment. Generally, the organisms occur on the surface of grapes and vine leaves (Wibowo e*t al.,* 1985) and as with acetic acid bacteria, the condition of the fruit has an influence. Their numbers are greatly reduced when working in new installations or in very clean conditions.

2.3.2 Malolactic Bacteria: Morphology and Physiology

Lactic acid bacteria are important in winemaking for two major reasons, they affect wine quality and they affect the economics of wine production (Wibowo e*t al.*, 1985). They are much smaller than yeasts, unicellular without a defined nucleus, are fermentative, aerotolerant, tolerant of high acidity and sensitive to sulfur dioxide in solution. The term 'malolactic bacteria' is usually used for those strains that prefer to metabolize malic acid over sugars and citric acid, and are more resistant to low pHs. The utilization of sugars as carbon and energy sources by lactic acid bacteria in wine has shown that there are species and strain differences in sugar utilization, but many species can metabolize pentoses, tartaric acid and glycerol.

Lactic acid bacteria are more exacting (fastidious) in their nutritional requirements than yeasts, and glucose and trehalose are generally preferred over other sugars (Liu, 2002). Morphology of the lactic bacteria found in wine is outlined in Table 2.3.1 below. They are broadly divided into two groups. Homofermentative

Table 2.3.1 *Comparison of major MLF bacteria in wine and cider*

Cell characteristic	*Oenococcus*	*Leuconostoc*	*Lactobacillus*	*Pediococcus*
Morphology	Spherical or lens-shaped cocci; occur in pairs and chains	Spherical or lens-shaped cocci; occur in pairs and chains	Long, slender rods, sometimes bent	Spherical cocci; occur in tetrads
Approx. size (μm)	0.6 × 1	0.6 × 1	0.8 × 5	0.4 ×1.4
Glucose fermentation	Heterolactic	Heterolactic	Both heterolactic and homolactic	Homolactic
Species found in wine and cider	*O. oeni*	*L. mesenteroides;*	About 16 species, including *Lb. brevis, Lb. buchneri, Lb. casei, Lb. cellobiosis, Lb. collinoides, Lb. hilgardii* and *Lb. plantarum*	*P. pentosaceus, P. damnosus, P. parvulus*

bacteria produce 80–90% lactic acid from glucose, and heterofermentative species produce between 30 and 50% lactic acid from glucose, also producing acetic and succinic acids, glycerol and ethanol (Jackson, 2000a). Heterofermentative bacteria also metabolize citric acid to acetic acid, lactic acid and carbon dioxide (Henick-Kling, 1995). In addition to being classed according to their products, they are also named according to their cell shape: 'coccus' for round and 'bacillus' for rods. Homofermentative cocci include *Pediococcus cerevisiae*, and heterofermentative cocci include the species *Oenococcus oeni*. *Lactobacillus casei* is an example of a homofermentative bacillus, whereas *Lactobacillus brevis* and *Lb. hilgardii* are heterofermentative. *Lactobacillus* and *Pediococcus* are capable of malolactic fermentation, but can produce undesirable characteristics – especially if the fermentation happens in the bottle. As with refermentations after bottling caused by the presence of yeasts, malolactic fermentations in the bottle can cause the production of carbon dioxide with off odors of vegetative, vinegar, mousy or geranium characteristics if sorbic acid is present.

With the introduction of molecular techniques, *Leuconostoc oenos* was reclassified as *Oenococcus oeni*, (Bartowsky, 2005). This species is generally preferred over other lactic acid bacteria in winemaking due to its acid tolerance and favorable flavor profile. It is microaerophilic, meaning that it grows best at low oxygen concentrations. *O. oeni* is heterofermentative and utilizes glucose via the phosphoketolase pathway, resulting in approximately one-sixth carbon dioxide, one-third ethanol, acetic acid or acetaldehyde and the remainder as lactic acid (Wibowo e*t al.,* 1985). However, if malic acid is present, this will be degraded before any glucose. Over centuries of selective pressure, *O. oeni* has become adapted to high ethanol concentrations ($<$15% v:v), low pH (as low as 2.9) and limited nutrient availability; conditions typical of a wine after alcoholic fermentation. Although *O. oeni* can tolerate this harsh environment, it can be a difficult and sometimes unreliable organism to work with. It is a fastidious organism and needs group B vitamins, an organic nitrogenous base and 11 amino acids, most of which can be derived from yeast autolysis. Lack of any one of these nutrients will inhibit growth.

2.3.3 Identification of Malolactic Bacteria

Lactic acid bacteria are Gram positive, catalase negative and fastidious. They require a medium rich in nutrients and will grow on glucose–yeast-extract agar and will be inhibited by penicillin. Isolation of lactic acid bacteria may also include the use of de Man Rogosa Sharpe agar (MRS) for the isolation of all three genera, *Leuconostoc*, *Pediococcus* and *Lactobacillus*, tomato juice agar and Irrmann agar. Wibowo and coworkers (1985) stated that no medium reliably recovers all of the malolactic species in a wine, and recommended the simultaneous plating of samples onto the three agars mentioned above. The incubation time and temperature are the same as that of acetic acid bacteria, but incubation of plates under an atmosphere enriched in carbon dioxide or nitrogen can encourage faster growth of the colonies of lactic acid bacteria (Fleet, 1994). Once isolated, a gram stain procedure can be performed and if the genera are all gram-positive, lactic acid bacteria can be rapidly and conveniently identified to species level with commercially available API 50 CH test galleries (Wibowo e*t al.,* 1985).

2.3.4 Factors Affecting the Growth of Malolactic Bacteria

The survival and growth of the bacteria are dependant on certain conditions, such as temperature, pH and sulfur dioxide levels. To encourage malolactic fermentation, the wine should have a pH of 3.3–3.5, no free SO_2, total SO_2 less than 50 mg/l and a temperature of 18–25 °C. Conditions that promote bacterial growth include extended skin contact, long lees contact with yeast autolysis and the inclusion of press fractions as the level of nutrients is increased. Carbonic maceration also favors growth. The yeast strain used should undergo

autolysis easily. When using indigenous bacteria (i.e. the population naturally present in the wine from grapes, barrels or lees), it is advisable to keep the wine temperature maintained at 18–20 °C. The presence of the bacteria can be very haphazard. Wineries vary greatly in the ease with which the MLF takes place. Often the fermentation is harder to start in newly established, clean or modern wineries. Once one tank has fermented, the lees may be used to inoculate other tanks if the wine showed no high increase in volatile acidity or other organoleptic defect. The bacteria also prefer an environment free of sorbic acid with low levels of glucose present (Morgan *et al.*, 2006). Factors affecting the growth of lactic acid bacteria are discussed in more detail below.

pH

Wibowo and coworkers (1985) stated that wine pH is one of the most important parameters that affect the behavior of lactic acid bacteria in wines and in wines above pH 3.5, spoilage flora of *Pediococcus* and *Lactobacillus* may grow. The pH of the medium needs to be considered in the selection of the bacterial strain as it affects the growth rate and yield, as well as the bacterial activity and the nature of the substrates involved. For most strains, minimal growth occurs at pH 3.0, and the lower the pH, the harder the malolactic fermentation is to start. At acidities between pH 3.0 and 4.0, *O. oeni* is the primary genus, different strains of which will dominate throughout MLF (Davis *et al.*, 1986). The optimum pH for the start of the fermentation is pH 3.5 to 3.8, and at pH 2.9 to 3.0 growth is possible, but slow. Inhibition of growth of *O.oeni* occurs above pH 4.5.

Sulfur Dioxide

The pH also affects the levels of sulfur dioxide, which influences both acetic and lactic acid bacteria. The higher the pH the lower the free sulfur dioxide, because the distribution of free or molecular sulfur dioxide in solution is a pH dependent equilibrium and it is the molecular form that is antimicrobial (for more detail on this see Chapter 2.5). Bacteria are more sensitive to SO_2 than yeasts, and so the wine is not sulfited after the alcoholic fermentation if malolactic fermentation is desired.

Although bound sulfur dioxide has little inhibitory activity against most yeast and acetic acid bacteria, at levels >50 mg/l it is believed to be inhibitory toward lactic acid bacteria (Fugelsang, 1997). Most lactic acid bacteria are sensitive to free SO_2 concentrations above 10 or 20 mg/l. Total SO_2 is usually kept below a maximum value of 70 mg/l (for reds) and 40 mg/l (for whites). Molecular levels of 0.6 mg/l are inhibitory, whereas levels of 1.2–1.8 mg/l are strongly inhibitory. Keeping molecular levels below 0.2 mg/l is desirable (Morgan *et al.*, 2006). It should be noted that *O. oeni* can degrade acetaldehyde. Thus, if SO_2 were previously added to the wine/must and subsequently became bound with acetaldehyde then the action of malolactic bacteria upon this bound complex will result in an increase in free SO_2. There is an interaction in effects of temperature and sulfur dioxide on bacterial growth. When no SO_2 is present in the wine the optimum temperature range for MLF is 23–25 °C (73–77 °F). However, this decreases with increasing concentrations of SO_2. Most strains of *O. oeni* either cease to grow or grow very slowly below 15 °C (59 °F). However, cells remain viable at low temperatures (Jackson, 2000a). Activity increases up to an optmum of 20–25 °C (Ribéreau-Gayon *et al.*, 2000), and bacteria start to die off above 30 °C (> 86 °F).

Contact with Yeast Lees

Lees provide nutrients for the bacteria, and by stirring the fine yeast lees, nutrients are released into solution even more effectively. Jackson (2000a) states that the polysaccharides released by yeast before and during the death phase at the end of fermentation stimulate growth. Substances that are toxic to the bacteria, such as

C10 and C12 fatty acids, which can be released during alcoholic fermentation, may inhibit growth if present in high enough concentrations, but this is unusual.

Temperature

This is the most easily monitored and controlled of the factors influencing the fermentation. Wines should be kept relatively warm if malolactic fermentation is required: the optimum temperature is between 20 and 37 °C in a laboratory culture. In wine (particularly when making white wine), it is not advisable to increase temperatures above 15 °C due to the risk of spoilage, but malolactic fermentation is unlikely to start below 20 °C (Morgan *et al.*, 2006). Depending on the temperature of the medium, the malolactic fermentation can take from a few days to several weeks. The fermentation may start with difficulty below 15 °C, but once started, it will carry on even if the temperature drops below 10 °C, but it may take several months to complete. As stated, temperature also has an effect on the resistance of the bacteria to sulfur dioxide.

Alcohol Concentration

The growth of lactic acid bacteria is also strongly influenced by the alcohol concentration, which appears to affect membrane function at higher concentrations. They are inhibited by levels above 13% v:v, and an 80% reduction in conversion of malic to lactic acid has been observed above this concentration (Jackson, 2000a). A few strains of lactic acid bacteria are able to grow in up to 15% v:v, but the higher the alcoholic strength, the longer the fermentation will take. Species differ in their tolerance, with *Lactobacillus* most resistant, and *O. oeni* most sensitive (Jackson, 2000a). Hence this must be taken into account when choosing a strain of bacteria. *O. oeni* appears to adapt to high alcohol environments over time, but loses this adaptation when returned to environments with lower alcohol concentrations.

Sorbic acid

This yeast inhibitor should not be added to a wine which is to (or may) undergo malolactic fermentation. The bacteria transform sorbate to 2,4-hexanediol, which reacts with ethanol to form an ester, 2-ethoxy-3,4-hexadiene. This has a strong smell of grass or geranium, and is the source of the 'geranium taint' (Jackson, 2000b).

2.3.5 Interactions Between Bacteria and Other Organisms

Interactions between coexisting yeast, usually *S. cerevisiae* and *O. oeni* (the most important MLF agent in wine) can cause problems with both fermentations (Lonvaud-Funel *et al*. 1988). *S. cerevisiae* releases ethanol, sulfur dioxide and medium chain fatty acids, which inhibit the bacteria. The utilization of complex nutrients such as amino acids by the yeast during the early stages of fermentation can complicate ensuing bacterial growth. Some authors recommend the addition of lactic nutrients to the wine if a coinoculation is to occur as yeasts tend to monopolize and consume most nutrients. Bacterial inhibition decreases towards the end of fermentation. This is probably connected with the death phase of yeast, as autolyzing cells release nutrients useful to the MLB. The growth of acetic acid bacteria during an alcoholic fermentation may favor *O. oeni*, as acetic acid is a known inhibitor of *S. cerevisiae*. Above pH 3.5, *Lactobacillus* spp. and *Pediococcus* spp. may have an advantage over *O. oeni*.

2.3.6 Spontaneous Malolactic Fermentation

Malolactic fermentation can occur before, during or after alcoholic fermentation. In traditional Spanish cider production, the two fermentations usually occur side by side (Section 2.8.5) (Blanco Gomis *et al.*, 2003), whereas in wine production, it is traditional for malolactic fermentation to occur after alcoholic fermentation, and frequently it happened automatically when wines were stored in barrels for ageing and maturation. However, using the natural bacterial population on the grapes and in the winery to conduct a spontaneous malolactic fermentation can be risky. The producer will not necessarily know which species is carrying out the fermentation, and the risks associated with this include degradation of glycerol to increase bitterness in the product, production of volatile phenols, production of pyridines, which lead to mousy odors, production of biogenic amines, degradation of arginine to ethyl carbamate and increased volatile acidity (du Toit, 2008).

Bacteria become resident in the wood or tartrate layer of cooperage, therefore a wine that has not been through, nor is intended to go through the malolactic fermentation cannot be made in a barrel which has previously been used for this purpose. Significant populations of bacteria are known to survive typical cleaning regimes that are applied to wooden casks or vats (see Section 2.8.5).

Lactic acid bacteria release enzymes that react with soluble substances in oak barrels, creating a wider range of flavors in a wine than would be produced in a sterile inert vessel. The MLF growth (lag) phase associated with spontaneous MLF (wild/uncultured strains) presents a time of increased risk from spoilage organisms due to the low SO_2 environment and the potential production of volatile acidity through the actions of other organisms or oxidation. Acetic acid is also produced during bacterial growth from sugar and organic acid degradation. Even under conditions chosen to stimulate development of the bacteria, the fermentation can take months to begin (Jackson, 2000a). Additionally, spontaneous malolactic fermentation in high pH and low SO_2 environments can be associated with unpleasant aromas and flavors, the source of which may be *Lactobacillus* and *Pediococcus* populations. The producer also runs the risk of introducing potential interference by bacteriophages (viral agents lethal to bacteria) present in a wild population.

When relying on wild bacteria, there can be no certainty of consistent results, but some producers feel that a spontaneous fermentation will add complexity and interest to their wines. Additionally, some areas in the world, which have been making wine in the same cellar (and region) for centuries, may have a resident population of spores and lactic acid bacteria, which produce consistent and favorable results for local producers. When using indigenous bacteria, it is advisable to keep the wine temperature maintained at 18–20 °C. The presence of the bacteria in wineries can be very variable, giving a wide variation in ease with which MLF takes place. Often the fermentation is harder to start in newly established, clean or modern wineries (for similar observations regarding traditional cider, see Swaffield *et al.* (1997) and Section 2.8.5).

2.3.7 Inoculation with MLB Starter Culture

In recent years, the introduction of commercial freeze dried bacterial cultures of *O. oeni* for direct inoculation into wine has ensured better control of the time of onset and the rate of MLF, reduced the potential for spoilage by other bacteria. Inoculating with a prepared culture avoids the problems associated with the bacterial lag phase by immediately providing the population necessary to conduct the fermentation. Commercially available preparations are normally freeze dried cultures of *O. oeni*, but some cultures contain more than one species of bacteria. Bacterial cultures are not easy to use as the bacteria are fastidious, but they are still more reliable than relying on natural populations (Morgan *et al.*, 2006). The winemaker also has more control over the flavor modifications. Inoculation of the bacteria is usually conducted after the wine has been fermented dry by yeast. This avoids the possibility of MLB metabolizing the sugar and producing unwanted products such as acetic acid. Some winemakers inoculate during or before the end of alcoholic fermentation, taking advantage

of the low alcohol, higher nutrient environment. In the event that the malolactic fermentation completes soon after alcoholic fermentation, sulfur dioxide can be added, and the wine is then protected from bacterial and oxidative attack.

It is important that the MLB have enough nutrients to develop. Fermenting yeast (*S. cerevisiae* var. '*bayanus*' in particular) can reduce the nutrients available to MLB considerably. Winemakers often add an MLB nutrient when inoculating with MLB to assist their development. When considering the timing of inoculation, the compatibility of yeast and LAB compatibility should also be considered. Inoculating grape musts with wine yeast and lactic acid bacteria concurrently in order to induce simultaneous alcoholic fermentation and malolactic fermentation can be an efficient alternative to overcome potential inhibition of bacteria in wines because of high ethanol concentrations and reduced nutrient content. The simultaneous inoculation of yeast and lactic acid bacteria was studied by Jussier *et al.* (2006) in cool climate Chardonnay must. Glucose, fructose, acetaldehyde, several organic acids and nitrogenous compounds were measured during the fermentations along with the final values of other key wine parameters. Sensory evaluation was done after 12 months of storage. No negative impacts of simultaneous AF/MLF on fermentation or sensory wine parameters were found. Acetic acid concentrations were slightly higher in wines after simultaneous AF/MLF, but the differences were not significant. Neither were any statistically significant differences found in pH or total acidity, nor in the concentrations of ethanol, acetaldehyde, glycerol, citric and lactic acids, and the nitrogen compounds arginine, ammonia, urea, citrulline and ornithine. Sensory evaluation by a semiexpert panel confirmed the similarity of the wines. Simultaneous inoculation of yeast and bacteria did, however, lead to considerable reductions in overall fermentation durations (Jussier *et al.*, 2006).

Another winemaking option is partial malolactic fermentation, which is used when the producer wishes to retain crisp acid and fruit character, but gain fullness and complexity from the bacterial metabolism. The volume of wine will be divided in a certain ratio, one portion goes through full MLF and the other no MLF. The two portions are then blended back together. The best method in providing bacterial stability therafter is to sterile filter the wine through a 0.45 μm (0.45 micron) membrane filter, as well as dosing with sulfur dioxide.

2.3.8 The Growth of Bacterial Populations

The growth of the lactic bacteria population may occur at the start of the alcoholic fermentation, during fermentation, at the end or later. The latent period in between is partly due to the formation of alcohol by the yeasts and can be prolonged by low temperatures after the alcoholic fermentation. Lactic acid bacteria require a certain population level (10^5 cells/ml) to be reached before they can begin malolactic fermentation, and inoculation will customarily achieve a million to 10 million cells per millitre (Jackson, 2000a). Due to the stress that inoculated bacteria undergo in adapting to the wine environment, inoculations of the bacteria may even be increased to population loads of at least 10^8 cells/ml. A bacterial population may simply be dormant and become active when conditions are more favorable. This explains the often seen delay of malolactic fermentation in bottled wines, as well as the increase in activity in cellared wines over the spring as cellar temperatures rise. Usually natural/indigenous MLF fermentations can have a lag phase of weeks to several months before they begin and this is prolonged at lower pH (Morgan *et al.*, 2006). The bacterial population will die back to 10^3–10^4 cfu/ml during alcoholic fermentation as cells lyse rapidly, but may rise to 10^6–10^8 cfu/ml once growth initiates substantially (Jackson, 2000a). After exponential growth, cell populations decline rapidly depending on the conditions (e.g. elevated temperatures or SO_2 will increase the rate). It is possible that the population of strains of *Lactobacillus* and *Pediococcus* may increase at this point. The malic acid breakdown usually occurs after the alcoholic fermentation when the bacterial population becomes greater than 10^7 cells/ml.

2.3.9 The Malolactic Fermentation Process

Malolactic fermentation is the conversion of (*S*)-(-)-malic acid to monocarboxylic (*S*)-(+)- or (*R*)-(-)-lactic acid and carbon dioxide, catalyzed by malate decarboxylase, and requiring the coenzyme NAD^+ and manganese (Mn^{2+}) (Du Toit, 2008). Only (*S*)-(-)-malic acid will be converted by MLB, but whether (*S*)-(+)- and/or (*R*)-(-)-lactic acid is produced depends on the bacterial strain and substrate involved. If *O. oeni* is the MLB, which is frequently the case, then the major product is (*S*)-(+)-lactic acid, as shown in Figure 2.3.1.

(*S*)-(-)-Malic acid concentration in grape juice varies depending upon the climatic regions from which the grapes are sourced; cool climate regions are often in the range 2–5 g/l, whereas in warm regions, grape juice often contains less than 2 g/l (Zoecklein *et al.*, 1995). Other substrates, such as residual sugars and citric acid are often broken down to obtain energy. Tartaric acid is only degraded in wines whose pH is above 4.

The malolactic fermentation is summarized in Figure 2.3.1. It consists of three steps:

- The uptake of malic acid from the external environment by a specific transporter, malate permease
- The decarboxylation of malic to lactic acid with an increase in internal pH of the cell
- The transport of lactic acid from the cell with one hydrogen ion, and an associated increase in proton motive force over the cell membrane.

The increase in proton motive force triggers the production of ATP within the cell (Du Toit, 2008). For every gram of malic acid metabolized, around two thirds is converted to lactic acid and a third to carbon dioxide, evidenced by the slight 'spritz' often found in wines undergoing the fermentation. The decarboxylation of malic acid (Figure 2.3.1) can reduce titratable acidity by between 1 and 4.6 g/l and increase pH by around 0.1–0.25.

Lactic acid bacteria are strictly fermentative. They have limited synthetic abilities, as stated previously, and are unable to produce heme proteins, cytochromes or catalase. Despite this, they are able to grow in the presence of limited amounts of oxygen, and detoxify oxygen radicals by accumulating large quantities of manganese ions, or by producing peroxidase enzymes. Manganese detoxifies superoxide by converting it to oxygen and peroxidase oxidizes peroxide to water (Section 5.8.4) (Jackson, 2000a). Although fermentative metabolism is inefficient in terms of energy production as discussed in Chapters 2.1 and 2.2, by metabolizing malic acid to lactic acid, acidity in the medium is reduced and the pH increased, which is beneficial for the bacteria.

Both malic acid and citric acid in the wine are metabolized by *O. oeni*. The cofermentation of citrate and glucose in *O. oeni* is physiologically important for this bacterium (Liu, 2002), and has been shown to enhance its growth rate and biomass yield, which result from increased ATP synthesis both by substrate level phosphorylation via acetate kinase and by a chemiosmotic mechanism (proton motive force). The growth stimulation of citrate–sugar cofermentation by the same mechanisms has also been reported in citrate-fermenting dairy bacteria (Nielsen and Richelieu, 1999). Malolactic bacteria follow a similar metabolic pathway to that of dairy lactic acid bacteria in the metabolism of citrate. Citrate is transformed to lactate, acetate, diacetyl, acetoin and 2,3-butanediol. A small amount of citrate is converted to aspartate via oxaloacetate and aspartate

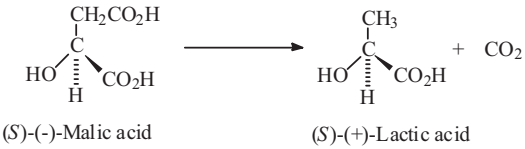

Figure 2.3.1 *Summary of MLF*

aminotransferase (Liu, 2002). From a winemaker's point of view, the cometabolism of citrate–sugar increases the formation of the volatile acid (acetate) in wine, which can affect the wine aroma detrimentally if present at excessive levels.

Ethyl carbamate (EC) is an animal carcinogen found in many fermented foods and beverages, including wine (Section 5.11.4). It is formed through the chemical reaction of ethanol and a precursor, such as citrulline, urea or carbamyl phosphate (Liu, 2002). Citrulline is an intermediate in the degradation of arginine by lactic acid bacteria. Arginine is a major amino acid present in wine: it is catabolized via the arginine deiminase pathway (ADI). Arginine degradation via the ADI pathway produces ATP, which is necessary for survival and growth of the bacteria. Carbamyl phosphate is also a precursor to pyrimidine and some bacteria can synthesize carbamyl phosphate from glutamine, bicarbonate and ATP, so this is potentially another source of ethyl carbamate. Factors affecting arginine degradation include LAB strain, pH, arginine concentration and sugar type. Citrulline excreted during arginine degradation, and ornithine, can also be catabolized by some wine LAB (Liu, 2002).

2.3.10 Production of Diacetyl

The most important enological significance associated with citrate fermentation is the production of diacetyl, an aroma compound with a buttery, nutty flavor note. It has been demonstrated that threshold values in different wines vary from 0.2 mg/l in Chardonnay wine to 0.9 mg/l in Pinot Noir and 2.8 mg/l in Cabernet Sauvignon wine. In general, wines that have undergone malolactic fermentation have higher concentrations of diacetyl (Martineau and Henick-Kling, 1995). The final level of diacetyl in wine is affected by a number of factors, such as bacterial strain, wine type, sulfur dioxide and oxygen (Nielsen and Richelieu, 1999). It should be pointed out that diacetyl is formed chemically from the oxidative decarboxylation of α-acetolactate, an unstable intermediary compound produced during citrate metabolism (Bartowsky *et al.,* 2002). *O. oeni*, as well as a number of other bacteria contain diacetyl reductase that converts the flavorful diacetyl to the much less flavorful acetoin and 2,3-butanediol. α-Acetolactic acid may decarboxylate spontaneously to acetoin and, in oxidizing conditions, also to diacetyl (Nielsen and Richelieu, 1999). In lactic bacteria-fermented dairy products, the latter reaction is now generally believed to be the only source of diacetyl.

After sulfiting, most wines are stored in tanks, barrels or bottles in a span ranging from a few months to several years. During this storage, SO_2 levels will gradually reduce as the molecule reacts reversibly or irreversibly with different compounds in the wine. Immediately after addition, some of the free SO_2 will combine with diacetyl. As the reaction is reversible, it can be the case that the diacetyl concentrations will increase later during storage as it is released once more into solution.

Oxygenation, high concentrations of citrate and sugars, lower temperature (18 °C), removal of yeast cells before malolactic fermentation and low inoculation rate all favor the production of diacetyl (Martineau and Henick-Kling, 1995). The presence of viable yeast cells during the fermentation, prolonged contact with bacteria and addition of SO_2 cause diacetyl reduction. Besides the microbial activity, the final diacetyl concentration in wine is also affected by the concentration of SO_2, which reduces it significantly. For example, addition of 80 mg of SO_2 per liter, which is within the range used in the wine industry, reduced the diacetyl concentration from initially 20 to as low as 5 mg/l, i.e. by 75% (Nielsen and Richelieu, 1999).

2.3.11 Microbial Stability

Under certain conditions, malolactic fermentation can increase the microbial stability of a wine. Some spoilage bacteria attack malic acid and by reducing its concentration, a more microbiologically stable wine will result.

Lactic acid bacteria also consume nutrients (amino acids, nitrogen bases, vitamins) and this reduction has been thought to increase microbial stability by limiting the potential growth of spoilage organisms. However, it has been shown that wines that have completed MLF can still support *O. oeni*, *Lactobacilus* spp. or *Pediococcus* spp., if SO_2 levels remain low (Morgan *et al.*, 2006). Malolactic fermentation can also raise the pH above 3.5, which provides a more favorable environment for spoilage organisms.

2.3.12 Monitoring Malolactic Fermentation

The progress of the malolactic fermentation can be monitored qualitatively in wines by tracking the decrease in malic acid and accompanying increase in lactic acid using paper or thin layer chromatography (Section 4.3.1). The usual criterion for completion of the malolactic fermentation, and subsequent addition of sulfur dioxide, is the exhaustion of malic acid. This may result in an incomplete citric acid degradation in the wine (Nielsen and Richelieu, 1999), leaving this as substrate for further bacterial activity if the wine is not sterile filtered. Other methods, for example chromatography (Sections 4.3.1 and 4.3.3), enzymatic/colorimetric analysis (Section 4.4.3) or infrared spectroscopy (Section 4.4.2) can also be used, but these are more complex and require capital investment into appropriate equipment. In the event that the producer is able to quantify the loss of malic acid, it is generally accepted that the MLF is complete when malic acid level falls below 0.1 g/l.

2.3.13 Finishing and/or Preventing Malolactic Fermentation

To prevent malolactic fermentation in wine, keeping maceration on skins and contact with yeast lees to a minimum, and maintaining relatively high levels of SO_2 at completion of the alcoholic fermentation, are required. Sterile filtration and pasteurization, if appropriate, are also good preventative measures, as this reduces bacterial load. Lowering pH (below 3.1 for whites, below 3.3 for reds) and storage temperatures (12 °C) will help prevent bacterial growth, as will sterile filtration to the 0.45 μm (micron) level. The use of a commercial antibacterial lysozyme (an enzyme that destroys the bacterial cell walls by catalyzing the hydrolysis of specific glucosidic links) can also help. In wine, an alcohol content of greater than 13% will inhibit malolactic fermentation.

Once malolactic fermentation is over, the wine should be racked, filtered and sulfited (40–50 mg/l to maintain the free levels at 20–30 mg/l). At this point, it is vital to clean all winemaking equipment thoroughly and maintain aseptic conditions in the winery.

Species associated with wine spoilage are generally members of *Lactobacillus* and *Pediococcus* genera. *Lactobacillus*, for example, can cause acescence (excessive acetic acid) by metabolizing residual sugar or tartaric acid. Both acetic acid and lactic acid bacteria are readily found in wines and the winery, but to minimize excessive growth leading to possible wine spoilage, measures should be taken to reduce contamination. Basic hygiene of winery equipment and the winery is essential and the proper storage of musts and wines will minimize spoilage. The removal of grape skins, a major source of bacteria coming into the winery, and the cleaning up of juice and wine spillages reduces the transfer of bacteria to the wine and equipment. Other then the winemaking conditions and the use of antimicrobial agents, a further method to minimize the growth of spoilage bacteria is the physical removal of such organisms by the way of filtration. The effectiveness of filtration depends on the correct set up being used, to ensure the filters are not compromised, and this is dependent on the wine style and the turbidity of the solution.

2.3.14 Production of Biogenic Amines by Malolactic Bacteria

Some lactic acid bacteria possess enzymes that decarboxylate amino acids to form the corresponding amines and carbon dioxide. Amines are toxic substances that have deleterious effects on human health if consumed in excessive amounts and some people can suffer severe allergic reactions from ingesting very low levels of amines in alcoholic beverages (Section 5.11.3). They are found in a range of fermented foods and beverages, such as fermented fish, cheeses, beer and meat products (Liu, 2002). *Lactobacilli*, *Lactococci* and *Leuconostoc* species isolated from beer, cheeses and meat starter cultures are known to produce histamine and tyramine via the decarboxylation of the corresponding amino acids, histidine and tyrosine. The major amines found in wine are histamine, tyramine, putrescine and phenylethylamine. The role of lactic bacteria and the malolactic fermentation in the biogenesis of amines has now been defined, since the decarboxylation of histidine to histamine and tyrosine to tyramine has been demonstrated with single strains of *Lactobacillus* and *Oenococcus* (Liu, 2002). Lactic bacteria vary in their ability to produce amines from amino acids, and in wine, it appears that the *Lactobacillus* and *Pediococcus* are the main producers. Histidine decarboxylase activity is also common in *Oenococcus*, which is primarily responsible for histamine formation.

Glyoxal and methylglyoxal found in wine are toxic compounds and also have implications for human health. It appears that *Oenococci* can produce glyoxal and methylglyoxal during malolactic fermentation. However, definitive studies are required to link the production of glyoxal and methylglyoxal with specific strains of *Oenococci* and other lactic bacteria.

2.3.15 Other Effects of MLF

It is generally accepted that the effect of the malolactic fermentation will vary according to the wine. It has a reputation as a destroyer of fruit character, red wine color and acid balance in low acid wines from warm areas. Also, MLF can be accompanied by by-products, sometimes of the sulfide or mercaptan type (cabbage, canned asparagus or green vegetable notes), or at other times diacetyl is formed, which has a buttery odor. Two consequences of deacidification during MLF are an increase in pH and a decrease in titratable acidity, which obviously affect taste, but have other, indirect consequences for the producer. As stated previously, an elevated wine pH can increase the susceptibility of wine to microbial spoilage. It can also decrease the efficacy of sulfur dioxide, and have implications for color and oxidative stability.

If a reduction in acidity is desired and the wine style is not driven by varietal aroma and flavors, malolactic fermentation may increase the quality of the wine. The malolactic fermentation is not encouraged in the making of wine from ‘aromatic’ varieties (those rich in terpenoid content) like Riesling, Gewürztraminer and the Muscat family or indeed any whites where primary (grape) aroma is important. Although malolactic fermentation traditionally occurred in red wine, some white wines, for example Chardonnay, can also benefit from the increased complexity. Base wines for sparkling wine production can be fairly acidic (pH 3.0), and most producers encourage malolactic fermentation in the base wine as it is impossible to remove bacteria during degorgement.

Malolactic fermentation can help to alleviate astringency in a number of ways. A decrease in acidity will decrease perceived astringency as the two sensations enhance each other. A raised pH may help polymerizing reactions to progress at a faster rate, and soften tannins and malolactic bacteria also metabolize a small percentage of the phenolics. A by-product of malolactic fermentation, ethyl lactate, often exceeds the sensory threshold of 150 mg/l giving fuller, enhanced mouthfeel.

Lactic acid bacteria are able to form exocellular polysaccharides (‘ropey’ wine) (for example, *Pediococcus* spp. produce a trimeric β-glucan, composed of three β-D-glucose units), but it seems to need a combination of factors including low sulfur dioxide and a mixed population of bacteria. In contrast to what is known

about the phenomenon in wine, a great deal of information is available on the exocellular polysaccharides in fermented dairy products. It appears that the bacteria in wine may be able to catabolize polysaccharides, which is one reason why 'ropey' wines are not common. *O. oeni* has been shown to possess extracellular β-(1-3)-glucanase activity and has the potential to degrade polysaccharides, such as β-glucans (Liu, 2002).

In conclusion, as Bartowski (2005) states:

> 'Living in wine can be very stressful for a cell; it requires a great deal of resilience to tolerate, amongst other things, high levels of ethanol and a low pH. What is required of O. oeni to survive and grow in such a hostile world? What distinguishes it biochemically and physiologically from most other bacteria, which struggle and perish in this habitat, and that includes the sturdiest of the other LAB? We do not have the answers yet, but we do have some ideas from research on other organisms, and work is well underway in several laboratories to address them for O. oeni.'

2.3.16 Malolactic Fermentation and Aroma

The impact of malolactic fermentation on the taste of wine as a result of deacidification is well recognized, but the effect on wine aroma and mouthfeel/body is ill-defined (Liu, 2002). Davis and coworkers (1985) reviewed the contribution of MLF to wine aroma and found no consistent impact of MLF on wine aroma. Nielsen and Richelieu (1999) cite studies (including that of Davis) that may be used to support the argument that malolactic fermentation does not significantly affect the flavor of wine other than to adjust the acidity. However, there are also those (e.g. McDaniel *et al.,* 1987) that state that similar wines which have undergone MLF can be distinguished fairly easily from those which have not. Swiegers and coworkers (2005) note that research in progress shows that bacteria can significantly modify components and sensory properties in wine. Since 1985, further work has been carried out to investigate the effect of MLF on sensory properties of wine using a more stringent panel training method, gas chromatography (GC)-olfactometry and GC-mass spectrometry (Liu, 2002). What is undeniable is that lactic acid bacteria produce a diverse range of products during their activities (including succinate, acetate, acetoin, lactate, diacetyl, mannitol, higher alcohols and a number of the biogenic amines) depending on available substrates and the strain of bacteria.

The compounds produced by lactic acid bacteria are claimed to modify or mask vegetal characters, and impart nutty, lactic and/or earthy aromas. It is probably safe to say that the process mellows the product and gives it complexity, but in some cases freshness, fruit and varietal aromas may be lost. The producer needs to consider these issues when deciding whether to carry out a malolactic fermentation, for example, where freshness and vivacity are important and in terpene-rich cultivars (like Riesling, Gewurztraminer and the Muscat types), the process is seldom recommended. In the case of barrel-fermented and aged Chardonnays (e.g. Burgundies) and red wines, where complexity is desirable, malolactic fermentation may enhance the quality and style of the wine considerably.

Diacetyl

Diacetyl (2,3-butanedione) is one of the major by-products of citric acid metabolism, which is closely linked to malolactic fermentation (du Toit, 2008). The compound imparts a buttery flavor to the wine, which benefits neutral cultivars, but is generally thought to have a negative impact on aromatic varieties. Threshold levels vary for different wines, depending on cultivar and style, as well as the personal preference of consumers. Jackson (2000a) states that 1–4 mg/l of diacetyl adds 'a desirable complexity' to the fragrance, but at more than 5 mg/l the aroma becomes overt and undesirable. On testing wines from 20 different regions, 28 different producers and eight vintages, Bartowsky *et al*. (2002) found that Chardonnay wines showed levels of diacetyl

between 0.3 to 0.6 mg/l, while reds (Cabernet Sauvignon, Merlot and Shiraz) showed 0.3 to 2.5 mg/l (mean value 1.1 mg/l). The extent of diacetyl formation strain also depends on strain (e.g. *Streptococci*), as well as oxygen and citric acid content.

Diacetyl appears to be a metabolic intermediary compound in the citric acid metabolism by *O. oeni* (Shimazu *et al.,* 1985). The higher the initial concentration of citric acid, the more diacetyl will be produced from MLF. Citric acid metabolism begins at the same time that malic acid degradation occurs, but the degradation of the citric acid is slower. Diacetyl concentration increases as citric acid is metabolized by the bacteria and decreases again when most of the citric acid has been consumed. Maximum diacetyl concentration tends to coincide with the exhaustion of malic acid during malolactic fermentation (Nielsen and Prahl, 1995). The amount produced depends on the bacterial strain and cell multiplication: the lower the cell multiplication, the lower the production of acetate and diacetyl. Under stressed slow growth conditions (where more diacetyl and less acetic acid are produced) the environmental conditions affecting LAB, the amount of citric acid available and the sulfur dioxide content, all influence the redox potential (oxygen content) of the wine. *S. cerevisiae* irreversibly reduces diacetyl, and sulfur dioxide binds reversibly with diacetyl. The addition of SO_2 will initially decrease the concentration of diacetyl, and increase the quantity of bound SO_2. When the diacetyl–SO_2 complex dissociates, however, the diacetyl and free SO_2 levels will increase. Semiaerobic (2–4 mg/l oxygen) malolactic fermentations yielded higher concentrations of diacetyl, whereas anaerobic ($<$0.2 mg/l oxygen) conditions yielded much lower concentrations. Also, it has been observed that the higher the pH, the lower the diacetyl production (Wibowo *et al.,* 1985).

Diacetyl is reduced irreversibly by lactic acid bacteria to acetoin and 2,3-butanediol, an odorless, but sweet-tasting higher alcohol. These two compounds have no influence on wine aroma when present in their normal concentrations in wine (de Revel *et al.,* 1989). For a high diacetyl concentration, the wine should be racked off the lees before inoculating with lactic acid bacteria and stabilized as soon as the malic acid is catabolized. Malolactic nutrients may be required in this case, since the necessary nutrients have been removed due to the racking/clarifying procedure. Since diacetyl is not considered desirable in red wines, malolactic fermentation is usually conducted on yeast lees, which results in the diacetyl being catabolized by the yeast (or the bacteria).

During malolactic fermentation in wine by *O. oeni*, the degradation of citric acid is delayed compared to the degradation of malic acid (Nielsen and Richelieu, 1999). The total production of diacetyl and acetoin during MLF by *O. oeni* is stimulated by increased citric acid concentrations in the wine. However, the production of the two compounds is strongly dependent on the redox potential and O_2 concentration of the wine (Nielsen and Richelieu, 1999). Maximum diacetyl concentration was found to occur when malic acid levels were exhausted. Once the maximum was reached, levels of diacetyl began to drop due to degradation of the compound by the bacteria. The authors noted that for control of the final diacetyl concentration in the wine, it is important to be aware of this coincidence of maximum diacetyl concentration and exhaustion of malic acid. If the wine is sulfited at this point, which is common at many wineries, all further microbiological activity stops. The irreversible reduction of the diacetyl will also then stop, because this can be accomplished only by living bacteria and yeast. If the buttery note from diacetyl is too overwhelming after exhaustion of the malic acid, the authors advise the delay of sulfuring until the diacetyl concentration has been reduced by the bacteria and yeast. Indeed, in the brewing of beer, it is usually necessary to allow a certain beer–yeast contact time after fermentation in order to remove excess diacetyl (Section 2.6.4). Sulfite added to wine reacts fast and rather strongly with diacetyl and also reduces the buttery flavor. However, in contrast to the microbiological reduction by bacteria and yeast, this reaction is reversible. If some of the SO_2 evaporates or combines with other compounds in the wine, the concentration of the flavor may later increase in the wine again. This should be kept in mind when the time of sulfitation after the MLF is decided and when the wine is stored in tanks or barrels and later bottled (Nielsen and Richelieu, 1999). The concentration of free diacetyl in wine containing SO_2 may also depend on the concentration of other SO_2-binding compounds,

such as acetaldehyde, α-ketoglutaric acid and pyruvic acid, which are substantially reduced during MLF. Several other factors can influence diacetyl formation and reduction. Low concentrations of sulfur dioxide, lower temperature (18 °C), and low inoculation rate favor the production of diacetyl (Swiegers *et al.*, 2005). Temperature also has a strong influence on the perception of the buttery aroma of diacetyl, and anything over 30 °C will lead to an overestimation of the actual diacetyl concentration. If one wishes to accentuate the buttery-nutty aroma of diacetyl in, say, a bottle of Chardonnay, it should be consumed at 20 °C rather than at 10 °C (Nielsen and Richelieu, 1999).

Hydrolysis of Grape Aroma Compounds

Detectable levels of β-glucosidase activity were found in 11 commercial preparations of wine *Oenococci* (Liu, 2002), suggesting that these bacteria have the potential to hydrolyse glycoconjugates to affect wine aroma and color. The sugar-bound monoterpenes are nonvolatile and flavorless. A glycosidase (e.g. β-glucosidase) hydrolyzes the sugar bound monoterpenes to release the volatile, aromatic monoterpenes as well as the sugar. The presence of glycosidase activity in lactic acid bacteria from sources other than wine is known. An α-glucosidase has been isolated in *Lactobacillus brevis* from beer, and a β-glucosidase was purified and characterized from *Leucenostoc mesenteroides* isolated from cassava. *Lb. plantarum* strains from cassava fermentation contain β-glucosidase that can degrade cyanogenic glycosides, which suggests that these enzymes may be used to detoxify food plants by way of enzymatic hydrolysis or fermentation. This area merits further study (Liu, 2002).

Increased levels of glycosidically bound grape-derived aroma norisoprenoids (α-ionone, for example) and monoterpenes, released through hydrolysis by bacterial endocellular β-glucosidase enzyme (du Toit, 2008) will contribute positively to wine aroma. Ugliano and coworkers (2003) studied the ability of four commercial preparations of *O. oeni* to hydrolyze wine aroma precursors by measuring the concentration of free and bound Muscat glycosides at the end of malolactic fermentation in model wines. They concluded that the *O. oeni* strains tested enhanced wine aroma through the hydrolysis of grape-derived bound secondary metabolites significantly. Another side effect of bacterial activity may be the modification of color in red wine. Anthocyanins are the main pigments of red grapes and young red wines, which are usually glycosidically bound to sugars, such as glucose (see, for example, Sections 2.9.2, 2.10.7, 2.11.2 and 5.8.6). A bacterial glycosidase (also referred to as anthocyanase) hydrolyzes the sugar bound anthocyanins to liberate the sugar and the corresponding anthocyanidin during malolactic fermentation, the latter spontaneously converting to brown or colorless compounds via condensation and other reactions (Liu, 2002).

Production of Volatile Phenols

Phenolic acids (mainly ferulic and *p*-coumaric acids) are natural constituents of grape juice and wine. These phenolic compounds can be decarboxylated microbially during fermentation into volatile phenols such as 4-ethylguaiacol and 4-ethylphenol (Liu, 2002). The volatile phenols can contribute to wine aroma positively or negatively, dependent on the concentration, due to their low detection thresholds and their distinctive flavors. It has been observed that the concentration of volatile phenols increased markedly during malolactic fermentation, suggesting that lactic acid bacteria might be involved. The metabolism of phenolic acids, *p*-coumaric acids, in particular by *Lb. plantarum* and *Brettanomyces* spp., have been well characterized, unlike the metabolism of phenolic acids by the more typical wine bacteria. *Lactobacilli* isolated from malt whisky fermentation can also decarboxylate phenolic acids into volatile phenols. However, phenolic compounds can favorably and unfavorably affect the physiology and growth of wine LAB, dependent on the concentration and type of phenolic compounds. (Liu, 2002).

Other By-products of Malolactic Fermentation

Esters, such as ethyl acetate and C_4 to C_{10} fatty acid ethyl esters, are largely, if not exclusively, responsible for the fruity aroma of wine. Yeasts are known to produce these esters during alcoholic fermentation (Jackson, 2000c), and there is some evidence to suggest that ethyl esters, such as ethyl acetate, ethyl lactate, ethyl hexanoate and ethyl octanoate, are formed by wine bacteria during malolactic fermentation. The ability of wine lactic acid bacteria to synthesize esters needs to be verified, but the bacteria and *Pseudomonas* of dairy origin are known to esterify alcohols and fatty acids. Dairy isolates of *Lactococci*, *Lactobacilli*, *Streptococci* and *Pseudomonas fragi* form ethyl butanoate and ethyl hexanoate from ethanol and butanoic and hexanoic acids (Liu, 2002). The concentrations of some esters decrease while others increase during storage of wines, presumably due to acid hydrolysis and chemical esterification.

Wine contains various volatile aldehydes that contribute important sensory properties, of which acetaldehyde is quantitatively the most abundant aroma compound present in wine (Jackson, 2000a). The aldehydes, including acetaldehyde, hexanal, *cis*-hexen-3-al and *trans*-hexen-2-al, cause the green, grassy and vegetative off aroma in wine, and can negatively impact on primary aroma from grapes. The removal of aldehydes in any wine except Sherry is beneficial to the quality of the product. Traditionally, sulfur dioxide is added to bind acetaldehyde when this compound is in excess, but it has been found that some lactic acid bacteria (specifically *O. oeni*) can catabolize acetaldehyde, converting it to ethanol and acetate (Liu, 2002), offering an alternative approach to sulfur addition. Lactic bacteria (for example, dairy *Lactococci* and *Lactobacilli)* may also produce acetaldehyde, but its production by *O. oeni* is still unclear (Liu, 2002).

Lactic acid bacteria can also metabolize glucose and fructose to acetic acid, lactic acid and mannitol, which may impart vinegary characteristics, and glycerol to acrolein, which is very bitter. Amino acids may be converted to the biogenic amines putrescine, cadaverine and histamine, which can cause allergic responses in some individuals, and at higher levels, have off-putting aromas (as their names suggest). Undesirable odors (cabbage, asparagus, canned vegetable) brought about by MLF are usually associated with the action of *Pediococci* or *Lactobacilli*, or with MLF occurring above pH 3.5, whereas malolactic fermentation by *O. oeni* below pH 3.5 is less likely to produce off odors (Jackson, 2000a).

Volatile sulfur compounds are produced via desulfuration of sulfur-containing amino acids, such as methionine and cysteine. The secondary reactions of amino acid catabolism involve the conversion of the above compounds (amines, α-keto acids and amino acids) to aldehydes. The reduction of the aldehydes to alcohols and/or their oxidation to acids constitutes the final reactions of amino acid transformation (Liu, 2002). Few studies have been conducted on the catabolism of amino acids other than arginine by lactic acid bacteria in wine, but this could have a significant impact on wine quality, given that a range of compounds can be produced.

In a study carried out by Zlotejablko and coworkers (2001), descriptive sensory profiles of Uruguayan Tannat wines after malolactic fermentation were compared with control wines where MLF was prevented. MLF led to a significant decrease ($P < 0.05$) in secondary descriptors such as 'berry fruit' and 'fresh vegetative,' as well as a decrease in related tertiary descriptors such as 'blackcurrant,' 'apricot,' 'cut green grass' and 'green pepper.' The main differences in the chemical composition found following MLF were an increase of the lactates (mainly ethyl lactate) over the sensory threshold, a significant decrease in ethyl esters and acetates, and a small increase of 4-vinylguaiacol and 4-vinylphenol (smoky aromas) with one of the MLF strains.

de Revel *et al.* (1999) studied lactic acid bacteria starter cultures in a white Sauvignon wine during partial malolactic fermentation in barrels and found carbonyl substances were formed during fast bacterial growth and metabolism of citric acid. Greater complexity was observed in the wood-matured wine after malolactic fermentation compared to a wine not having undergone bacterial development, especially regarding buttered, spiced, roasted, vanilla and smoked notes. The intensity of grape varietal character also decreased significantly.

2.3.17 Lactic Spoilage

As discussed earlier in this section, uncontrolled metabolism by lactic acid bacteria as well as other substrates may lead to the production of off odors within the wine. The fermentation of tartaric acid by bacteria occurs only in very high pH wines (above pH 3.5) with low free sulfite levels. Tartaric acid is converted into lactic acid, acetic acid and carbon dioxide, accompanied by a significant reduction in total acidity. This fault is known as '*tourne*,' and is primarily caused by *Lb. brevis*. The wine will appear cloudy with silky waves, gassy and dull in color, and red wine will gain a brownish tinge. The wine becomes very flabby and can appear 'mousy' in extreme cases (Jackson, 2000b).

Lactic acid bacteria may ferment glycerol in musts made from immature rotted grapes, with the effect being particularly noticeable in the press and lees wines, which have the lowest acidity. Glycerol is converted to lactic and acetic acid, causing the wine to taste thin and acidic. Acrolein, a bitter tasting compound, may also be formed, and putrid odors may develop. This spoilage (known as '*amertume*') was common in Burgundy at the end of the nineteenth century, but is now rare due to better pest control in the vineyard and the widespread use of sulfur dioxide. A fault known as 'lactic souring' occurs when sugars are attacked by any lactic acid bacteria, and on wines with stuck fermentations (particularly those which have overheated) or on high pH wines (over pH 3.3) with residual sugars on which the malolactic fermentation is attempted (Morgan *et al.*, 2006). The sugars are converted to lactic and acetic acids, giving the wine a sweet/sour taint. The risk of lactic souring can be reduced by good prefermentation clarification with sulfur dioxide addition and good fermentation control, fermenting the wine to dryness. Lactic souring often occurs after a normal alcoholic fermentation, as the nonfermentable sugars remaining are attacked. The resulting increase in acidity is slight, and the gain in volatile acidity is only 0.1–0.4 g/l. There is little clouding, so the spoilage often passes unnoticed, except for a slight loss of quality in the wine. To prevent this, wines of low acidity should be racked, clarified and sulfited as early as possible after the alcoholic fermentation (Morgan *et al.*, 2006).

During an apparently normal malolactic fermentation, the lactic bacteria surround themselves with a mucillaginous layer of polysaccharides (β-1,3-glucans) that connects them and makes the wine appear oily and flowing in a viscous fashion ('ropiness'). There is no increase in volatile acidity. This condition is rare in table wines, as it only occurs in wines with no sulfur dioxide (e.g. those destined for distillation). Strains of *O. oeni* and *Pediococcus* species have been isolated from ropey wines, but they are not always present (Jackson, 2000b). Sometimes a combination of microorganisms in combination with lactic bacteria may lead to a fault known as 'mousiness.' The sensitivity of individuals to this taste varies greatly, but it is most unpleasant to some people; often becoming apparent some time after the wine has left the mouth, and being very persistent. There is no known cure. The causative organisms are certain lactic bacteria including *Lb. brevis, Lb. cellobiose*, *Pediococcus* and/or *Brettanomyces* yeasts. These organisms are sensitive to free sulfur dioxide, and so this fault does not occur if wines are normally sulfited. The transformation of the antifungal agent sorbic acid by lactic acid bacteria to many other compounds may lead to the wine smelling of crushed geranium leaves. There is no cure for the 'geranium fault,' but it can be prevented by using sorbic acid only in the presence of 20–30 mg/l free sulfur dioxide (Morgan *et al.*, 2006). The sorbic acid will inhibit yeast development, and the sulfur dioxide will prevent lactic bacteria growth.

In conclusion, the current consensus would appear to be that malolactic fermentation can affect wine aroma, even if the effect is as subtle as giving greater complexity. It would also appear that the impact of MLF on wine flavor varies with wine LAB and wine type. It is certainly the case that further research is required to relate the wine attributes altered during MLF to the production and/or degradation of a specific chemical compounds by wine LAB (Liu, 2002). This information will help a producer to match the right strain of wine LAB to the right style of beverage being produced so as to maximize or minimize a particular flavor attribute.

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2.4
Acetic and Other Fermentations

2.4.1 Introduction

The fermentation pathway, along with glycolysis, is one of the most ancient metabolic processes, as it appeared when the planetary environment was strictly anaerobic, and all life was prokaryotic The enzymes for these ancient pathways are located in the cytoplasm, and not in the mitochondria (Purves *et al.*, 2001). At some point in the very distant past, cells on Earth gained the ability to photosynthesize and molecular oxygen was added to the atmosphere, rendering most environments aerobic. Further evolution of cells in these new environments led to the incorporation of mitochondria through endosymbiosis, and the development of the citric acid cycle and the electron transport chain on and in the mitochondrial membranes. Respiration, therefore, is a relatively recent evolutionary development compared to fermentation.

The organisms that are likely to be encountered during a beverage production process will incorporate a wide variety of different metabolic styles, and many different fermentations are carried out by various bacteria and eukaryotic body cells distinguished in most cases by the end product. However, microorganisms that are not deliberately introduced to the fermentation medium by the producer will normally cause spoilage of the product or problems with the production process.

Microbial spoilage occurs when microorganisms break down desirable constituents in fermented beverages and produce undesirable substances. These changes may appear as turbidity, sediments, gassiness, color change and sensory changes (off odor and off taste). In almost all cases the damage is irreparable and so prevention, rather than cure, is the rule. Microorganisms causing spoilage during brewing and beer processing are limited to a few genera of bacteria, wild yeasts and molds. Beer is an unfavorable growth medium for most microorganisms due to the alcohol content, low pH and the presence of hop constituents, as well as the lack of nutrients, but a few species will survive, and have deleterious effects on flavor and shelf life (Goldammer, 2000). Wine, with an even higher alcohol content and similarly low pH is even less of an environment conducive to the growth of microorganisms. However, a number of specialized organisms are able to withstand the conditions, and complacency in the winemaking process will lead to high volatile (acetic) acidity or excessive acetaldehyde. It is not only the wine that can sustain the growth of unwanted microorganisms, but the equipment, including barrels, storage tanks, pumps, pipes and filters. Microorganisms which cause spoilage are either aerobes: (require the presence of oxygen in air to reproduce) e.g. acetic bacteria and yeasts; or facultative anaerobes: these can reproduce without the presence of oxygen, e.g. lactic bacteria.

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

The other factors that affect the growth of microorganisms in fermented beverages are the acidity in solution (only *Acetobacter* grow actively at pH 3.0–4.0), alcohol concentration, temperature and the use of preservatives such as sulfur dioxide. The existence of fermentable sugars (above 10g/l) in a medium facilitates the risk of spoilage – specifically by yeast. Growth factors, nutrients such as amino acids and vitamins, are important in determining the growth of microorganisms. In wine, there are many opportunities for microbial spoilage, but the winemaker has key controls at their disposal to ensure they are minimized. The conditions necessary for proliferation and spoilage in a winery can be avoided with good hygiene, the use of antiseptic agents like sulfur dioxide and management of storage conditions including higher temperatures and exposure to oxygen. These are discussed in more detail later on in this section.

2.4.2 Acetic Acid Bacteria

Although no known pathogenic bacteria can survive in wine media, many others can grow and flourish. Two of the most common bacteria found in the winery environment are acetic and lactic acid bacteria. There is an almost permanent presence of acetic acid bacteria during every stage of winemaking and a change in the type of bacteria present occurs during fermentation and storage. *Acetobacter* or acetic acid bacteria are able to oxidize ethanol to acetic acid, a process vital to vinegar production. It was thought originally that exposure to air was required for their growth, but it has been found they can use hydrogen acceptors other than oxygen so they can remain viable for years in anaerobic conditions such as barrels or bottled wine (Jackson, 2000). There are several genera of acetic acid bacteria, but the only one to jeopardize wine is *Acetobacter* (Ribéreau-Gayon *et al.*, 2000). This bacterium is capable of generating acetic acid in the absence of sulfur dioxide and in the presence of oxygen. In addition, it can esterify acetic acid and ethanol to ethyl acetate, which produces the unpleasant organoleptic characteristics of acescence (nail polish remover and vinegar odor). This organism is also responsible for elevated volatile acidity in wines exposed to air where it develops on the surface as a white layer (bloom).

The ability of acetic acid bacteria to metabolize sugar is atypical (Jackson, 2000). The pentose phosphate pathway is used by these organisms exclusively for the oxidation of sugar to pyruvate sugars may also be oxidized to gluconic and mono- and diketogluconic acids. In addition to oxidizing ethanol to acetic acid, acetic acid bacteria can also oxidize other alcohols to their corresponding acids, for example butanol to butanoic acid. In addition, polyols (molecules with more than one alcohol function, like glycerol) can be oxidized to ketones (dihydroxyacetone results from metabolism of glycerol). Members of the genus *Acetobacter* can further oxidize acetic acid to carbon dioxide and water, which is known as overoxidation.

Acetic bacteria can grow on the surface of the wine as a thin white film or as a thicker, more oily layer. It is a very serious problem, especially for red wines produced in warm regions. 'Mother of vinegar,' a thick, hard, viscous mass, is formed by an acetic bacteria which is rarely found in wine, but often found in wineries on dripping taps and leaking wine tank valves.

Acetic or ethanoic acid is the principal component of 'volatile acidity' or 'VA,' so called because its concentration is measured in wine by distillation followed by titration with a base. It is acrid and bitter to the palate and detectable at doses above 0.75 g/l as a vinegary taint, though lower concentrations will considerably 'thin' a wine. The maximum legal limit (UK) is 1.08 g/l in white and rosé wines, and 1.2 g/l in red wines (Robinson, 2006). The production of acetic acid is usually accompanied by its combination with alcohol to form ethyl acetate. This has a characteristic (pear drop) aroma, which is usually easier to detect in spoiled wines than the acetic acid itself (recognisable at 0.16 g/l). It is common for a wine to gain 0.1–0.2 g/l VA (as acetic acid) during the alcoholic fermentation, due to the action of wild yeasts, especially at high temperatures. Another 0.1g/l may be produced during the malolactic fermentation, particularly if the

temperature and pH of the wine are high. Any increase above 0.3 g/l indicates lactic or acetic spoilage. VA is detectable in white wines at around 0.4 g/l and in red wines at around 0.5 g/l (Morgan *et al.*, 2006).

It is recognized that the acetic acid bacteria form a distinct family of Gram-negative rod-shaped bacteria characterized by their ability to oxidize ethanol to acetic acid (Jackson, 2000). Acetic acid bacteria from the *Acetobacteraceae* family are divided into the genera *Acetobacter* and *Gluconobacter*. There are over 10 different species identified belonging to the genera, but *Gluconobacter oxydans, Acetobacter aceti* and *Acetobacter pasteurianus* are the most frequently encountered in enology (Ribéreau-Gayon *et al.*, 2000). Goldammer (2000) stated that for all practical purposes there are only seven common genera of bacterial contaminants in the brewery, and the Gram-negative spoilage bacteria in beer include *Acetobacter* spp*, Acetomonas* spp*, Zymomonas* spp*., Enterobacteriaceae* spp. and *Pectinatus* spp. The concentration and species of acetic acid bacteria introduced into the winery via the grapes can vary depending on the quality of the fruit. On good quality red or white grapes the main species found is usually that of *G. oxydans*, whereas on spoiled fruit, infected with *Botrytis cinerea,* the main bacteria species is that of *A. aceti* with the presence of *A. pasteurianus* in smaller quantities. *G. oxydans* prefers glucose for its growth and this species is not tolerant to alcohol, which explains why it is more readily found in the must and on good quality fruit, because once the fruit becomes spoiled and there is fermentation caused either by the presence of wild yeasts on the fruit or by controlled fermentation in the winery, alcohol levels rise. The other main area of acetic acid bacteria contamination, except for poor hygiene of equipment, is that of the barrel. Used barrels, due to the nature of the absorbency of the wood, retain wine and small quantities of bacteria. During maturation there is a small ingress of oxygen into the barrel (about 30 mg/l per year) which prevents the complete destruction of the population which would occur if conditions were strictly anaerobic, e.g. in bottles (Ribéreau-Gayon *et al.,* 2000). Insects such as fruit fly (*Drosophila*) will carry acetic bacteria onto damaged berries. Acetic bacteria are also present on all cellar surfaces, and even in normally sulfited wines. The numbers of acetic bacteria will often increase greatly in the pomace in red wines, particularly in open tanks where the pomace is not blanketed with carbon dioxide and is seldom immersed in fermenting must. Another factor increasing the risk of acetic spoilage is high temperature. Spoilage is twice as fast at 28 °C as at 23 °C and twice as fast at 23 °C as at 18 °C. The reproduction of acetic bacteria is greatly reduced at pH below 3.0 but rapid at pH above 3.2. Wines with high alcohol levels are less prone to spoilage (Morgan *et al.,* 2006).

Although acetic acid bacteria are classified as strictly aerobic, the ability of *Acetobacter* to grow under semianaerobic conditions and produce acetic acid emphasizes the importance of careful handling of the product during operations which may expose wine to air. Because of resistance to SO_2, control is by minimizing air contact at wine surfaces and keeping temperatures low.

Identification of microorgansims, particularly bacteria, is not easy and producers more often than not do not have access to the methods necessary for the task. The isolation of acetic acid bacteria requires an appropriate medium (glucose–yeast-extract agar with calcium carbonate is a common one), which is then incubated aerobically, usually for two to four days at a temperature of between 25 and 30 °C (Ribéreau-Gayon *et al.*, 2000). It is necessary to incorporate cycloheximide or pimaricin into the medium to inhibit the growth of yeasts, and the calcium carbonate neutralizes the acid resulting from the acetic acid bacteria. This also aids with identification, as the acid solubilizes the calcium carbonate leaving clear areas around each colony forming unit. Further additions can be made to the media to inhibit the growth of lactic acid bacteria making the isolation of acetic acid bacteria easier, and penicillin can be used for this purpose. Another addition that can be made to the media is that of ethanol and the strong aroma of acetic acid arising from ethanol oxidation is a good indication of the presence of acetic acid bacteria (Morgan *et al.*, 2006). If yeasts are not present, a simple Gram stain procedure can be performed on the isolate. The family of *Acetobacteraceae* are Gram-negative rods due to the constituents of their cell walls and this differentiates them from lactic acid bacteria, which are Gram-positive. Once isolated the acetic acid bacteria can be further identified to species level by their abilities to oxidize calcium D,L-lactate, overoxidize ethanol, form water soluble pigments, oxidize glycerol,

Table 2.4.1 *Characteristics of acetic acid bacteria and* Gluconobacter oxydans

	Acetobacter			*Gluconobacter*
Characteristic	*A. aceti*	*A. pasteuranus*	*A. hansenii*	*G. oxydans*
Overoxidation of ethanol	+	+	+	−
Growth on ethanol	+	some	+	−
Growth on sodium acetate	+	some	−	−
Ketogenesis from glycerol	+	−	+	+
Ketogenesis from sorbitol	+	−	+	
Ketogenesis from mannitol	some	−	+	
Oxidation of lactate to CO_2 and H_2O	+	+	+	−
2-Ketogluconic acid produced from glucose	+	some	some	+
5-Ketogluconic acid produced from glucose	+	−	some	+
2,5-Diketogluconic acid produced from glucose	−	−	−	some

produce 5-ketogluconic acid from glucose and the ability to grow on media containing ethanol and sodium acetate as sole carbon sources (see Table 2.4.1).

2.4.3 Lactic Acid Bacteria

Lactic acid bacteria, which affect both beer and wine as spoilage organisms, can be identified initially by using a Gram stain. *Lactobacillus* spp. and *Pediococcus* spp. are both Gram-positive. *Pediococcus* spp. metabolize a number of substrates including malic acid and/or sugars with different end products and consequences for wine quality. They are less likely to grow if substrates have been depleted by a previous malolactic fermentation. *Lactobacillus* is associated with wine spoilage, and is notorious for producing excessive acetic acid by metabolizing sugar or tartaric acid, which can cause yeast inhibition and lead to stuck fermentations. *Pediococcus* is one of the common lactic acid bacteria found in wine and its entire species are homofermentative (transform sugar exclusively into lactic acid). They may produce increased volatile acidity and polysaccharides that cause undesirable texture defects. Strains of *Pediococcus damnosus* produce histamine (a biogenic amine), which can pose a health hazard for individuals sensitive to the compound (see Section 5.11.3). Lactic souring, *tourne*, *amertume,* 'ropiness' and 'mousiness' are all wine faults caused, in whole or in part, by lactic acid bacteria (Jackson, 2000). These faults and other activities of lactic acid bacteria are discussed in detail in Section 2.3.1.

2.4.4 The Activities of Yeasts other than *Saccharomyces*

A spoilage yeast in a brewing context is defined as any yeast other than the pitching yeast (Goldammer, 2000). Spoilage yeasts can be isolated at all stages of the brewing process from raw materials, wort, pitching yeast and fermenting beer, through to the packaged product and the dispense system. They produce unintended flavors, including hydrogen sulfide, estery, acidic, fatty acid and phenolic or medicinal notes. Turbidity is another effect caused by growth of wild yeasts that remain after the culture yeast has been removed by filtering or fining. In the presence of air, some spoilage yeasts can grow rapidly and form a film on the surface of the beer, which can cause haze. Other effects may include primary yeast fermentation and separation difficulties,

significantly lower terminal gravities, and a higher alcohol content in the finished beer. The lower terminal gravities are due to the ability of wild yeast to ferment sugars (such as maltotetraose and dextrins) not used by the primary yeast. A spoilage yeast infection is usually more of a problem for brewers not having a pure culture yeast propagation system than for those who do. In a winemaking context, a spoilage yeast is any yeast (including *S. cerevisiae*) that ferments residual sugars once a wine has been bottled, as it will in all likelihood be spoiled. A number of different genera of yeast have been implicated in wine spoilage.

Pichia spp. are ascospore-producing yeasts which are capable of fermenting low levels of residual sugar, but are inhibited by alcohol levels of near 10% at lower temperatures. At higher temperatures, growth may be found in products of up to 13% alcohol. They are generally weak fermenters, but unrestricted growth imparts an aldehydic character to wine (Zoecklein *et al.*, 1995). The ascospores have a Saturn-shape and form pseudomycelium. *Hansenula* strains are reported to form large amounts of acetic acid, (around 1–2 g/l), and volatile esters, particularly ethyl acetate (up to 2 g/l) as well as isoamyl acetate (a pleasant, fruity banana odor at low concentrations) under aerobic conditions. In the early stages of fermentation, the formation of these volatile esters has been known to add limited flavor and bouquet to the wine. Boulton and Quain (2001) note that an infection by *Pichia* will be limited to the initial aerobic phase of fermentation, but the organisms can spoil unpasteurized draught beer, forming a haze and surface films. *P. membranifaciens* is known to give a 'sauerkraut' flavor.

Epiphytic yeasts, such as *Kloeckera apiculata* and *Metschnikowia pulcherrima*, are known to produce 2-aminoacetophenone as a result of their metabolism of sugars, as well as acetic acid, ethyl acetate and diacetyl (Jackson, 2000). The first compound has been implicated in the 'atypical aging aroma' in white wines, for which various descriptors exist, 'mothball' and 'wet wool' being amongst them. With *Hanseniaspora/ Kloeckera* known to add quantities of glycerol, esters and acetoin to the complexity of the final product, depending upon the strain present, a selection program with the aim of finding suitable apiculate yeast strains for use in fermentation would give the benefit of reduction of undesirable products to levels below the spoilage threshold and the increase of levels of desirable products not produced in sufficient levels by *S. cerevisiae.*

Zygosaccharomyces spp. are likely to be found in high sugar concentrations and are therefore commonly associated with grape juice concentrates that are often used to adjust color and sugar in final wine blends. It is far more common in white and rosé wines than red, for this reason, and because growth is somewhat suppressed by polyphenols. The species can grow in a large range of sugar levels, high alcohol concentrations and is resistant to yeast inhibitors like sulfur dioxide, sorbic acid and diethyl dicarbonate (Jackson, 2000). It generates floccular and granular deposits, which look a little like sand, if there is an infestation in a bottle. This spoilage yeast may produce carbon dioxide and turbidity once the wine has been bottled. Recent research has demonstrated that temperature is the principle factor affecting the development of *Zygosaccharomyces*, and as low a concentration as 1 cfu/10 l of wine is sufficient to induce spoilage (Jackson, 2000). Boulton and Quain (2001) state that this organism is infamous for spoiling soft drinks, fruit juices and high sugar products.

Brettanomyces species are notorious spoilage organisms on the one hand, but essential fermentative organisms for certain types of beers, on the other hand. *Brettanomyces bruxellensis (*synonym *Dekkera bruxellensis)* is an alcohol-tolerant asporogenic wild yeast isolated from British spontaneously fermented beer at the beginning of last century. It gives rise to an organoleptically disastrous condition known as 'brett' when it infects beers and wines, yet under different conditions it provides much of the sensory character associated with certain spontaneously fermented beers, such as the lambic beers of Belgium (Section 2.6.7). *Brettanomyces* species have been recognized as serious spoilage yeasts in wine for around 40 years, and depending on the species can produce either mousy taints, smoky, spicy, medicinal and woody taints, or apple cider odors, high levels of acetic acid and haziness. The influence of *Brettanomyces* and *Dekkera* on winemaking has been reviewed by Oelofse *et al.* (2008).

Brettanomyces spp. include nine different species of which those found in wine include *B. intermidius*, and *B. lambicus* (Jackson, 2000), both of which species produce 2-acetyltetrahydropyridines responsible for the

'mouse' taint. Boulton and Quain (2001) state that *Brettanomyces* is notable for causing off flavor in bottle-conditioned beers, and that the species succeeds *Saccharomyces* in the spontaneous fermentation of wort (lambic and gueuze: see Section 2.6.7). The name *Dekkera* refers to the ascospore-forming (sporogenous) form of this yeast, and *Brettanomyces* used for the non-spore-forming type. It is most well known for producing high concentrations of volatile acids, esters and the volatile phenols 4-ethylphenol, 4-vinylphenol and 4-ethylguaiacol, which are largely responsible for the infamous 'brett' characteristic. The yeast is capable of using many different carbon sources, including sugars extracted from wood and forming unpleasant secondary metabolites (Jackson, 2000), including the classic 4-ethylphenol (Band aid or Elastoplast), isovaleric acid (rancid/vomitous) and all three of 2-acetyl-1-pyrroline (ACPY), 2-acetlyltetrahydopyridine (ACTPY) and ethyltetrahydropyridine (ETPY), responsible for mousy off flavors (Herezstyn, 1986). It would seem that the yeast generally leads to a typical 'brett' aroma, with an associated degrading of varietal and fermentation character: that is a loss of typicity. However, low levels of 'brett' may also add complexity to certain robust styles of wines. *Brettanomyces*, however, is not easily controllable, and neither are the levels of products that result from its fermentative pathways. Jackson (2000) noted that the taint may also derive from synthesis of isobutyric, isovaleric and 2-methylbutyric acids. The organism is capable of growing in both red and white wines, but the polyphenol composition, higher pH and cooperage use seem to encourage development in red wine particularly. Populations of the yeast are ubiquitous in vineyards and wineries throughout the world, and the species was originally isolated from high strength 'stock' beer (see Section 2.6.13). Fruit flies (*Drosophila melanogaster*) can also be carriers of *Brettanomyces*.

The belief is that the main infection route in wine is through old barrels (Jackson, 2000), but it is just as likely to be from an original population on the grapes. Microbial analyses of older and recently bottled wines demonstrated the ability of certain wine microbial species to survive and grow in the bottle. Among them, *B. bruxellensis* was predominant, necessitating effective methods for removing these microorganisms before bottling (Renouf *et al.*, 2007). It is only during the wine ageing process that *Brettanomyces* cells slowly increase in number, which makes its presence difficult to detect before the sensory effects are noticed. Another factor that may enhance risk of infection is the presence of residual sugars left over at the end of fermentation as a result of high initial sugar levels at the start. Also, the addition of diammonium phosphate as a supplementary nitrogen source for yeasts has also become routine in winemaking, which means that amino acids are less likely to be metabolized, and may therefore form a nitrogen source for *Brettanomyces.*

2.4.5 Film Forming Yeasts

Yeasts can also ferment sugars, as well as other substrates, before a product has been bottled, during bulk storage, if the conditions are conducive to growth. Yeast can form films on the surface of wine, and other beverages, under aerobic conditions. The growth of any film forming yeast in wine is detrimental to quality (Loureiro and Malfeita-Ferreira, 2003). Film forming yeasts require oxygen for growth and include the genera *Hansenula*, *Kloeckera*, *Pichia*, *Metchnikowia* and *Debaryomyces*, as well as strains of *S. cerevisia*. They produce acetic acid, 2-phenylethanol and ethyl acetate under oxidative conditions, from ethanol as well as glycerol and organic acids. Uncontrolled growth results in decreased alcohol levels and total acidity/pH changes (Zoecklein *et al.*, 1995). The process of growth in a surface film is, of course, crucial to the production of certain sherries and other wines, where they are known as 'flor' and where they oxidize ethanol to produce acetaldehyde and at the same time protect the wine beneath the film from oxidation (Sections 2.10.3 and 2.10.4). Flor may be caused by several yeast species including strains of *S. cerevisiae* and *Zygosaccharomyces fermentati*, and species of *Candida*, *Hansenula* and *Pichia* can be associated harmlessly with flor films, but are a cause of spoilage when growing alone. Flor yeast growth in a production context is, of course, carefully monitored. Sherry production is discussed in more detail in Sections 2.10.1–2.10.3.

In wine, *Candida* spp. typically grow as surface yeasts, producing chalky white films on the surface of low alcohol wines. In the presence of oxygen they are fast growers, using ethyl alcohol in addition to wine acids as carbon sources, meaning *Candida* spp. have the potential to produce significant concentrations of volatile compounds, such as acetic acid, ethyl acetate and 2-phenylethanol in wines.

After the alcoholic fermentation, all the surfaces in the winery are covered with very large populations of yeasts. They should all be cleaned, if not disinfected, thoroughly. This is especially important if dealing with wines with residual sugar.

2.4.6 Molds

Molds are non-chlorophyll-bearing plants that range in size from a single spore to large cell aggregates. Commonly occurring types are species of *Mucor*, *Penicillium*, *Aspergillus*, *Cladosporium* and *Rhizopus*. Molds are normally aerobic organisms and can grow over a wide pH and temperature range, although most species prefer an acid pH (Goldammer, 2003) and higher temperatures.

In a study of tainted wines in the early 1990s, the most common off odor in wine was 2,4,6-trichloroanisole (TCA), notorious as the compound responsible for cork taint (Jackson, 2000). The moldy odor may originate from the growth of bacteria and filamentous fungi such as *Penicillium roqueforti*, *P. citrinum* and *Aspergillus versicolor* on corks. The role of *Penicillium* in the formation of TCA has been noted (Jackson, 2000). Further discussion on cork taint and its analytical assessment can be found in Section 4.2.4.

2.4.7 Prevention of Spoilage

The current trends in the wine industry are for wines with lower levels of sulfur dioxide and fewer processes such as sterile filtration. Although this is laudable if the goal of the producer is to aim for the most natural product possible, it is also risky, and should be coupled with increased hygiene controls and good manufacturing practices to minimize contamination.

It remains the case that one of the most effective methods of preventing growth of microorganisms relies on limiting microbial activity by addition of SO_2, to which both film yeasts and lactic acid bacteria are very susceptible (Sections 2.5.2 and 2.5.3). The main reason these groups are not as much of a problem in white wines compared to red wines is the higher concentration of free SO_2 in white wines. In red wine, a greater proportion of SO_2 is bound (e.g. to phenolic compounds) and is hence is less active. Maintaining low pH has a strong inhibitory effect on lactic acid bacteria, especially in the presence of SO_2. *Lactobacillus* and *Pediococcus* are more pH sensitive than *Leuconostoc*. SO_2 addition is not always effective against fermentative yeasts in sweet wines because of their higher tolerance to it and also because of the production of acetaldehyde, which then binds the free SO_2. Maintaining free SO_2 at moderate to high levels, and filtration to 0.45 μm is the only sure way of preventing refermentation of any sugar present in a bottled wine.

Filtration techniques (Sections 2.6.9 and 2.9.4) were evaluated over several years after bottling by microbial analysis and by volatile phenols measurements. The smaller the pore size, the more microbes were eliminated. Elimination of bacteria required a 0.3 μm filter, but a 1.0 μm filter was efficient for yeast elimination. It has been argued that filtration (especially tighter membrane filtrations) affect the quality of products. Renouf and coworkers (2007), for example, found that volatile phenol concentrations were lower in less tightly filtered wines and in unfiltered wines than in more tightly filtered wines.

Sorbic acid or its salts, such as potassium sorbate, are inhibitory to fermentative yeasts and are sometimes added to sweet wines at levels up to 200 mg/l. Sorbic acid should always be used in conjunction with sulfur

dioxide, and producers should note the possibility of formation of 2-ethoxy-3,4-hexadiene, which has a strong geranium smell, by lactic acid bacteria against which sorbic acid is ineffective (Section 2.5.4) (Jackson, 2000).

Excluding oxygen, especially in finished wines, is essential for maintaining the quality of the product. Film yeasts and acetic acid bacteria require atmospheric oxygen (apparently at low levels) so effective sealing of containers and inert gas cover are important strategies to control their growth. It is important to monitor the tops of tanks, especially if they are on ullage (air space) and do not have an inert gas cover. If a growth is detected prompt action should be taken. To further prevent the growth of acetic acid bacteria in a product, pasteurization or sterile filtering coupled with completely sterile conditions will eliminate them completely. Although a certain amount of acetic acid is formed by yeasts and lactic bacteria, acetic spoilage as such can only occur if the wine is in contact with oxygen for the duration of a few days, especially at low temperatures. Aeration on racking is not enough to make a substantial difference, as an increase of 0.1 g/l of acetic acid requires 0.24 l of air per liter of wine (Morgan *et al.*, 2006). Sulfiting is not totally effective, as the free sulfur dioxide on the surface of a partially filled tank will rapidly be combined or oxidized.

Temperature is an important factor affecting microbial activity. The activity of bacteria is very slow or nonexistent at temperatures below 15 °C, and yeast are inhibited, so storage of products at low temperatures is advisable. Pasteurization may also be used to sterilize products. In a winemaking context, heating to 60 °C for 5 mins or 70 °C for 6 s is sufficient. Short periods of heating at high temperatures are better than longer periods at lower temperatures due to the chemical and physical impacts of prolonged heating on the flavor and aroma of wine (Morgan *et al.*, 2006).

Timing and magnitude of sulfur dioxide additions are important in prevention of *Brettanomyces*, as small incremental doses of sulfur may encourage the selection of sulfur-resistant strains of *Brettanomyces*, which dominate during storage and aging. Sanitation has been shown to be an important factor in keeping wild spoilage yeasts from the final product, as reservoirs of wild yeast, bacteria and spores of both are found on production equipment. Wooden cooperage, which is fairly common in the production of quality red wines, is a well-known route for infection as *Brettanomyces.* The yeast produces β-glucosidase which allows it to metabolize cellobiose, a compound that results from the toasting process, present in both old and new barrels. The enzyme cleaves the disaccharide cellobiose to produce glucose molecules, which are then used for growth.

Many winemakers consider that the taint caused by powdery mildew in white wine results from the mycelium of the fungus itself, and thus minimizing taint is based on minimizing the quantity of the mycelium in the juice. The application of a high pressure, high volume water spray in the vineyard the day before harvest is reported to have a positive effect in minimizing mycelium levels. Juice from powdery mildew-affected fruit should be settled at the lowest temperature possible to achieve rapid settling, and pectolytic enzymes should be used (Morgan *et al.*, 2006).

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2.5

Preservatives Used in the Production of Alcoholic Beverages

2.5.1 Introduction

Preservatives are added to beverages in order to protect them from microbial and oxidative effects, and to help retain their original quality as long as possible (in the case of wine, this may be decades). Generally, additives allowed in beverages and foodstuffs are strictly regulated, and anything added to a product in order to preserve it would be subject to exactly the same regulation. There are nonadditive methods, such as pasteurization, that will extend the shelf life of a product beyond a usual 'fresh period,' but generally speaking, these methods only work in the short term. It is also the case that heating can alter the organoleptic qualities of the product, which is usually undesirable. Other methods of removing microbial content include different forms of filtration, which are discussed in detail in Sections 2.4.7, 2.6.9, 2.8.5, 2.8.6 and 2.9.4. Cost of machinery, volume of product, packaging preferences, even distribution (climate/transport/storage and shelf-time/retail outlet type), all influence the method chosen to preserve the product.

2.5.2 Sulfur Dioxide (SO_2)

Sulfur dioxide has a long history of use in preservation of foods and beverages. It is formed by burning sulfur (32 g of sulfur reacts with 32 g of oxygen to give 64 g of sulfur dioxide), and dissolves in water and ethanol, depending on the temperature. In Europe, the permitted sulfur additives for wine are sulfur dioxide (E220), potassium bisulfite (E228) and potassium metabisulfite (E224) (Bird, 2005). Interestingly, SO_2 is produced in small quantities (between 12 and 64 mg/l) by yeast during fermentation (Jackson, 2000a). The formation of SO_2 by *S. cerevisiae* is a strain characteristic, with the majority of strains (80%) producing less than 10 mg/l of SO_2 and a few strains producing more than 30 mg/l, suggesting that completely sulfite-free wine is unlikely to be produced without some form of removal. The amount produced also depends on the wine environment and tends to be higher in highly clarified musts with low levels of suspended solids (Romano and Suzzi, 1993). Biological sulfite is always bound and is produced slowly so the effect on wine is difficult to assess, although there is some evidence to suggest that some of the sulfite-producing yeast strains act to stabilize the wines in a similar way to the addition of sulfur to the must. Sulfur dioxide per se can be produced by burning

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

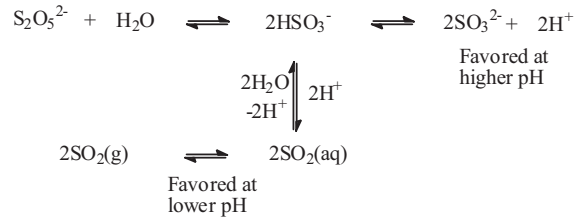


Figure 2.5.1 *Various forms of free sulfur dioxide in solution*

elemental sulfur in air, but nowadays is more likely to be obtained from a cylinder of compressed liquid (see Section 5.9.2). Sulfur dioxide exists in both 'free' and 'bound' forms in solution: the free forms are molecular SO_2 (SO_2), sulfite (SO_3^{2-}), and bisulfite (HSO_3^-) (Rotter, 2008) (Figure 2.5.1). Around 2% exists as the free dissolved gas in wine, with most of it occurring as bisulfite ions (Jackson, 2000). The bound forms include a range of compounds in which the molecule becomes associated with, or permanently bonded to, another molecule like an aldehyde or a polyphenol. The total sulfur dioxide content of a wine is the sum of the free and the bound concentrations.

Potassium metabisulfite dissociates in water to potassium ions (K^+) and singly ionized bisulfite (HSO_3^-). An equilibrium forms between the hydrogen ions present in the medium, sulfite, bisulfite and molecular sulfur dioxide, of which at wine pH around 2% exists as the free dissolved gas (Jackson, 2000a). The position of equilibrium and hence the concentration of each species depends the pH and to a lesser degree the temperature of the environment (Figures 2.5.1 and 2.5.2). Free molecular SO_2 is the form that is most active against microorganisms and it is present in greater concentrations at lower pH. Sulfur dioxide is thus far more effective as an antimicrobial if the environment is acidic (Jackson, 2000b), and a lowering of pH by even 0.1 units can greatly enhance the efficacy of its action.

Bound (combined) sulfur dioxide is bonded to sugars, phenolics, some minor wine acids and, especially, acetaldehyde (ethanal) (Figure 2.5.3). When sulfur dioxide combines with acetaldehyde, it is rendered

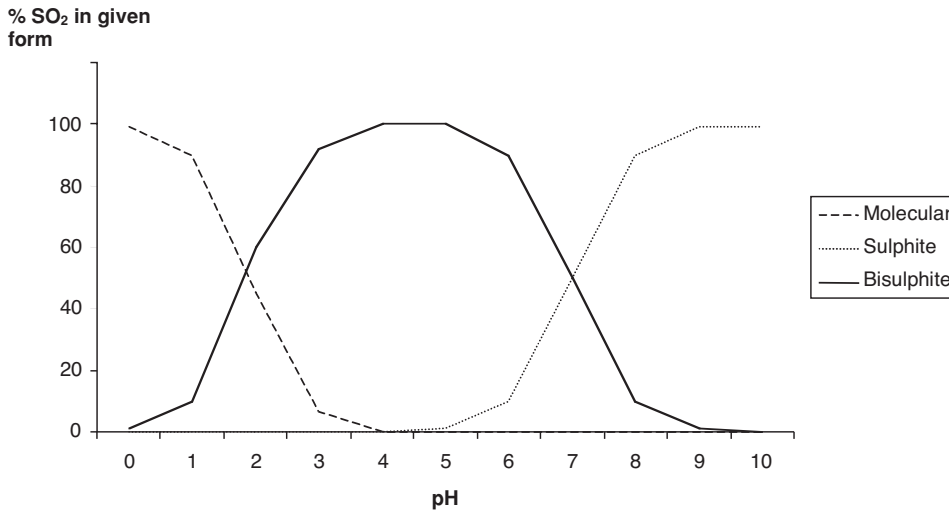


Figure 2.5.2 *Effect of pH on forms of free sulfur dioxide*

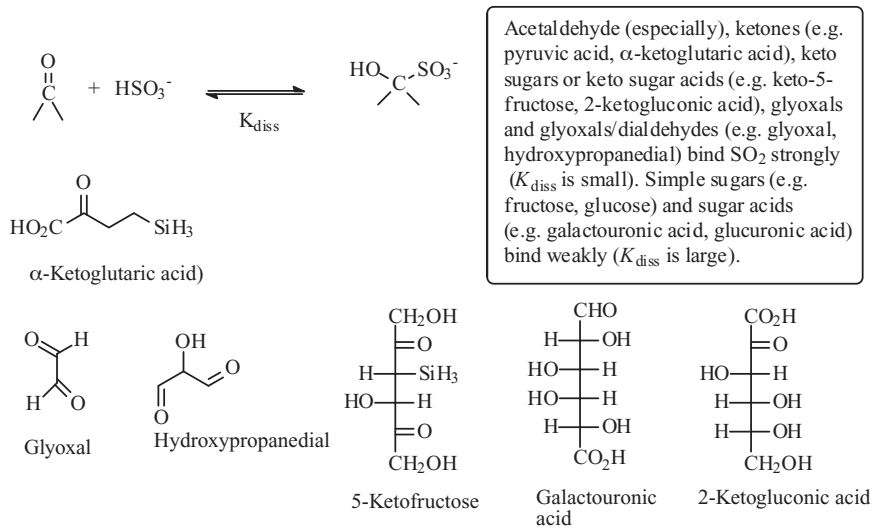


Figure 2.5.3 *Binding of SO_2 species by carbonyl compounds, with examples of carbonyl compounds found in grape must and wine*

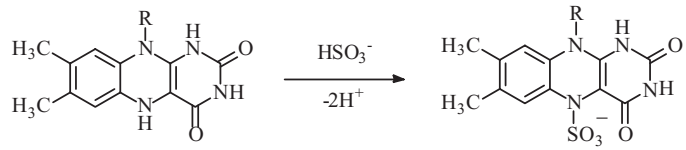
permanently inactive because the bisulfite complex formed shows little tendency to dissociate (it has a high dissociation constant, K_{diss}). Most producers assume that around 50% of the SO_2 they add will become bound when the total SO_2 is below 100 mg/l. Ketonic acids (for example, α-ketoglutaric acid, pyruvic acid and galacturonic acid) also bind to SO_2 (Figure 2.5.3). Increased ketonic acid levels may be the result of nutritional deficiency or stress during fermentation, and different yeast strains also produce varying levels of these products. Keto sugars, keto sugar acids and glyoxals/dialdehydes (Figure 2.5.3) are found in higher concentrations in grapes infected by *Botrytis cinerea* (e.g. Sauternes, Tokay and Trockenbeerenauslese wines) and so these will may make a significant contribution to the binding of SO_2 species in the sweet wines, as some will survive fermentation. Low fermentation temperatures, anaerobic fermentations, addition of ammonium salts and use of nonketogenic yeast strains may all help minimize production of ketones and aldehydes, and therefore the bound fraction of sulfur dioxide.

2.5.3 The Roles of SO_2

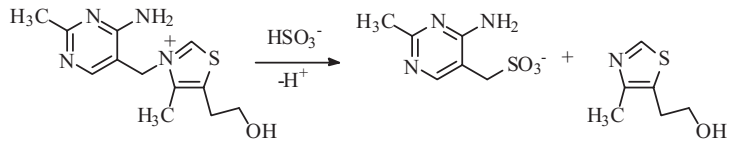
Sulfur dioxide has a wide spectrum antimicrobial activity: about 1.5 mg/l concentration of molecular sulfur dioxide is enough to inhibit most yeast and spoilage bacteria (Jackson, 2000b). The toxic action of the molecule is thought to include inhibition of metabolism by reaction with coenzymes and by its reaction with disulfide bonds in protein, thus leading to denaturing and destruction of tertiary structure (Figure 2.5.4). The suphur dioxide in Figure 2.5.4 is depicted as bisulfite, which at biological pH is the most likely species. Some organisms are more susceptible than others: acetic bacteria are most sensitive, followed by lactic bacteria and so-called 'wild' yeasts, then cultured yeast (e.g. *S. cerevisiae*).

As an antimicrobial, sulfur dioxide has a strong effect when in the free form (hence the importance of constantly monitoring free SO_2 at all stages) which affects the majority of natural yeasts commonly found on grapes (Table 2.5.1). Sulfur dioxide is the only effective antimicrobial agent against lactic acid bacteria,

Destruction of protein tertiary structure by disruption of disulfide bridges of CYS residues



Inhibition of flavoproteins (some dehydrogenases, such as D-glucose dehydrogenase and oxidases, such as amino acid oxidases) by forming a stable adduct via 5-sulfonation. Shown here for reduced form of flavin coenzyme



Destruction of thiamine pyrophosphate moeities, required as a coenzyme of pyruvate decarboxylase and other enzymes. Shown here for thiamine.

Figure 2.5.4 *Possible ways in which sulfites may inhibit growth of microorganisms*

Table 2.5.1 *Profile of the most common yeasts found in grape juice fermentations conducted in Emillia Romagna region, Italy*

	Yeast frequency in untreated grape juice				
	No SO_2		With SO_2		
Species	Population start of fermentation	Population at end of fermentation	Population at start of fermentation	Population at end of fermentation	Sulphur Dioxide resistance
Kloeckera	very high	very high	absent	absent	none
Hanseniaspora	very high	very high	absent	absent	none
Metschinikowia	medium	low	absent	absent	none
Hansensula	medium	absent	absent	absent	none
Pichia	medium	absent	absent	absent	none
S. cerevisiae	low*	low	high	very high	very high
Saccharamycodes	low	low	low	low	very high
Torulaspora	low	low	low	low	very high
Zygosaccharamyces	low	low	low	low	very high
Schizocaccharomyces	low	rare	rare	rare	very high
Brettanomyces	low	low	low	low	very high

Source: After Romano and Suzzi (1993).
*Until inoculated

and greatly inhibits the growth of non-*Saccharomyces* yeasts, resulting in the variation of the aromatic characteristics of fermented media. *S. cerevisiae* has a very high resistance to SO_2, and dosing with this preservative allows this species to dominate the fermentation, thus SO_2 inhibits wild yeast growth and favors the added culture (Table 2.5.1) (Romano and Suzzi, 1993). Sulfur dioxide is also used to 'stun' wild yeasts before the alcoholic fermentation to allow enough time for static settling. It is used at higher doses after the alcoholic fermentation to protect wines from microbial spoilage. A 2% solution is often used to clean filters and for sanitation purposes in the production area (Morgan *et al.,* 2006).

Molecular SO_2 is most effective against lactic acid bacteria, but the ionic and bound forms also have an effect. Evidence suggests that while levels of free SO_2 between 1 and 10 mg/l are sufficient to inhibit lactic bacteria, in practice much depends on the specific species of bacteria, the pH and the amount of insoluble solids present in solution (Morgan *et al.*, 2006). Lower pH values and increasing ethanol concentrations work synergistically to enhance the inhibitory effect of SO_2. Evidence suggests that acetic acid bacteria are better controlled by ensuring an absence of oxygen rather than by the use of SO_2.

Sulfur dioxide also acts as an antioxidant, but does not react directly with O_2, rather with hydrogen peroxide (H_2O_2) and acetaldehyde formed during oxidation of phenolic compounds. During this process cinnamic acid derivatives, such as caffeic and caftaric acid, are enzymatically oxidized to their corresponding quinones (Section 5.8.7), which results in the browning (via the formation of polymers) of juice exposed to O_2. In wine the same reaction is possible, but alcohol inhibits oxidative enzymes and thus the oxidation that takes place is chemical in nature, and is a much slower process.

Acetaldehyde is the compound that binds to SO_2 most rapidly, and sulfur dioxide is said to 'freshen' the aroma by effectively removing it from solution. The main factors influencing acetaldehyde concentrations during fermentation are the yeast strain, the presence of other microorganisms and the juice thiamine content. If acetaldehyde (a yeast inhibitor) is produced early on in the alcoholic fermentation, sulfur dioxide will combine with it and allow the fermentation to proceed normally. After the fermentation, acetaldehyde can be produced by oxidation of ethanol or by spoilage microorganisms.

Oxidative enzymes are responsible for premature ageing reactions (like browning) in certain products, and the loss of aromas. As an antioxidant, sulfur dioxide rapidly inhibits catalytic action of tyrosinases (or polyphenoloxidases), which cause the browning of juices and musts. Catechol oxidase is a membrane-derived component of grapes, which can pass into juice following damage to berries at harvesting and crushing. Laccase is a strongly oxidizing enzyme secreted into the berry by *Botrytis cinerea*. SO_2 also decolorizes and stabilizes oxidation products against polymerization, by combining irreversibly with phenolic quinones (if present) to form colorless addition products. This makes these constituents unavailable as substrates for polyphenol oxidases.

Sulfur dioxide increases cell membrane permeability and helps substances (particularly phenolics) dissolve into the must. Sulfur dioxide is said to suppress tastes of rot, earth and mustiness, and accentuate aromatic qualities in wines, but if used incorrectly or in excess, it can be detected in the bouquet. It binds to anthocyanins, and temporarily bleaches them by neutralizing the positive charge on the central oxygen heterocycle (ring B – see Section 5.8.6, Figure 5.8.6), but this association is not permanent, and when free sulfur dioxide is removed from solution, the portion attached to the anthocyanidins will dissociate, and the juice or wine will regain its red color.

Liquid sulfur dioxide is sold as a gas compressed to liquid state. It is the cheapest way to buy sulfur dioxide and is easy to use, but presents a potential health hazard since high concentrations of SO_2 are toxic (Section 5.9.2) and dosing musts or wines this way can be inaccurate and wasteful. Sulfur dioxide from cylinders is often dissolved in water prior to utilization to give saturated 'sulfurous acid' solutions.

More commonly, sulfur dioxide is introduced to a medium as potassium or sodium metabisulfite ($K_2S_2O_5$ or $Na_2S_2O_5$), also known as 'Campden tablets' if sold in tablet form. It is a colorless powder and an almost universally used additive to wine. Potassium or sodium metabisulfite both give approximately 57% and 67%

(respectively) free SO_2 when dissolved, i.e. on dissolution, 1 kg metabisulfite will produce around 570 g sulfur dioxide (Bird, 2005). Metabisulfite can be used as a 2.5%, 5% or 10% solution.

Sulfur matches or candles can be burnt to produce sulfur dioxide, but this is only recommended for fumigating wooden barrels or casks; it should never be used with plastic or stainless steel vessels (Morgan *et al.*, 2006).

In white wine production, sulfur dioxide is usually added to the must as soon as it has been pressed. Sometimes sulfur dioxide is added to the vintage before pressing (e.g. in vintages which are very sensitive to oxidation), but it is recommended to destem first as phenolic extraction is increased. The amount of sulfur dioxide to be added before alcoholic fermentation depends on the ripeness, health and temperature of the vintage. Sulfur dioxide has a very low toxicity for humans if used at correct levels (Bird, 2005), however there has been an increasing consumer focus on the ingestion of SO_2 from foods/drinks. Between 10 and 20% of the population is hypersensitive to sulfur dioxide and some of these will develop headaches and gastric upsets even with wines containing normal doses (Section 5.9.2). Recommended doses vary a great deal, but due to health concerns (especially regarding asthmatics), the use of SO_2 is strictly controlled, with total additions being restricted by law to (for example) 160 mg/l for reds and 210 mg/l for dry whites (Section 5.9.5). Consideration should be given to the state of health of the crop, e.g. if levels of rot are high, more SO_2 will be needed to combat laccase activity. Addition of the sulfur dioxide should be homogeneous to ensure protection of all juice or wine fractions.

In winemaking, after alcoholic fermentation, unless a malolactic fermentation is required, 50–75 mg/l of sulfur dioxide is added on first racking (Morgan *et al.,* 2006). The free level is measured after a few days, and more sulfur dioxide may be added so as to maintain the free sulfur dioxide levels at 5 to 15 mg/l for red wines, 20–30 mg/l for dry whites and 60–80 mg/l for sweet whites. This dose depends on the temperature of storage and the pH of the wine. There is no maximum legal limit for the free sulfur dioxide content of wine, but the maximum total sulfur dioxide content in Europe is controlled by law (Section 5.9.5). The maximum total sulfur dioxide content in most countries for dry white and rosé wines is around 210 mg/l, whereas that for white or rosé wines with more than 5 g/l residual sugar is 260 mg/l (Morgan *et al.*, 2006) (see also Section 5.9.5). The addition of SO_2 is particularly important for wine being aged in wood as it is difficult to ensure complete air exclusion from all barrels with a resulting risk of film yeast growth.

As the sulfur dioxide content of wines must be below legal limits (Section 5.9.5), it is important to monitor levels of total SO_2 throughout the production of wine. Measurement of total SO_2 in wine is usually done by a modified Ripper method, in which the sulfur is titrated with iodine (Section 4.6.3). Once all the sulfur dioxide has reacted with the iodine, any more iodine added to the wine will turn a starch indicator solution blue. Sulfur dioxide can also be determined by aspiration and titration of aspirated, oxidized sulfur dioxide with sodium hydroxide (Section 4.6.3) (Iland, *et al.,* 2004).

2.5.4 Sorbic Acid

Sorbic acid is a short chain fatty acid used as a chemical preservative, primarily as a fungicide. Sorbic acid inhibits fermentative yeasts (but not oxidative film yeasts) so is useful as a fermentation control. It has little effect on lactic or acetic bacteria, and is relatively ineffective against *Zygosaccharomyces bailli* (Jackson, 2000c). The pure compound has a flavor similar to butter, but if impurities are present, it can seem rancid. It will reinforce the antimicrobial effects of sulfur dioxide, but has a low threshold value (135 mg/l), and some tasters perceive it at 50 mg/l, so should be used with care. pH affects the efficacy of this compound as a preservative. At pH 3.1, 150 mg/l is adequate for antimicrobial action, whereas at pH 3.5, the effective dose is more than 200 mg/l (Morgan *et al.*, 2006). Lactic acid bacteria can convert it to 2-ethoxyhexa-3,5-diene which has a ‘geranium leaf’ aroma (Jackson, 2000d) (Figure 2.5.5). It has no antioxidant action. It is sold

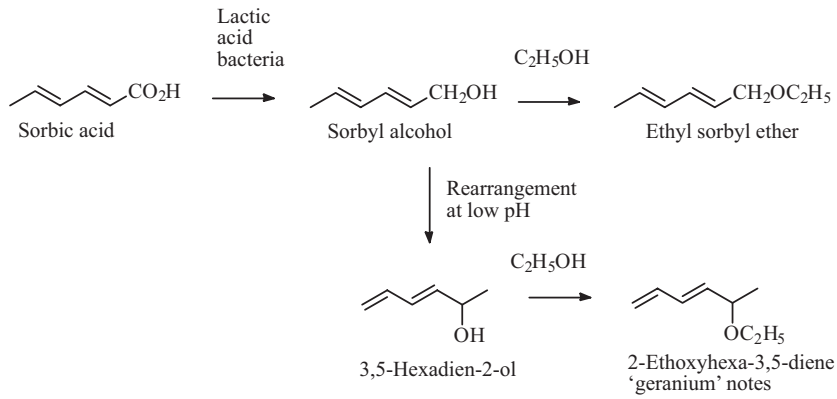


Figure 2.5.5 *Formation of malodorous products from sorbic acid*

either as sorbic acid, which is poorly soluble, or as potassium sorbate (270g KS ≡ 200g SA). Sorbic acid is only used in conjunction with SO_2, and in wines with residual sugar and a low yeast count (<100 cells/ml).

2.5.5 DMDC (Velcorin)

Diethyl dicarbonate, a preservative used in a number of beverages in the past, was formerly marketed as Baycovin. At higher pH and higher temperatures, this compound is hydrolyzed to ethanol and carbon dioxide. One of the breakdown byproducts is ethyl carbamate, which is carcinogenic (Section 5.11.5). Dimethyl dicarbonate (DMDC) was developed as a replacement for DEDC, and cannot form ethyl carbamate, but forms methyl carbamate on breakdown, which so far has not been found to be carcinogenic. It is a sterilant, which has been used with dealcoholized wines, and may have some application in sterile bottling without the use of membrane filters. Recent studies of the inhibitory effect of DMDC added to red wine media (12% ethanol v:v; pH ∼ 3.50) inoculated with cultured yeasts and bacterial populations showed that it was (at the maximum legally permitted dose: 200 mg/l) an efficient preservative against low contamination rates of yeasts (Costa *et al.*, 2008). DMDC at the 100 mg/l level was effective against initial inocula of 500 cfu/ml of *Dekkera bruxellensis*, *Lachanacea thermotolerans*, *Pichia guilliermondii*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Zygoascus hellenicus* and *Zygosaccharomyces bailii*, but not against all of them when inoculated at 10^6 cfu/ml. A combination of potassium metabisulfite (100 mg/l ≡ 1 mg/l of SO_2) and DMDC (100 mg/l) was effective against yeast populations of 10^6 cfu/ml, but did not fully kill *S. cerevisiae* or *S. pombe*. On the negative side, DMDC is ineffective against lactic acid and acetic acid bacteria (Costa *et al.*, 2008), is very corrosive, is not water soluble (Jackson, 2000c), and the equipment needed for using it is cost prohibitive for most small wineries.

2.5.6 Ascorbic Acid (Vitamin C)

Ascorbic acid or vitamin C is a monobasic acid with a lactone ring occurring between carbons 1 and 4 (Zoecklein *et al*., 1995). It has long been used in the wine industry as an antioxidant, the reason being the ability of the acid to rapidly remove molecular oxygen from juice or wine. Ascorbic acid has been licensed for use in winemaking in the USA since the mid 1950s and other 'new world' countries, where it

has been added to wine prebottling as a 'preservative.' The reaction between ascorbate and oxygen takes place almost 1700 times more rapidly than between sulfur dioxide (SO_2) and O_2. Ascorbic acid occurs in grapes at low concentrations (up to 50 mg/l) and it is rapidly oxidized during racking and crushing of grapes, but may be added to must or wine by the winemaker (maximum legal addition is 150 mg/l) (Morgan *et al.,* 2006). It serves to help preserve fragile aromas and prevent browning. It has no curative effect and no antimicrobial action. Ascorbic acid may reduce oxidized phenols (quinones) back again to phenols, but by-products of this reaction are dehydroascorbic acid and H_2O_2, the latter being a potent oxidizing agent (Jackson, 2000b).

The formation of H_2O_2 means that sufficient free SO_2 has to be present in the wine to react with it so as to prevent further oxidation. Zoecklein and cowriters (1995) suggests that ascorbic acid has no obvious antimicrobial or antioxidant benefit, and raises concerns that it may in fact have the opposite effects due to its reactivity with molecular oxygen. It has the ability to catalyze oxidation of SO_2 and should only be used where wines already have low levels of SO_2 (less than 100mg/l). It should not be used as an alternative to SO_2. Recent research certainly supports this view, showing that ascorbic acid seems at first to act as an antioxidant, but when its concentrations are low, it can enhance oxidation in an artificial wine medium (Bradshaw *et al.,* 2004). By-products other than dehydroascorbic acid and H_2O_2 have been identified and could lead to ascorbic acid enhancing the yellow color of white wine. This should prompt winemakers to reassess the use of ascorbic acid, as well as SO_2 levels in wine. It is usually recommended that ascorbic acid is used only in conjunction with free SO_2, which with H_2O_2 forms H_2SO_4 (sulfuric acid) (Bradshaw *et al.,* 2004). Ascorbic acid is mostly used for limiting oxidation before alcoholic fermentation (50 mg/l), protecting the wine against ferric haze, and protecting the wine from oxidation when bottling (the 'bottle sickness' period is reduced). Ascorbic acid is also widely used in fruit juices, beer and sparkling drinks. The concentration of ascorbic acid, even if moderately high, does not adversely affect quality.

2.5.7 Pimaricin (Natamycin)

Natamycin, also known as pimaricin, is a substance produced by the bacterium *Streptomyces natalensis* in order to prevent growth of competing yeasts and moulds. Pure natamycin has no color, odor or taste (http://www.dsm.com/en_US/downloads/dfs/Fact_sheet_Natamycin_v6.pdf).

Natamycin is widely used in the food industry in juices, wine and yoghurt, and may be used in South Africa in wine, but not in the EU as yet. It hydrolyzes after use, and loses its effect (Ribéreau-Gayon *et al.*, 2006). As natamycin is a 'natural ingredient,' it is also used in organic and biological cheeses. Natamycin binds to ergosterol, a building block in cell walls of yeasts and molds, causing breach of the cell wall. Bacteria do not contain ergosterol and are therefore not affected. Natamycin is effective in small quantities against most yeasts and molds that may occur on food products.

2.5.8 Other Preservatives

Stead (1993) assessed the potential of hydroxycinnamic acids (including caffeic, coumaric and ferulic acids) on the growth of three wine spoilage strains of *Lactobacillus collinoides* and one of *Lb. brevis* in tomato broth containing 5% ethanol at pH 4.8. At concentrations of 500 and 1000 mg/l, all three compounds markedly inhibited growth; coumaric and ferulic acids were more effective than caffeic acid. At a concentration of 100 mg/l, however, all compounds stimulated growth. In general, the strains of *Lb. collinoides* were more susceptible both to inhibition and stimulation than *Lb. brevis*.

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2.6
Beer

A man who doesn't care about the beer he drinks may as well not care about the bread he eats.

—Michael Jackson, 1977

2.6.1 The Basic Brewing Processes

Nowadays, the term beer refers to any drink that is produced by the fermentation of sugars derived mostly or entirely from malted cereals (principal of which is barley) and flavored with hops. Other flavorings, such as cherries, citrus, coriander, juniper and spruce are also used for certain beer styles (see Section 2.6.13). Some staple drinks of Africa, Asia and South America: fermented beverages based on maize, rice or sorghum, and which do not involve flavoring with hops, are discussed in Chapter 2.7.

The basic source of fermentable sugars in the brewing of beer is malted barley grains, although other malted cereal grains, notably wheat and rye, may be used, but usually in conjunction with malted barley. Unmalted cereals, such as barley, wheat, torrified wheat, flaked maize, rice and others, are sometimes used as adjuncts (minor ingredients) in the brewing of certain types of beers or in others to effect particular characteristics in the finished product (Section 2.6.2). Also, some larger breweries use industrial enzymes to produce fermentable sugars directly from the starch of unmalted grains such as rice, thus avoiding the malting process for at least some of the mash (Section 2.6.2). Additionally, malt extracts, syrups and a variety of brewing sugars are used (along with malted barley) in some breweries (Section 2.6.3).

The basic stages of brewing are shown in Figure 2.6.1, which refers to batch (or 'gyle') brewing (see Section 2.6.5 for continuous fermentation). Malting and mashing can be considered as the first stages in brewing. Malting is the controlled germination of barley (and other cereal) seeds. It leads to 'modification' of the barley constituents: certain chemical changes, caused by the action of released enzymes, occur in the germinating grains (Section 2.6.2). Kilning (and/or roasting) then stops the modification processes and causes other effects (flavoring and coloring), depending on the temperatures (Section 2.6.2). Many breweries buy their barley already malted by large malting companies, such as Baird, Munton and Fison, Pauls and Simpson (all UK), Dewolf-Cosyns, Dingemans (Belgium), Meusdoerffer, Weyermann (Germany), Cargill (Australia), Gambrinus (Canada), Briess, Schreier (USA) and Malteries Franco-Belges (France). A few breweries still

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

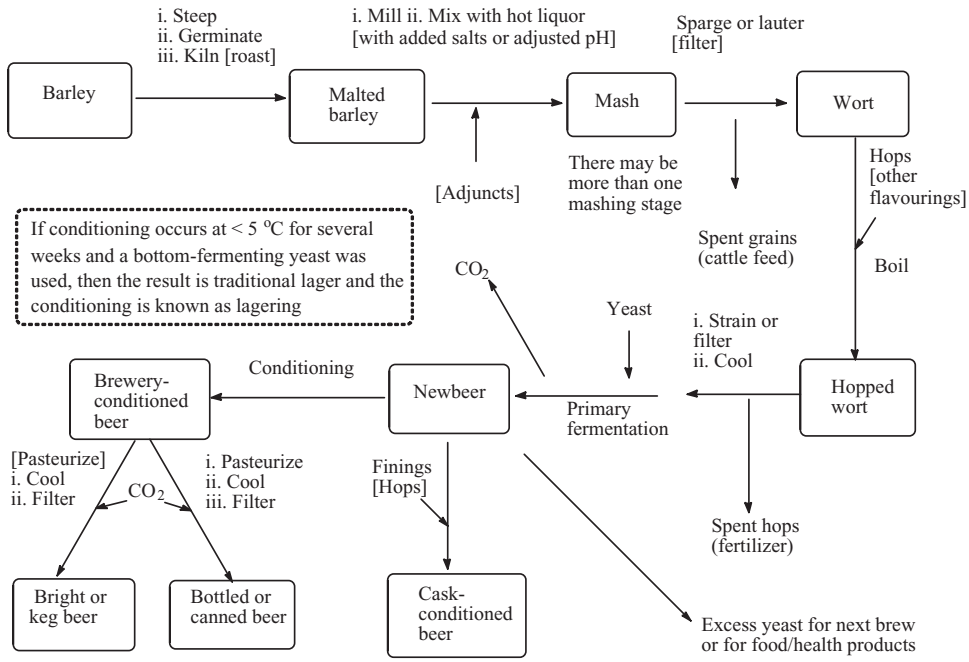


Figure 2.6.1 *The basic brewing processes (optional items are given in square brackets)*

malt their own barley, an example being Cooper's of Adelaide, Australia. However, the big majority of breweries buy malt from large malting companies or are supplied with malt from a malting company under the same ownership as the brewery.

Whatever the source of malt, the brewery will require the best quality barley, carefully malted to certain specifications, depending on the intended product. Quality control analysis of a malt sample will be carried out in order to ensure that these specifications are met (Bamforth, 2002a). Likewise, analysis of the brewery's water (liquor) supply, especially with regard to Ca^{2+}, SO_4^{2-} and HCO_3^- content, will be performed on a regular basis. This is to ensure that the mashing process occurs at the optimum pH (~5.2) and that the correct balance of salts is present for the type of beer being brewed (Section 2.6.2).

After mashing, the hot liquid (now called wort or gyle) is run off or pumped into a brewing kettle or 'copper.' The barley husks act as a filter bed, but the wort can be clarified by further filtration if necessary. The husks are then rinsed with a fine spray of a predetermined volume of hot liquor at a predetermined temperature. This is known as sparging or lautering; it optimizes the extraction of sugars from the malted grains. At this stage, the unhopped wort may be analyzed, at least for its original gravity (OG), so that the extraction efficiency can be determined (Section 2.6.2).

The wort is boiled with hops (and any other flavorings), filtered and then cooled rapidly, as it is transferred to the fermentation vessel. The boiling process not only stops enzyme action, sterilizes the wort and adds flavors, but also coagulates some proteins that might otherwise cause the beer to be cloudy (Section 2.6.3).

Yeast is added to the cool, hopped wort and fermentation is conducted at a temperature and for a period of time that depends on the type of beer being brewed (Section 2.6.4).

The fermentation progress can be monitored simply by measuring the drop in specific gravity (attenuation) (Section 2.6.4), but more specific tests can be performed, according to the circumstances. Excess yeast is

separated from the liquid as required; it can be compressed and used in a future brew, or can be used to make health products. After a period of primary fermentation, the young ('green') beer is run off or pumped into conditioning tanks where it continues fermentation; for traditional lager beers, this process can occur over several weeks at low temperatures (∼5 °C or 41 °F).

Alternatively, the young beer can be transferred directly to the casks for further fermentation ('cask-conditioning'). Finings and hops ('dry hopping') can be added at this stage. Extensive analytical and sensory tests may be carried out on the beer at any stage of the fermentation process, before and after the beer leaves the brewery. Most beers destined for can or bottle are pasteurized, chill filtered and then artificially carbonated, although many bottle-conditioned beers exist (Sections 2.6.10 and 2.6.13). Many brewery-conditioned beers intended for serving on draught are chill filtered, but not pasteurized (Section 2.6.9).

For detailed discussions on various aspects of beer production, the reader is referred to Hough *et al.* (1982), Fix (1999), Hornsey (1999), Hughes and Baxter (c06+bib+0006), Iserentant (2003) and Paterson *et al.* (2003).

2.6.2 Malting and Mashing

Barley is the most important of the many cereals that are used in brewing. *Hordeum vulgare* and *H. distichon* are the botanical names of the six-rowed and two-rowed species of the plant (respectively) producing the seeds (grains, corns or kernels) that are so valuable to the brewer. The superiority of barley over other cereals is a result of the higher starch to protein ratio and lower moisture content of its unmalted seed. Additionally, barley is rather easier to grow than wheat, rye or maize: it is more tolerant of inclement weather and is less liable to fungal infection. It is also easier to malt than other cereals.

Two-rowed barley is grown in areas with temperate growing seasons all around the world, from northern USA and Canada to central and western Europe, including England and Scotland. Six-rowed barley is grown in warmer climates, such as mid western USA. This produces grains with higher protein contents than two-rowed types. Six-rowed barley grains are used in brewing, but certain varieties of two-rowed barley (often winter sown varieties) form the mainstay of quality in the brewing industry. These include Harrington (USA), Maris Otter (UK), Scarlett (Germany), Alexis and Prisma (Belgium). Other popular two-rowed malting varieties include Chariot, Fanfare, Gallatin, Golden Promise, Halcyon, Merit, Optic, Pearl, Pipkin and Regina and two popular six-rowed varieties (USA and Canada) are Legacy and Robust. Some of these barley varieties (e.g. Optic and Pearl) are also used to produce malt whisky (Section 3.2.2). Many of these varieties are grown by farmers under contract from the large malting companies, who then malt the harvested crop according to specifications required by particular breweries, as described next.

Figure 2.6.2 shows a section through a typical barley grain. Such a grain contains 78–83% total carbohydrates (63–65% starch, 3–8% β-glucans, 4–7% pentosans and 4–5% cellulose), 10–12% proteins and amino acids, 2–3% lipids and 2% minerals. Apart from these, water and flavonoid phenols (including proanthocyanidins) and low concentrations of many organic compounds are present.

Brewers prefer their malted barley with a high starch, low protein and low moisture content. When the batches of harvested barley arrive at the malting plant, the maltster will immediately apply a number of tests to representative samples of the grains from different batches. These are:

1. Visual tests (for maturity, signs of disease or damage and identity)
2. Determination of protein and moisture content, by near infrared spectroscopy(Section 4.4.2)
3. Viability tests, such as the tetrazolium dye test.

The last-named test uses triphenyltetrazolium chloride (TTC) to determine the viability of cereal grain embryos. Intense, even staining of the aleurone layer is an indicator of viability; it predicts that the sample of

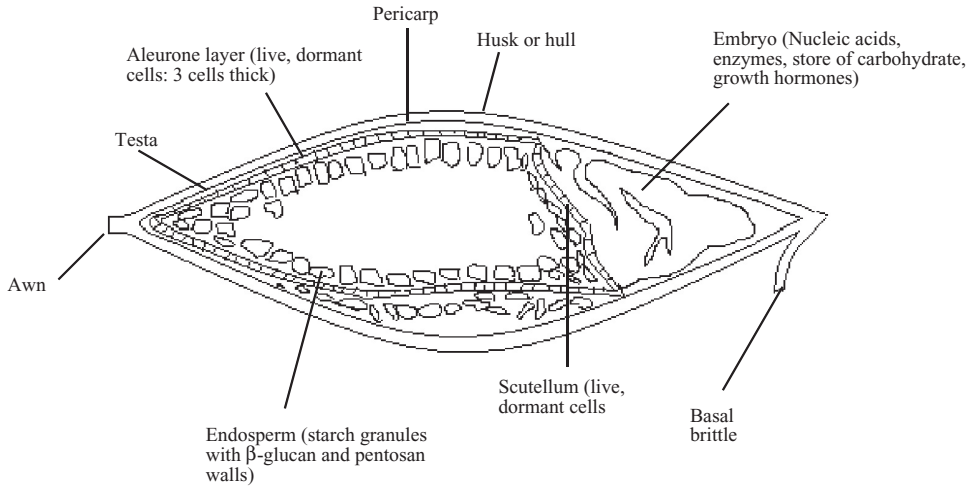


Figure 2.6.2 *Schematic cross section through a barley grain*

barley has good malting qualities and will undergo extensive modification during malting (Briggs *et al.*, 2009). Colorless, soluble TTC is reduced to a scarlet insoluble formazan product at sites on cereal cell mitochondria, including cytochrome oxidase, salicyl hydroxamic acid (SHAM)-sensitive oxidase and succinate oxidase. Barley with poor malting potential undergoes poor or patchy staining of the aleurone layer, because of faulty mitochondrial function, hence the intensity of tetrazolium staining of aleurone layers can be used as a guide to the breeding of barleys with superior malting qualities.

If these tests give satisfactory results, the barley will be accepted and will then be transferred to silos for storage. The maltster will then perform both physiological/anatomical tests and specific chemical tests on samples of grain. The former include germinative capacity, germinative vigor and germinative energy tests; these will be carried out periodically, so that the maltster can assess when seed dormancy is over and the grains are ready for malting. The results of these tests may indicate the type of malting regime that can be applied. Specific tests include moisture assessment, nitrogen content determination (Section 4.6.3), β-glucan assessment (Section 4.4.3) and analysis for fungal toxins (especially deoxynivalenal, DON, from infection by *Fusarium* species) (Section 5.11.4) and pesticide residues (Section 5.10.2).

The concentrations of fungal toxins and pesticide residues must be below regulatory limits: levels of DON may be decreased by spraying the barley grains with sodium bisulfite solution prior to malting (Lake *et al.*, 2007). Apart from the above analyses, pale ale brewers require low levels of the dimethyl sulfide (DMS) precursor *S*-methyl methionine (SSM) (Section 2.6.4) in the malt and may request its assessment. Once satisfied, the maltster will carry out a pilot (small scale) malting before committing larger quantities. A typical barley specification prior to malting is given below:

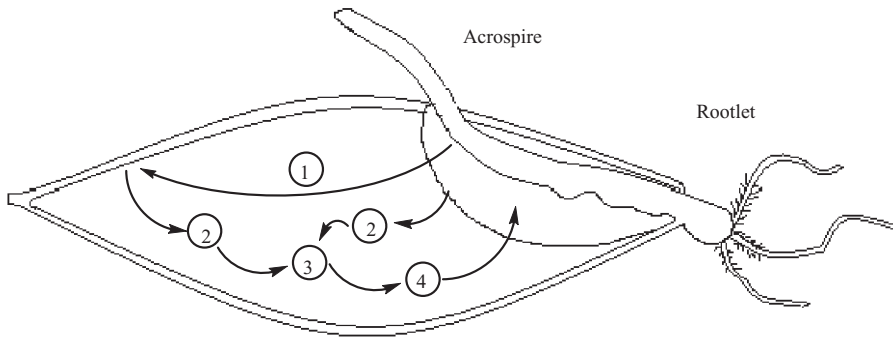
Germinative capacity >96% after 72 h
Germinative energy 95% after 72 h
Water content 12.0–13.0%
Protein content 9.0–11.5%
β-glucan content < 4%
Pesticide and fungal toxin residues within statutory limits

Purity of barley variety >99%
The malting process consists of the following basic steps:

- washing
- soaking/aerating
- germinating
- drying (and/or roasting for brewing dark beers)

There are many ways in which each of these steps can be performed, and indeed a particular malting plant may use different combinations of methods for particular kinds of malt. Modern malting plants, apart from being partially or fully automated and generally working with hundreds of tonnes of grain at a time, are designed to occupy minimal space, to waste less energy and water, to promote maximum hygiene and ease of cleaning and to produce uniformly high quality malt. Traditional malting houses, which include a germination floor (they are often known as 'floor maltings') are now quite scarce, but include small scale operations associated with a small number of Scotch malt whisky distilleries (see Chapter 3.2, Figures 3.2.2 and 3.2.5; see also this section, Figure 2.6.5). They are much more labor intensive than modern malting houses and are probably more wasteful on energy and water, but they do produce excellent malt and they have an unmistakably romantic aura attached to them. The first two stages of malting, washing/steeping and germination, along with a description of the biochemical and physiological changes that occur during these stages, are outlined next for both traditional and modern malting plants. This is followed by an outline of the final malting stages of drying (kilning) and roasting.

In the steeping vessel, the washed grains are subjected to a cycle of spraying and aeration. After about 24 h, the water content of the grains reaches 45% or so and small roots ('chits') appear as first signs of germination. In traditional malting plants, the grains are transferred to the malting floor to a depth of about 25 cm in order to continue germination for 5–8 days, depending on the degree of modification required. Germination causes profound biochemical changes to the barley grains, as summarized in Figure 2.6.3. During germination, the first scene of action is the embryo. Influx of water promotes respiration of the embryo carbohydrates, which provides energy for growth. As these carbohydrates are depleted, the scutellum cells produce hormones called



1. Gibberellin release from embryo on steeping in water. 2. Release of hydrolytic enzymes from a leurone layer and scutellum. 3. Hydrolysis of cell wall carbohydrates, proteins and some starch (to maltose and glucose) 4. Transport of sugars to embryo for metabolism (respiration) and growth.

Figure 2.6.3 *Barley germination: summary of events*

gibberellins, which are released into the endosperm, where they stimulate the aleurone cells to produce a variety of enzymes, as summarized below:

- Cytolytic enzymes: β-glucanases and cytases, for degrading the endosperm starch cell walls (composed of ~75% β-glucans, 20% pentosans (arabinoxylans) and 5% proteins)
- Carbohydrases: α- and β-amylases mainly, for degrading starch into maltose and other sugars such as dextrins (α-glucans)
- Proteolytic enzymes (proteases): proteinases and peptidases, for degrading proteins into α-amino acids and small peptides
- Oxidoreductase enzymes: thioredoxin (actually a thioredoxin-thioredoxin reductase-NADPH complex) for reducing disulfide bridges of proteins (e.g. hordeins, α-amylase subtilisin inhibitor), thereby destroying their tertiary structures and making them more susceptible to hydrolysis by proteases.
- Acidity regulating enzymes: phosphatase.

The actions of these enzymes during germination cause 'modification' of the barley: β-glucan polymers, proteins and a little starch are degraded to soluble products of lower molecular weights. On the other hand, the maltster takes care to avoid overmodification: the aim is not to produce new barley plants.

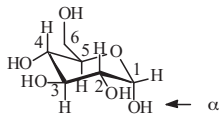
Excessive quantities of soluble β-glucan oligomers (smaller polymers) in the wort can cause processing problems and can also lead to haze problems in the finished beer (Section 2.6.9). In view of this it would seem desirable that degradation of β-glucan polymers and release of soluble β-glucans is minimized and/or extensive soluble β-glucan degradation (to β-D-glucose) is promoted during malting and mashing.

In practice, during malting, degradation of β-glucans in the cell wall to soluble oligomers is limited, whereas degradation of pentosans (arabinoxylans) is much more extensive. Experiments mimicking the action of hydrolytic enzymes on cell wall constituents support this observation. Addition of purified enzymes to purified barley cell walls have indicated that the action of β-glucanase enzymes on β-glucan polymers is hampered by the presence of pentosan polymers, and especially because of the numbers of acetyl and feruloyl sidechains in these polymers (Kanauchi and Bamforth, 2002). In fact, xylanases were the most effective releasers of β-glucans, presumably because these enzymes were able to release all of the pentosan from the cell walls. Also, esterases that hydrolyze acetyl and feruloyl groups released some β-glucan (but not pentosan), suggesting that these side chains play an important role in the inaccessibility of the β-glucans. No more than 15% of total glucan present in the purified cell walls was released by various enzymes, under these conditions.

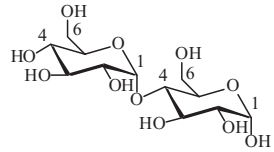
Up to 40% of barley proteins are degraded during germination. Again, excess protein content in the beer can lead to hazes, especially if the polyphenol content (Section 2.6.9) is also high. On the other hand, starch degradation is only slight – a typical malt has 59% starch and 8% sugars, compared with 65% and 2%, respectively, in unmalted barley. This is despite the fact that α-amylase and associated enzymes are synthesized in malted barley; the α-amylase activity of raw barley is typically less than 10% that of malted barley. The major degradation of starch occurs during mashing, hence the hydrolytic reactions will be dealt with there (see Figure 2.6.10). Structural characteristics α- and β-D-glucose, of α-glucans, β-glucans and starch are illustrated in Figure 2.6.4.

Germination time depends on barley characteristics, temperature and aeration of the malt bed, but is usually around five days. Regular turning of the malt grains during germination ensures uniform modification. In a traditional floor malting plant, this is known as 'grubbing' and is performed manually using a large wooden fork (Figure 2.6.5).

Some of the more modern malting houses use the saladin box type (or rectangular enclosed floor type) (Figure 2.6.5) for germination or the revolving drum type for both germination and kilning (see Section 3.2.3). Uniform germination is achieved by regular use of mechanical screws or rotation, respectively. The



α-D-Glucopyranose (α -glc), a monosaccharide and the monomer of starch



α-D-Glucopyranosyl (1-4) α-D-glucopyranose (α-glc (1-4) α-glc), better known as maltose: a disaccharide and the major fermentable sugar of malt. The ring hydrogens have been omitted, for clarity

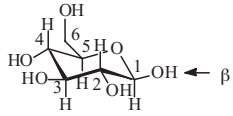
....α-glc (1-4) α-glc (1-4) α-glc(1-4) α-glc....

Amylose (linear)

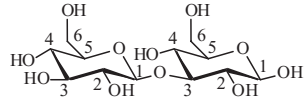
....α-glc (1-4) α-glc (1-4) α-glc(1-4) α-glc....
|
(1-6)
|
α-glc (1-4) α-glc (1-4) α-glc

Amylopectin (branched)

Starch (a poly (α-glucan). Amylose sugars are derived from the hydrolysis of amylose, whereas the branched dextrins (α-glucans) are produced by hydrolysis of amylopectin. Dextrins are only partially fermentable by yeasts



β-D-Glucopyranose (β–glc), a monosaccharide and monomer of cellulose and β-glucans



β-D-Glucopyranosyl (1-3) β-D-glucopyranose (β–glc (1-3) β–glc), a disaccharide β-glucan

β-glc
(3-1)
|
β–glc (1-4) β–glc (1-4) β– glc (1-4) β–glc

A β-glucan. β-glucans are constituents of plant cell walls. In cereals the links are mostly 1-4 and 1-3, but 1-6 links are important in fungal β-glucans.

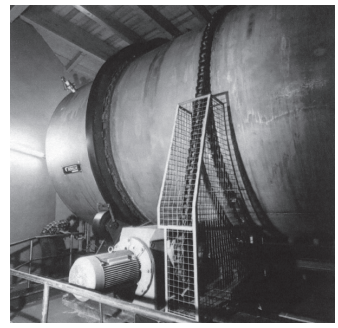
Figure 2.6.4 *Glucose based carbohydrates in barley and malt*

main advantages of these types of malting techniques are that they demand less space than traditional floor malting, require less labor and use less energy and water.

Tower malting is an even more modern technique, best described by consideration of the malting plant owned by the Bavaria Brewery (Lieshout, the Netherlands) at Eemshaven on the Dutch coast. The malting process (which went into operation in 2005) is performed in steel sectionalized towers. The grains are steeped in the top section (which can accommodate batches of 440 tonnes of barley), known as the 'Ecosteep' (Figure 2.6.5) because of its economical use of water. The section below is comprised of three rotatory germination units, each capable of taking 440 tonnes of grains. Rotation is reckoned to result in uniform germination and modification of the grains. The bottom section is a single floor rotating kiln deck for hot air kilning. Malt houses like this are able to produce almost 140 000 tonnes of malt a year, the malt being of very uniform high quality because of the rotating floors and gentle screw conveyors that discharge and spread the grains. Major advantages of lower water usage (30% less than floor maltings) and better sanitation are claimed.

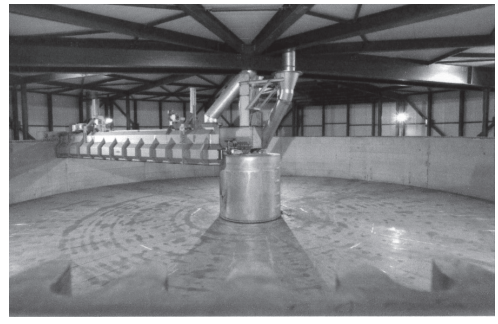
The least modified malt is preferred for traditional Pilsner style beers, whereas rather more highly modified malts are used for pale ales. The extent of modification of malt grains can be assessed using a friabilimeter, consisting of a rubber roller and a rotating sieve drum, through which the grains are passed. Satisfactorily modified malt will break into small pieces and filter through the sieve, the percentage by weight that does this being regarded as a measure of proper modification. The expectation is 90% friability or better. Alternatively, the brewer can take a representative sample of malt grains and boil them in water for 30 min. This loosens

(a) Grubbing the malt on Kilchoman distillery malting floor



(b) Grain washing drum

(c) Conical steep



(d) ECO-steep

(e) Germbox horizontal germination plant

Figure 2.6.5 *Traditional and modern maltings equipment. Photograph (a) by kind courtesy of Kilchoman distillery, Islay. Photographs (b)-(e) by kind courtesy of Bühler GmbH, Germany*

the husks and allows inspection of the length of the acrospires of, say, 100 grains; the longer the acrospire in relation to the grain length, the greater the modification. This gives the brewer a general picture of extent of modification and may also indicate cases of overmodification, where the acrospire is longer than the grain.

Germination stops when the malted barley grains are moved to the kiln and dried, usually in a stream of hot air. This mild heating (85–90 °C) shrivels the rootlets (which fall off the grains) and partially inactivates

some of the enzymes that promoted modification during germination. It also produces a very small amount of coloring substances, so the resulting beer brewed from these malts alone will be a pale to full gold. These are the base malts; not only are they used to brew pale beers, but they also form the main cereal ingredient of most other beers (Section 2.6.1). During mashing, most of the enzymes are supplied by these malts.

Whilst malting necessarily results in the formation of acrospires and rootlets, growth of large rootlets leads to significant loss (*ca.* 4%) of barley weight upon kilning, known as 'malting loss.' At the present time, potassium bromate is used by some maltsters to inhibit excessive rootlet growth during malting. A pilot plant screening experiment using *Lactobacillus plantarum* 15GR as rootlet inhibitor resulted in a 50% reduction in malting loss, but produced malted barley of similar quality to untreated barley (Schehl *et al.*, 2007). The *L. pantarum* strain performed significantly better than potassium bromate under these conditions (and is probably considerably safer).

Stronger kilning, or roasting at higher temperatures, often in a slotted, rotating drum, produces colored malts for brewing darker beers. The heat causes production of highly colored substances, caramelization and the formation of flavor compounds, via the 'Maillard reaction' (Figure 2.6.6). Caramelization of sugars causes the production of yellow-brown polymers and pleasantly aroma active furans, isomaltol and maltol. However, most of the color (called 'nonenzymic browning') and flavor of dark malts arise from the Maillard reaction, which is actually a complex multitude of reactions that begin with sugars and α-amino acids, amines or peptides. Products include those similar to caramelization, plus strongly antioxidant 'reductones,' Strecker aldehydes, nitrogen heterocycles and mellanoidins, brown pigments that are also antioxidants (Coghe *et al.*, 2004).

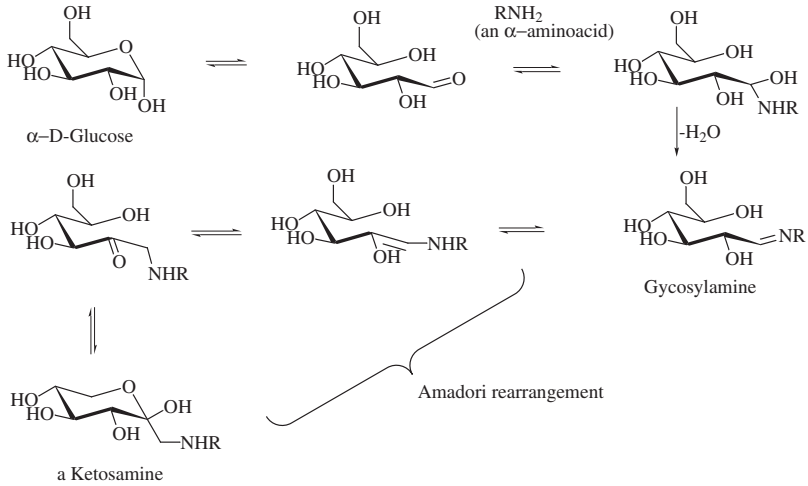
Many of these compounds are flavor active, some being more attractive than others (Figure 2.6.6). To minimize burnt flavors, some breweries, such as Diebels Altbier Brauerei of Issum, Germany, have requested dehusking of the barley grains after malting, but before roasting. Prolonged exposure to high temperatures disables more of the enzymes, so that the diastatic power (ability to hydrolyze starch molecules via amylases), measured in °Lintner (°L) (Section 4.6.3), of most colored malts is low: zero for the darkest malts. A typical pale malt would be expected to have around 130 °L of diastatic power (Bamforth, 2002b). Consequently, the highly colored malts must be used in conjunction with base malts.

Amber, mild ale, Münchner and Wiener malts are the palest of the colored malts. These still have considerable diastatic power, typically 40–70 °L (Fix, 1999b), and may be used as the major cereal in the 'grain bill' of certain beers (Section 2.6.13). They are produced from green malt by ramping the kiln temperature from 65 °C (when the moisture content has reached ∼10%) to 85 °C, the depth of color depending on the ramping programme and the time spent at higher temperatures.

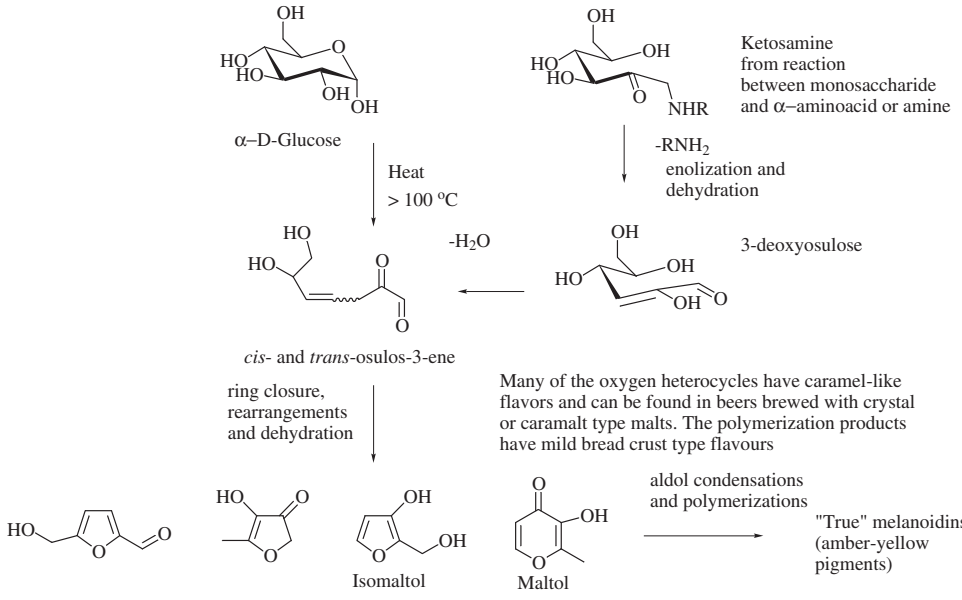
Next are the caramel and crystal malts: these have a wide range of colors and other characteristics depending on the roasting temperatures and the humidity and duration of heating. They are made by sending the green malt directly to the roaster, where firstly the surface moisture is dried off at ∼50 °C for a few minutes, then a temperature of 65–75 °C is applied for 45 minutes to stimulate saccharification and crystallization. Next, the temperature is increased to 80 °C for about the same period of time in order to dry the grains to about 5% moisture and to start color/flavor reactions. Finally, the latter processes are continued at temperatures of 120–160 °C for a period of time, depending on the depth of color and caramelization required. Two hours at 135 °C is typical for crystal malt.

Chocolate and black malts are the most highly colored of all malts, although roast (unmalted) barley gives the strongest color of any adjunct (Section 2.6.13). Brown, chocolate and black malts are all produced by roasting pale malts from the kiln. A gradual increase in the roaster temperature up to 230 °C is applied; the higher the final temperature or the longer the time spent in the higher temperature range, the greater the carbonization and the darker the malt will be.

The degree of malt coloration can be estimated by visible spectrophotometry or colorimetry (Section 4.4.3) and is expressed on scales devised by the American Society of Brewing Chemists (Standard Research

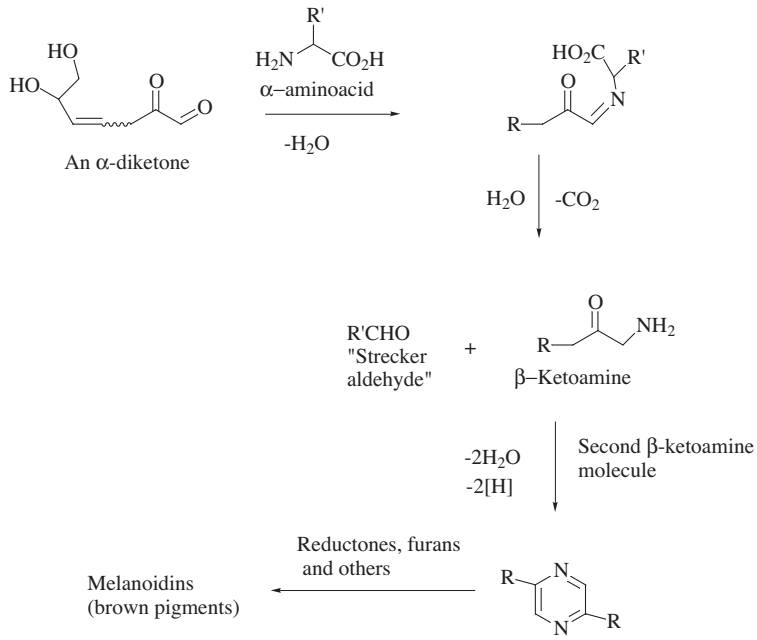


(a) Formation of ketosamine



(b) Formation of reductones, furans, pyrones and non-nitrogenous melanoidins

Figure 2.6.6 *Summary of the Maillard reaction. Single tautomers and single configurations are written for most structures, for simplicity*



(c) Strecker degradation, formation of pyrazines and melanoidins

Figure 2.6.6 (Continued)

Method, SRM, similar to the old Lovibond scale) or the European Brewing Convention (EBC). Table 2.6.1 displays typical characteristics of common malts. It should be kept in mind that these characteristics will differ somewhat between maltsters and from season to season.

In recent years, there has been considerable interest in the malting of cereals other than barley and wheat, such as sorghum or millett (*Sorghum bicolor* L. (Moench) and buckwheat (*Fagopyrum esculentum*), which are infrequent components of European beers, but are commonly used to make indigenous brews of Africa and Asia (Section 2.7.2). It has been found that a five day malting period at 26 °C led to an optimum sorghum malt regarding starch modification, protein degradation and lipid degradation. Addition of such malt to a sorghum flour porridge (which is often the way indigenous African brews are mashed and fermented) resulted in an overall sixfold increase in carbohydrate digestibility and a fourfold increase in free amino acid content (Correia *et al.*, 2008). Malted indigenous Botswana surghum varieties were shown to have β-amylase as the major contributor of diastatic power, ranging from 55% to 88% (Letsididi *et al.*, 2008). Limit dextrinase was also high: up to 231 U/g for the variety Sefofu. The malted grains of several varieties showed desirable amylolytic properties, indicating their potential use in the production of lager type beers.

Common buckwheat (with or without hulls), when malted at 16.5–20.2 °C showed similar free amino acid and soluble nitrogen contents to those of malted barley. At 20.2 °C germination, the major diastatic power contributor was again β-amylase (∼127 U/g wet weight): optimum α-amylase activity was ∼48 U/g (wet weight) using hull-less buckwheat at 16.5 °C germination. Maximum apparent fermentability was 56% with buckwheat germinated at 20.2 °C. Although fermentability and amylolytic levels were

Table 2.6.1 *Some characteristics of common barley malts*

Malt type	Approx. kilning or roasting temp/°C	Colour/°SRM/ Lovibond*	Diastatic power/° Lintner§	H_2O content/%	Total protein content/%	SMM# content/mg g^{-1} malt
Pilsener	65–85¶	1.4–1.8	90–100	3.5	11.5	8–10
Pale	65–85¶	2.5–3.5	125–135	3.0	10.0	1–2
Wiener	65–85¶	3.0–4.0	50–70	4.0	11.5	–
Mild ale	65–85¶	3.5–4.5	55–70	3.0	10.5	1–2
Münchener	65–105¶	4.0–7.0	40–65	4.5	11.5	–
Caramalt/crystal	80–160	10–150	–	4.5–9.5	10.0–11.5	–
Chocolate	Up to 230	300–500	–	3.5–5.0	11.5	–
Black	Up to 230	500–600	–	3.5	11.5	–

¶The darker malts of this category are held in kiln at higher average temperatures
*Determined according to a standard reference method of the American Society of Brewing Chemists (ASBC) (Section 4.4.3). Approximate conversion of SRM to EBC (European) units: EBC = 2.6(SRM – 0.46)
§A measure of the malt's enzyme power, according to a standard procedure of the ASBC (Section 4.6.3)
#SMM = *S*-methylmethionine, the major dimethyl sulfide (DMS) precursor

generally low compared with malted barley, buckwheat malt is considered to have potential as a brewing ingredient (Wijngaard *et al.*, 2004), particularly with regard to gluten-free beers (Section 5.11.3).

The next stage in brewing is mashing: the malted barley grains are put through a screening machine (to remove stones and other debris) before being crushed ('milled') and the resulting grist is mixed with hot brewing liquor. This mash is left for a period of time at a fixed temperature, or is taken through a number of temperature stages ('rests') by a temperature ramping process (Figure 2.6.7). Cereal adjuncts can be added at the mixing stage and the acidity and mineral content of the liquor can be adjusted prior to mixing.

During mashing, starch is hydrolyzed by α- and β-amylase to soluble sugars (mainly maltose and α-glucans or dextrins). Proteins and β-glucans are hydrolyzed to soluble products by proteinases and β-glucanases, respectively. Additionally, there is believed to be some reaction between proteins and carbohydrates to give glycoproteins, useful for haze stability and foam stability ('head retention'). It has been observed that the native barley hydrophobic protein, lipid transfer protein 1 (LPT1), which is a poor foam promoter, is transformed during malting and mashing to a modified structure, which is a good foam promoter (Leisengang and Stahl, 2005).

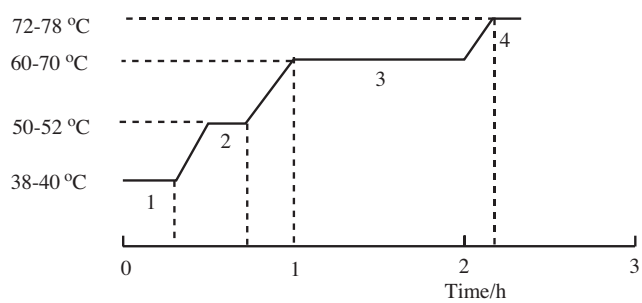
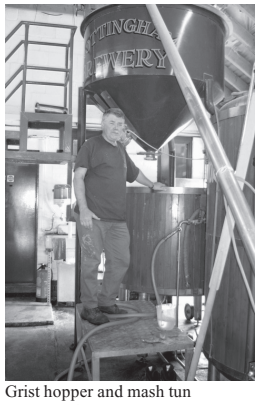


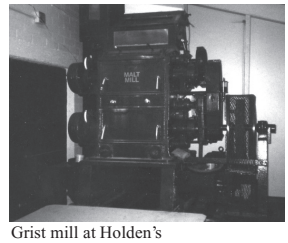
Figure 2.6.7 *Typical stepwise infusion mashing program*

Mashing lasts for about an hour or more depending on the regime, after which time, the sweet liquor (wort) is run off through the grains into the brewing kettle, ready for the boiling stage. The spent grains are rinsed through with hot liquor ('sparging') in order to maximize sugar extraction. Most of the above factors are discussed in more detail in the next paragraphs.

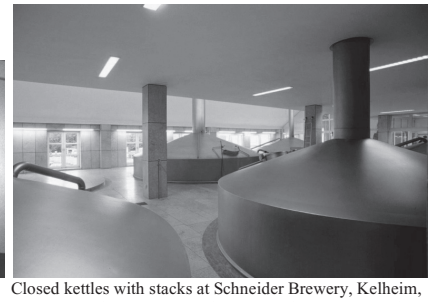
Coarse milling tends to be favored by smaller breweries: the grains are cracked, but the hulls are left intact to act as a filter bed for the wort. Some malting companies are able to supply microbreweries with small bags (10 kg) of crushed pale malt. Adjuncts such as flaked maize and rice hulls can aid this filtration process. Figure 2.6.8



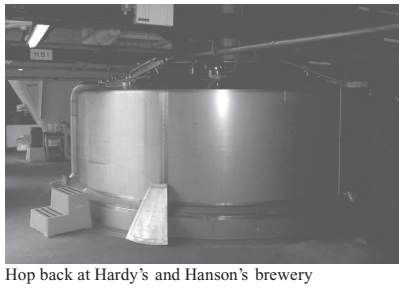
Grist hopper and mash tun at Nottingham Brewery (Nottingham, UK).



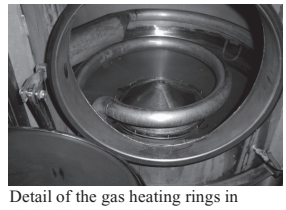
Grist mill at Holden's Brewery (Woodsetton, UK)



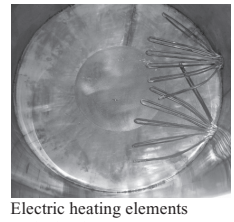
Closed kettles with stacks at Schneider Brewery, Kelheim, Germany. *Photo courtesy of G. Schneider & Sohn GmbH.*



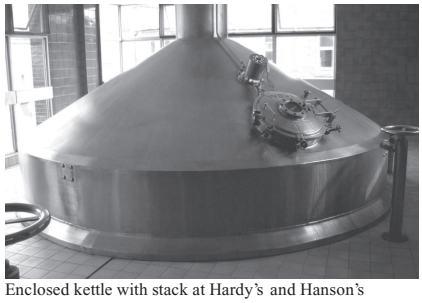
Hop back at Hardy's and Hanson's brewery (Kimberley, UK). The copper is directly above.



Detail of the gas heating rings in the kettle at Nottingham Brewery.



Electric heating elements in the kettle at Magpie Brewery, Nottingham.



Enclosed kettle with stack at Hardy's and Hanson's brewery.

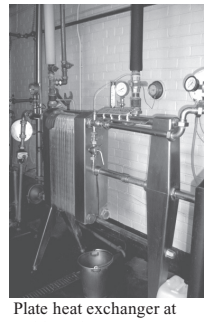


Plate heat exchanger at Holden's brewery.

Figure 2.6.8 *Some prefermentation brewery equipment. Photo courtesy of Luc Bohez from Brouwerij Lindemans, Belgium. Photo courtesy of G. Schneider & Sohn, Nürnberg, Germany*

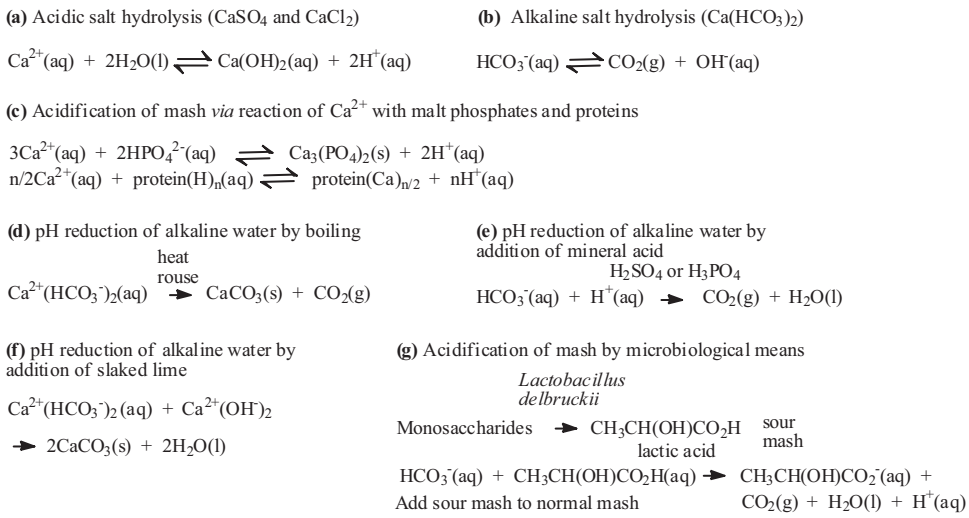


Figure 2.6.9 *Salt hydrolysis, effect of Ca^{2+} on mash acidity and methods for reducing liquor or mash pH*

shows malt mills, mash tuns and related equipment at a number of breweries. Many larger breweries prefer to mill the malt grains into a fine powder. Because of the larger surface area, this results in a more effective conversion of starch to sugars. Following this, a more efficient extraction of the sugars from the insoluble residue can then be obtained by mash or membrane filtration (Kunze, 1996; Schneider and Weisser, 2004).

The mineral content and pH of the mashing liquor are of prime importance, not only because of the influence that these have on the mashing process itself, but also because this liquid will be the medium for all subsequent brewing stages. The hydrolytic enzymes in the mash require a medium pH of between 4.5 and 5.0 for maximum activity, and it is generally held that the maximum mash pH should be 5.4 (Fix, 1999). Because of salt hydrolysis, the presence of significant concentrations of minerals (dissolved salts) in the liquor can cause the mash pH to deviate either way from the optimum range (Figure 2.6.9). Now, a mash of pale malts only and distilled water will have a pH close to 6.0, whereas a mash with substantial quantities of colored or roast malts in its grain bill may have a pH of less than 5.0. Consequently, acidic salts in the liquor will be beneficial in the brewing of pale beers, whereas alkaline salts will be more suitable for dark beers. Acidic salts (in permanently hard water) include $CaSO_4$, $CaCl_2$ and $MgSO_4$; alkaline salts (in temporarily hard water) include $Ca(HCO_3)_2$, $Mg(HCO_3)_2$ and $CaCO_3$. The mineral content of brewing liquor will depend on the local geology and, if obtained from the local water company, on the water treatment implemented by that company.

Experience has shown that certain locations produce their best beer styles in accord with the mineral profile of the local water, as outlined in Table 2.6.2 However, 'incompatible' beer styles have been brewed in such areas for many years (or even centuries) by adjustment of mineral content and pH; indeed some of the best pale ales are brewed in London and some of the best Pilsners in Dortmund, despite the high HCO_3^- content of the local water in these two cities.

The most important salts are $CaSO_4$ and $Ca(HCO_3)_2$, although salts of Mg^{2+}, Na^+ and Cl^- may also be significant. Calcium ions in the brewing liquor are of benefit to brewers for many reasons:

- Reaction with malt phosphates and nonhydrolytic proteins lowers mash pH, which in turn enhances hydrolytic enzyme activity
- Reaction with nonhydrolytic proteins causes precipitation, thereby enhancing clarity
- Calcium ions protect α-amylase from inhibition (denaturation) by heat

Table 2.6.2 *Approximate mineral composition (in mg/l or ppm) of famous brewing liquors (major ionic species only)*

Location	$[Ca^{2+}]$	$[SO_4^{2-}]$	$[HCO_3^-]$	$[Mg^{2+}]$	$[Cl^-]$	Most famous beer style
Plzen (Pilsen)	10	5	15	2	5	Pilsener (pale lager)
München (Munich)	70	10	150	18	2	Münchner (dark lager)
London	60	30	160			Porter (dark ale)
Dublin	50	80	160	4	19	Stout (black ale)
Wien§ (Vienna)	200	120	120			Wiener (amber lager)
Dortmund	220	120	270			Pale lager
Burton–upon–Trent*§	250	640	200	60	40	Pale ale

*Applies to much of the English Midlands
§Also contains ~60 mg/l of Na

- Oxalates are precipitated by Ca^{2+}, hence minimizing hazes
- Reduced pH reduces extraction of undesirable polyphenols and silicates from the malt grains
- Reduced pH gives the wort and subsequent beer greater resistance to microbiological infection
- The production of colored substances during the boiling stage (see Figure 2.6.6) is inhibited by Ca^{2+} ions
- Calcium ions aid yeast flocculation, thereby enhancing clarity.

Calcium deficient brewing liquors intended for pale beers are adjusted by addition of gypsum ($CaSO_4.2H_2O$) or calcium chloride dihydrate ($CaCl_2.2H_2O$), whereas liquors intended for dark beers are adjusted with chalk ($CaCO_3$). A total Ca^{2+} concentration of around 150 mg/l (ppm) is the usual aim. Countering the effect of calcium ions, high concentrations of bicarbonate are responsible for high pH brewing liquors. If such untreated liquors are used to brew pale beers, several problems can arise. Firstly, hydrolytic enzyme action is impaired in mashes of high pH, giving wort with lower sugar content and higher protein content, which can lead to haze problems. Secondly, hop character is more pronounced – often to the point of astringency – in the finished beer. Other problems may arise, but these are the main two.

Some of the more common methods used by brewers to lower liquor pH, by removal of HCO_3^-, are outlined in Figure 2.6.9 (d–g). On the positive side, high pH water is suitable for the brewing of dark beers. During mashing, the alkalinity of the liquor is partially offset by the higher acidity of the dark malts. Also, the presence of bicarbonate in the medium seems to decrease the astringency of some of the Maillard products that are necessarily present in dark beers. This gives a certain mellowness of flavor, provided that the 'hop rate' is low.

At the start of mashing ('mashing in'), the liquor and the grist are often mixed in the proportion of around 2.5 l of liquor to each kg of grist (~1 gal/3 lb), although the extremes are from ~3.5 l/kg (thin mash) to ~1.5 l/kg (thick mash). Cereal adjuncts that possess gelatinized starch (flaked maize, torrified barley or torrified wheat, for example) can be included in the grist at mashing in. Others, such as rice, unmalted barley or wheat and sorghum need to be treated in the cereal cooker before mixing with the mash – see 'double infusion.'

There are many mashing regimes, but all of them use temperatures in excess of 60 °C for a period of time, in order to enable the saccharification enzymes (the amylases and limit dextrinase) to hydrolyze starch to soluble sugars. The most common mashing methods are:

- Stepwise infusion
- Infusion
- Decoction
- Double infusion.

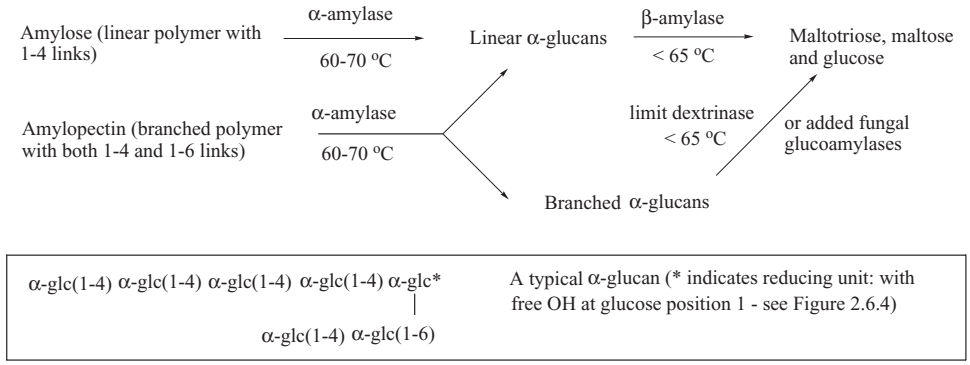


Figure 2.6.10 *Scheme for saccharification of starch during mashing, showing a typical branched α-glucan (dextran)*

In practice, there are many variations within these methods, but their basic characteristics are discussed next.

Stepwise infusion takes the mash through a series of temperature controlled stages, with ramping and rest periods that are designed to optimize sequentially the conditions for different enzymes. Figure 2.6.7 shows a typical program, although some breweries may skip one or more rest and temperature ramps and times will vary between breweries or between different beer styles. Equilibration of pH occurs during stage 1, along with cell wall and protein hydrolysis, catalyzed by β-glucanases and xylanases, and carboxypeptidases and endopeptidases, respectively. This continues into stage 2, where proteolytic activity reaches a maximum. The α-amino acids produced here will provide nutrition for the yeast during fermentation, and some of the peptides will contribute to head retention in the finished beer. Stage 2 is also responsible for a certain degree of starch cell gelatinization, and saccharification begins here.

A further increase in temperature to stage 3 inactivates the proteolytic enzymes and provides the most favorable conditions for the saccharification enzymes α-amylase, β-amylase and limit dextrinase. The enzyme α-amylase works best at temperatures close to 65 °C, but is relatively heat tolerant and is still viable at 70 °C or higher. It hydrolyzes the polymeric starch molecules randomly at (1→4) links to produce oligomeric (medium sized molecules) α-glucans (dextrins), which cannot be fermented by normal brewing yeasts (Figure 2.6.10). The enzyme β-amylase is much more heat sensitive and is inactivated at temperatures above 65 °C. It hydrolyses the α-glucans at alternative (1→4) links, but cannot hydrolyze (1→6) links; it thus produces maltotriose, maltose and glucose, and residual branched α-glucans ('limit dextrins') containing from seven up to 25 glucose units (Figure 2.6.9). The third enzyme, limit dextrinase (LD), is normally present in much lower concentrations than either of the two amylases (McCaffery *et al.*, 2004). Hence its action is limited, so that typically ~20 % of the starch in the mash remains as nonfermentable dextrins.

Performing stage 3 at 60 °C allows β-amylase and limit dextrinase maximum efficiency and leads to highly fermentable low dextrin worts, used for the brewing of 'light' (lite) beers and 'diet Pils' (Section 5.7.2), especially if the pH of the mash is initially lowered (e.g. by the addition of $CaCl_2$), and then readjusted. Addition of certain reducing agents (e.g. dithiothreitol) also leads to inceased LD activity. Some brewers add glucoamylase or pullulanase (of fungal origin) to the mash in order to accentuate the hydrolysis of branched α-glucans (Section 5.7.2). At the other extreme, performing stage 3 at 70 °C (or higher) disables both β-amylase and limit dextrinase and produces a high dextrin wort, suitable for brewing certain low alcohol beers (Chapter 2.13).

Finally, the temperature can be raised to stage 4, where some of the α-glucans react with residual proteins to give glycoproteins, substances that are known to increase beer foam stability (Ishibashi *et al.*, 1997) (Section 2.6.9).

In practice, it has been found that for well-modified malt at 62 °C, the saccharification stage 3 requires only 20 min rest time for production of maximum sugars and free amino nitrogen (FAN) (Mitzscherling *et al.*, 2007). Indeed, a longer rest time at this temperature results in a mash with unnecessarily high levels of soluble β-glucans, which may lead to filtration and haze problems further on in the brewing process (Kühlbeck *et al.*, 2005).

Infusion mashing is the traditional method for ales. The mash is held at a fixed temperature (∼65 °C) for 1–2 h, thus giving the amylases optimum conditions for starch hydrolysis. This method is especially good with low protein malts (from two-rowed barley), traditionally used in the brewing of pale ales. However, nowadays the infusion mash is also used in the brewing of many lagers, where more modified, low protein malts are used in place of the traditional undermodified, higher protein malts.

Decoction mashing, however, is the traditional method for lager beers; it involves heating a part of the mash to boiling point (>100 °C) and mixing this back into the mash bulk, so that a predetermined temperature is achieved, typically ∼50 °C, for protein degradation. This process can be carried out twice (double decoction) or three times (triple decoction), achieving a different rest temperature, by varying the bulk mash:boiled mash ratio. As mentioned already, decoction is the traditional method for lagers, where undermodified, high protein and enzymatically weak malts were once the norm. Decoction mashes are still used in many breweries in southern Germany and Central Europe.

Double infusion mashing is used in the production of high adjunct beers, where the adjuncts are unmalted (ungelatinized) cereals, such as rice, maize (corn) or sorghum. These adjuncts provide cheaper and low flavor extracts, but they must be subjected to a cereal cooker to gelatinize the starch cells before use. This adjunct wort is then mixed with a high diastatic malt wort (often produced from six-row barley), which has sufficient enzymes for saccharification of the whole mixture. High adjunct beers are a common product of many breweries on the North American continent.

On completion of the mashing process (by whatever method), the wort is separated from the grains, leaving a considerable extract as residue on the small particles. Sparging is the name for the process of rinsing the grains through with hot liquor (74–78 °C, but no higher) in order to maximize extraction. Lautering is the name for the whole process, filtering plus rinsing. The sparge liquor should be about the same volume as the mash liquor, too much (especially if pH >5.5) extracts polyphenols, lipids and other undesirable substances, which can cause astringency, haze and staling problems (Section 2.6.12) in the finished beer.

Many brewers simply filter the wort and sparge washings through the bed of broken hulls and particles, ready for the next stage, boiling. Rice hulls, added at the start of mashing, may help in the lautering of wheat mash grains, because these do not possess their own hulls. Brewers in larger breweries will often use mash filtration, having milled the malted barley into a powder for the mash (Schneider and Weisser, 2004); this gives clear wort. Brewers are undecided on the influence of wort clarity on the characteristics of the finished beer, but clear wort may benefit pale beers, whereas turbid wort may be better for full-bodied dark beers. Recent research has shown that beers brewed from clear and turbid worts show little difference in quality (Kühlbeck *et al.*, 2006). However, linoleic acid levels were higher in beer brewed from turbid wort, a condition that may cause a premature onset of staling in the packaged beer if a long shelf life product is desired (Section 2.6.12).

2.6.3 Boiling the Wort

Boiling of the wort is an important part of the brewing process; only some local brews of certain countries around the Baltic Sea (see Sahti, Section 2.6.13) and some beverages based on rice or sorghum

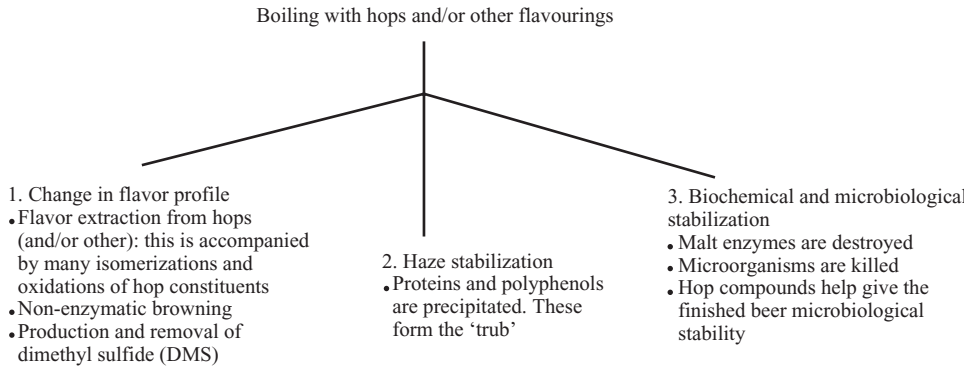


Figure 2.6.11 *Effects of boiling the wort*

(Sections 2.7.1 and 2.7.2) do not use boiled wort. The major ways in which boiling affects the wort are shown in Figure 2.6.11; these will be discussed in greater detail in the next paragraphs, but much the greatest emphasis will be placed upon flavor profile, bitterness, protein coagulation and clarity.

The brewing vessel in which boiling is carried out is known as the kettle, the wort boiler or the copper. The last term pays homage to the fact that for many years copper was the metal of choice for construction of such vessels, because of its superior heat conduction and malleability. Nowadays, stainless kettles are popular; they are less easily corroded ('pitted') than copper and are easier to clean, but have inferior thermal conductivity. There is also a tendency for insulating layers of air bubbles to form at the wort/steel interface and for wort solids to bake onto the steel surface at the air/wort/steel interface. Both of these problems can be minimized by the inclusion of impellers in the kettle, for agitation of the boiling wort. Direct heating of the kettle, using natural gas or electrical heating elements, tends to be limited to craft breweries and microbreweries. If too great a thermal load is applied without sufficient agitation, there is the likelihood of some charring and the formation of unwanted bitter Maillard products (Section 2.6.2, Figure 2.6.6). Kettles in most breweries are heated by either high pressure hot water or steam conducted from an external heating system known as a callandria. This is done via an external jacket at the base of the kettle or by way of internal pipes or central radiating panels ('star heaters'). Many kettles are supplied with impellers or other forms of wort agitators, such as fountain devices. Other kettles are designed to produce a 'whirlpool' action (discussed later). Some examples of kettles can be seen in Figure 2.6.8.

Whatever the design of the kettle, it is essential that the wort is boiled vigorously, for maximum extraction of bittering substances from the hops, for maximum formation of 'hot trub' (a precipitate of proteins and polyphenols, mostly) and to drive off unwanted volatile compounds, especially sulfur compounds. The kettle should also be energy efficient, with low operating and maintenance costs. It should be easy to clean. High pressure kettles are probably the most energy efficient, but undesirable volatile compounds cannot escape unless the kettle possesses a condensate trap in the stack.

In recent years, many European brewers have replaced or supplemented their conventional externally heated copper whirlpool systems with energy saving systems. These include Merlin, Schoko and Ziemann systems (Mitter *et al.*, 2007). In the first system, the wort is boiled, transferred to a whirlpool and then boiled again before cooling, etc. In the second two systems, the wort is boiled, transferred to a whirlpool and then to either a flash evaporator (Schoko) or vacuum evaporator (Ziemann) prior to cooling, etc. These systems give worts with very similar bittering units and iso-α-acid concentrations as conventional systems, although levels of aroma substances can be lower, a situation that can be rectified by incremental addition of hops, as in conventional boiling.

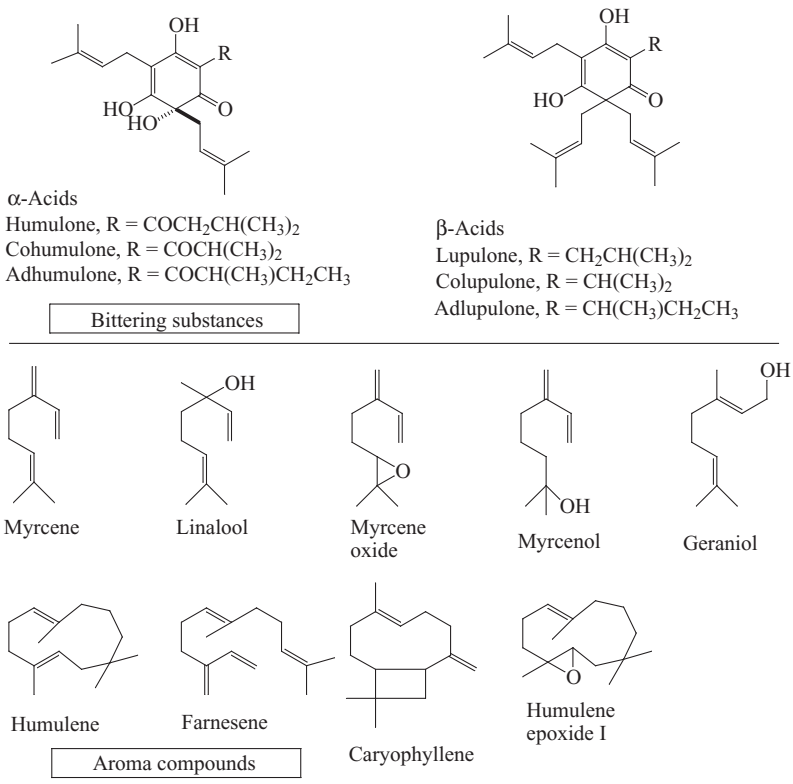


Figure 2.6.12 *Some bittering and aroma constituents of hops*

An important aspect of boiling is the extraction of bittering substances and aroma compounds from the flavoring agent, by far the most important of which is the hop flower or cone, which comes from the female hop plant (*Humulus lupulus*). The hop flowers contain substantial amounts of resins (for bitterness) and essential oils (for aroma and flavor). Both the resin and oil are composed of numerous compounds, many of which belong to the terpenoid or norisoprenoid families of natural products (Figure 2.6.12). The phenolic norisoprenoids, known collectively as α-acids and β-acids, are the principle source of bitterness, whereas monoterpene and sesquiterpene hydrocarbons and their oxygenated derivatives are the main source of aroma (Figure 2.6.12).

The hop is a perennial climbing plant that thrives in temperate climates. It is grown throughout the world, the largest producers being Germany, USA, Russian Federation, Czech Republic, UK, Slovenia and Poland. There are many varieties, but these can be divided into categories according to their α-acid content. Low (∼5%) α-acid varieties are often known as aroma or noble varieties and include Cascade (USA), Crystal (USA), East Kent Goldings (UK), Fuggles (UK), Hallertau (Germany), Lubelski (Poland), Saaz (Czech Republic, Germany) and Styrian Goldings (Slovenia). Moderate to high α-acid (8–15%) varieties, known as bittering hops, include Brewer's Gold (UK), Columbus (USA), Galena (USA), Northern Brewer (Germany, UK) and Wye Target (UK).

Typical α-acid and β-acid compositions, as well as aroma compound compositions, are given for some common hop varieties in Table 2.6.3. Cohumulone (Figure 2.6.12) is reckoned to be the most aggressive bittering agent of the three main α-acids, especially if the hop cones are added late in the boiling stage.

Table 2.6.3 *Bittering and aroma constituents of hops Data from Haunold (1998)*

Variety	Origin	α–Acids/% of total acids	α/βAcid ratio	Cohumulene/ % of total α–acids	Myrcene	Humulene	Farnesene	Caryophyllene
					/% total essential oil			
Cascade	USA	5.8	1.0	38	52	13	10	4
Crystal	USA	4.2	0.8	22	45	25	0	6
East Kent Goldings	UK	4.8	2.0	20	50	30	0	5
Fuggles	UK	5.0	1.5	20	30	35	2?	10
Galena	USA	13	1.8	43	58	13	0	5
Hallertau	Germany	4.0	1.0	25	35	37	0	10
Northern Brewer	UK/ Germany	9.0	2.2	33	33	27	0	9
Saaz	Czech Rep/ Germany	4.0	1.0	25	30	25	12	6
Wye Target	UK	12	2.2	38	63	13	0	5

This is why the percentage cohumulone data is included in Table 2.6.3. Myrcene is one of the most pungent of the aroma compounds, whereas humulene is much more subtle, but each compound will make its own contribution and together they make up the aroma profile of a particular hop variety.

During boiling and fermentation, large quantities of aroma compounds are lost, through evaporation and by numerous chemical transformations. However, given a particular hop variety and a fixed brewing regime, the hop character of the finished beer should be reasonably constant from brew to brew. Many beers are produced using a single hop variety, but others are brewed with two or more varieties, for a particular hop character. It is quite common for high α-acid hops to be added at the start of boiling ('first wort hops'), with aroma hops being added toward the end of boiling, or for aroma hop only brews, hops being added at the start and toward the end of the boiling. The aim here is to achieve an acceptable bitterness, whilst preserving an attractive aroma. This aim can also be realized by the use of isomerized hop oils, produced by low pressure steam distillation, at the conditioning stage, or by the dry hopping of cask-conditioned beers.

After harvesting, hops are dried and compressed packed into pockets or bags; many breweries use only hop cones to produce their beers, although relatively small bags of vacuum packed hops, with longer shelf lives, are available for small scale or home brewers.

However, because the compression packing ruptures many of the lupulin glands in the hop cones, oxidative degradations and other transformations occur during prolonged storage. Loss of α-acids, in particular, can be extensive (∼50%) after 12 months storage, even at low temperatures. In the extreme, the carbonyl side chains of α-acids (Figure 2.6.12) are oxidized to short chain carboxylic acids, which have definite cheesy notes. The loss of α-acids during storage, may be partially compensated by the survival of β-acids, in varieties with low α-acid:β-acid ratios (especially the aroma hops). Also, many of the hydrocarbon aroma compounds are oxidized on storage to oxygenated derivatives (Figure 2.6.12).

The appearance of hops flowers changes markedly with time. Aged hops lose much of their fresh green character and take on a much more yellow appearance; bags of such hops will often be found to have deposits of yellow or even orange-brown powder at the bottom. In general, for ordinary beer, the quality will not be as good as fresh hops, although lambic beer (Section 2.6.7) is traditionally brewed with old hops.

Recently, it has been discovered, using reversed phase HPLC with tandem mass spectrometric detection to determine stilbene phenols in six American hop varieties, that *cis*-resveratrol can be found only in aged hops, whereas *cis*- and *trans*-piceids, as well as *trans*-resveratrol were found in both fresh and aged (leaf and pelleted) hops (Jerkovic and Collin, 2009). Thus, the concentration of *cis*-resveratrol can be used as an indicator of hop freshness.

Some brewers use hop powders, pellets or extracts to supplement, or to replace entirely, hop cones in the kettle. Hop pellets do not deteriorate so rapidly as cones, especially if kept at 0–5°C. They give a higher degree of α-acid extraction/isomerization than cones and also simplify the use of a whirlpool in place of a hopback. Some hop pellets are made from mechanically enriched powder: these are especially useful in the dry hopping of cask-conditioned ales. Also, some pellets are made from hop powder containing ∼1% (w:w) of $Mg(OH)_2$ or $Ca(OH)_2$. They are known as stabilized hop pellets because a substantial α- and β-acids are converted to their anions, which are more resistant to oxidation. Hop extracts are also used by some breweries to supplement or replace hop cones in the kettle; this also aids whirlpool separation. Nowadays, many hop extracts are made by using supercritical carbon dioxide (at *ca.* 10 °C and 50 atm. pressure) as the extraction fluid, rather than organic solvents. These extractions can be fractionated: aroma compounds extract first, followed by β-acids and finally α-acids. The first (essential oil) fraction is a useful (and much faster) alternative to cones or pellets in dry hopping. Hop oil, produced by the low pressure (∼0.02 mm Hg) steam distillation of hop powder at 25 °C, can be added to bright beer in conditioning tanks and can also be used for the dry hopping of cask-conditioned beers.

During boiling, many of the resin and essential oil constituents decrease in concentration, presumably because of loss in vapors to the atmosphere, but some may also undergo isomerization or decomposition;

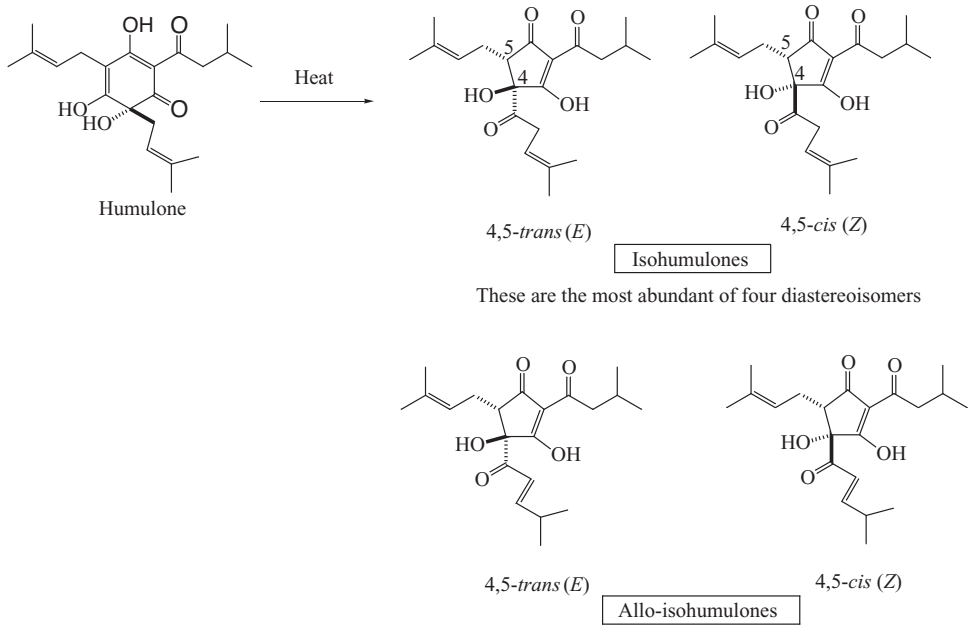


Figure 2.6.13 *Isomerization of humulone during boiling of hopped wort*

terpenoids and norisoprenoids are generally prone to rearrangement when heated under mildly acidic conditions. β-myrcene and linalool undergo a quadratic-like drop in concentration with boiling time, whereas the decrease in concentration of many others (such as β-caryophyllene, β-eudesmol, β-farnesene, geraniol, α-humulene, humulene oxide I and humulenol) with time follows a more linear curve (Kishimoto *et al.*, 2005). The norisoprenoid β-damascenone, on the other hand, was found to increase its concentration slightly during boiling and then markedly during the whirlpool step.

The principle isomerization is the conversion of α-acids to the much more soluble (and hence more potent flavoring) iso-α-acids, by a ring contracting rearrangement, as illustrated for humulone-isohumulone, in Figure 2.6.13. Many other isomerization products are formed (see Figure 2.6.13 for two examples) and other reactions, such as hydrolysis and oxidation occur in abundance (Hough *et al.*, 1982). Many of these conversions are incomplete at the end of the boil (∼1.5 h), particularly with respect to the formation of iso-α-acids, so that % utilization (≡ [quantity of iso-α-acids formed/quantity of α-acids used] × 100) is rarely greater than 40% when hop cones are used. Much higher utilizations are possible when isomerized hop extracts are added to fermented wort.

Consistent bitterness is an important commercial factor in brewing, especially for larger breweries with global markets. Bitterness may be expressed as International Bittering Units (IBU) and may be determined by standard solvent extraction/colorimetric methods (Section 4.4.3) of the European Brewing Convention (EBC) or American Society of Brewing Chemists (ASBC). Small scale brewers can estimate bitterness by calculating α-acid units from the mass of hop cones used in the brew and assuming 10% utilization, after 5 min boil, 20% after 30 min and 30% after boiling for 1 h. The final isomerized α-acid units can be converted to IBUs by multiplying by 75. Although this is only approximate, it is probably sufficiently close for most brews. Sensory analysis does not always relate to bitterness units in blind tastings and certainly the quality of hop flavor bears no relationship to IBUs (Fix, 1999c). Other flavoring agents, such as coriander (Section 2.6.13), can be added at the boiling stage, as can brewing sugars, syrups and caramel.

The second important aspect of boiling is the precipitation of proteins, polyphenols and lipids, such as long chain fatty acids and esters. Removal of proteins and polyphenols is important for haze stability in the finished beer (Section 2.6.9), whereas removal of lipids will reduce the risk of premature 'staling' (Section 2.6.12).

Very soon after application of heat, a brown scum forms on the surface of the wort. Once boiling starts, this scum breaks up into lumps that circulate within the boiling wort. It is known as 'hot trub.' The general opinion is that it is important to rid the hopped wort of the hot trub before the fermentation step. The three major methods for doing this are the hopback, the coolship and the whirlpool.

The hopback is a vessel with a perforated false bottom (rather like a mash or lauter tun), such that when the hopped wort is discharged into the vessel, the spent hop cones provide a filter bed (thickness ~15–60 cm), filtering both the hops and the trub. Some brewers provide a bed of fresh hops on the false bottom before discharge of the hot wort, thus aiding filtration and enhancing hop aroma. The hopback should not allow hop material through (this may block the plate heat exchanger during cooling) and filtration of a whole batch ('gyle') should take only 30 minutes or so, including a final rinse or sparge with hot liquor. Many larger breweries use hop separators that incorporate screw conveyers to compress the sparged hops before expulsion. A typical hopback is shown in Figure 2.6.8; it is located below the kettles.

The coolship is the simplest vessel for the removal of trub, and it (at least partially) cools the wort prior to fermentation. It is a long, wide, shallow vessel, usually of stainless steel. Typically hot wort is left in the coolship for about 3 h and is then pumped off the settled trub through plate heat exchangers. Less commonly, the wort is left to cool fully in the coolship (~12 h), before being transferred to fermentation vessels. Here, the risk of infection may be minimized by the use of sterile air ventilators. Coolships are still used in a very few UK breweries and some breweries in Belgium (see Section 2.6.7 and Figure 2.6.18) and southern Germany.

The whirlpool tank, pioneered by Molson Breweries in Canada, is now the most widely used vessel for the removal of hot trub, especially in breweries that use hop powder, pellets or extracts in the boil. Whirlpool tanks are insulated stainless steel vessels with a vent or stack. They are cylindrical vessels with floors that are flat, conical or inverted conical. The standard height to diameter ratio is between 0.3:1 and 1.5:1, with the most popular being around 0.7:1. The hot wort enters the tank at an angle of about 25° to the vertical wall, usually about one third tank height, at a speed of between *ca.* 3.6 and 15 m/s (~12–50 ft/s). The complex circulatory fluid movements ultimately impose a centrifugal force on the trub particles, which are deposited at the center of the tank floor. Larger breweries incorporate whirlpool tanks with vibrating screen filters and wort return pumps in order to minimize an otherwise substantial product loss; other breweries use combined kettle/whirlpools. These usually involve pumping the hot wort from the bottom of the kettle to just above the surface of the wort, tangentially. Alternatively, an impeller, driven by a high speed motor with a clutched gear drive, can be used to create the whirlpool.

Hot trub is composed mostly of proteins (derived from the malt), but it also contains some polyphenols (from hops and malt), lipids and minerals, such as Zn^{2+}. The presence of some of these substances in the wort can lead to haze and premature staling problems in the finished beer. In order to reduce the amount of potential haze forming substances in beer, some brewers add certain fining agents ('kettle finings') to the boiling wort. The most common of these is Irish Moss, dried fronds of the red marine alga, *Chondrus crispus*. Carrageenan, a polysaccharide consisting of α-D-galactose and other monosaccharides, with alternating 1→3 and 1→4 links, is responsible for the precipitating action of Irish Moss. Purified carrageenan can be used in place of Irish Moss. A substantial number of monosaccharide side chain OH groups are esterified with sulfate groups, hence carrageenan molecules are negatively charged. Haze active proteins carry an excess of positively charged sites (side chains of the α-amino acids arginine and lysine, mainly) at wort pH (~5), so when mixed with carrageenan, the protein colloidal sol flocculates. A danger of using Irish Moss or carrageenan is the possibility of removing foam stabilizing glycoproteins, along with the haze active proteins.

More recently, silica xerogel, polyvinylpolypyrrolidone (PVPP) and polyvinylpyrrolidone have been shown to be shown to be more or less specific in their interaction with polyphenols, thus removing, at the boiling stage, crucial haze forming agents, without affecting glycoprotein concentrations too much (Mitchell *et al.*, 2005; Leiper *et al.*, 2002).

The third benefit of boiling is the removal of unwanted volatile compounds, including organic sulfur compounds, many of which have low odor threshold values (OTV) (Section 4.7.3) and are unpleasant. A good many organosulfur compounds can be found in hop oils (Hough *et al.*, 1982b) and many others are degradation products of these and possibly of organosulfur pesticide residues (Section 5.10.2). A normal, vigorous and well ventilated boiling procedure should dispel most of these into the atmosphere. High protein malts (e.g. from six-row barley) can contain relatively high levels of *S*-methyl methionine (SMM), a nonvolatile α-aminoacid derivative. This compound undergoes thermal decomposition to the volatile dimethyl sulfide (DMS) (Figure 2.6.14), which has an unpleasant odor in all but the lowest concentrations. DMS is produced during the kilning of malt (Section 2.6.2), where most of it is vaporized. This is why the more highly kilned pale malts and darker roasted malts contain the least SMM and DMS. Similarly, during boiling, DMS formed from SSM is vaporized and, in well ventilated kettles, is dissipated into the atmosphere. Residual SSM can decompose to DMS after boiling has finished, if the cooling rate is low (as in coolships), thus allowing organoleptically detectable levels of DMS to remain in the beer, even after fermentation (which reduces DMS content). This is more likely if high SSM malts are used. In the brewing of pale ales, this is rarely a problem, as low SSM malts (e.g. from Maris Otter barley) are usually the choice. Also, a certain concentration of DMS in the beer is considered desirable for many pale lagers, which otherwise may taste rather insipid. Wort is relatively rich in flavor active Strecker aldehydes, such as 2-methylpropanal, 3-methylbutanal, 2-methylbutanal, benzaldehyde, phenylactetaldehyde and other compounds, such as methional, that can produce off flavors in stored beer (De Schutter *et al.*, 2008). A 60 min boiling period can reduce significantly the concentrations of many of these volatile components, although some, such as acetylfuran, β-damascenone (desirable for white wine, but not for beer), methional and phenylacetaldehyde, tend to persist at high levels after boiling (De Schutter *et al.*, 2008).

Cooling the hot wort is necessary before the yeast is added ('pitched'). For top fermentation (ales), pitching temperature should be >15 °C, whereas for bottom fermentation (lager) a temperature of around 8–10 °C is normally desirable. The original method was to transfer the wort to coolships. These are still used in a number of breweries, but usually in conjunction with heat exchange type wort coolers, which are much more rapid and much smaller cooling systems. The major disadvantages of coolships are the possibility of infection and the large space required. The main idea behind heat exchangers is the rapid cooling of wort caused by the transfer of heat, though a highly conducting wall, to a coolant fluid, often cold water. The resulting hot water (∼70 °C) can be used for the next mash or for cleaning purposes. Vertical shell type heat exchangers and tube coolers are still in use, but plate heat exchangers are by far the most common (Figure 2.6.8). Using cold water coolant, the wort will emerge from the exchanger at about 15 °C, suitable for top fermentation. A two stage cooling process, using firstly cold water and then water–ethylene glycol, is needed to bring the wort temperature down to 8 °C, ready for bottom fermentation.

During cooling of wort, further precipitation occurs; this is known as 'cold trub' or 'cold break.' It occurs throughout the yeast of the brewing process and consists of coagulated particles of protein–polyphenol complexes, which would otherwise cause haze (Section 2.6.9) in the finished beer. Some North American breweries encourage a good formation of cold trub, by running off the newly fermenting wort a day or so after pitching the yeast. Others cool the wort to ∼2 °C so that it forms slush ice. The temperature is then raised to 8–10 °C and the wort is run off the sediment into a fermenting vessel before pitching. However, recent research suggests that the presence of a small amount of trub particles has a beneficial effect on fermentation: trub-containing worts were observed to ferment more rapidly to lower final gravity, lower pH and higher

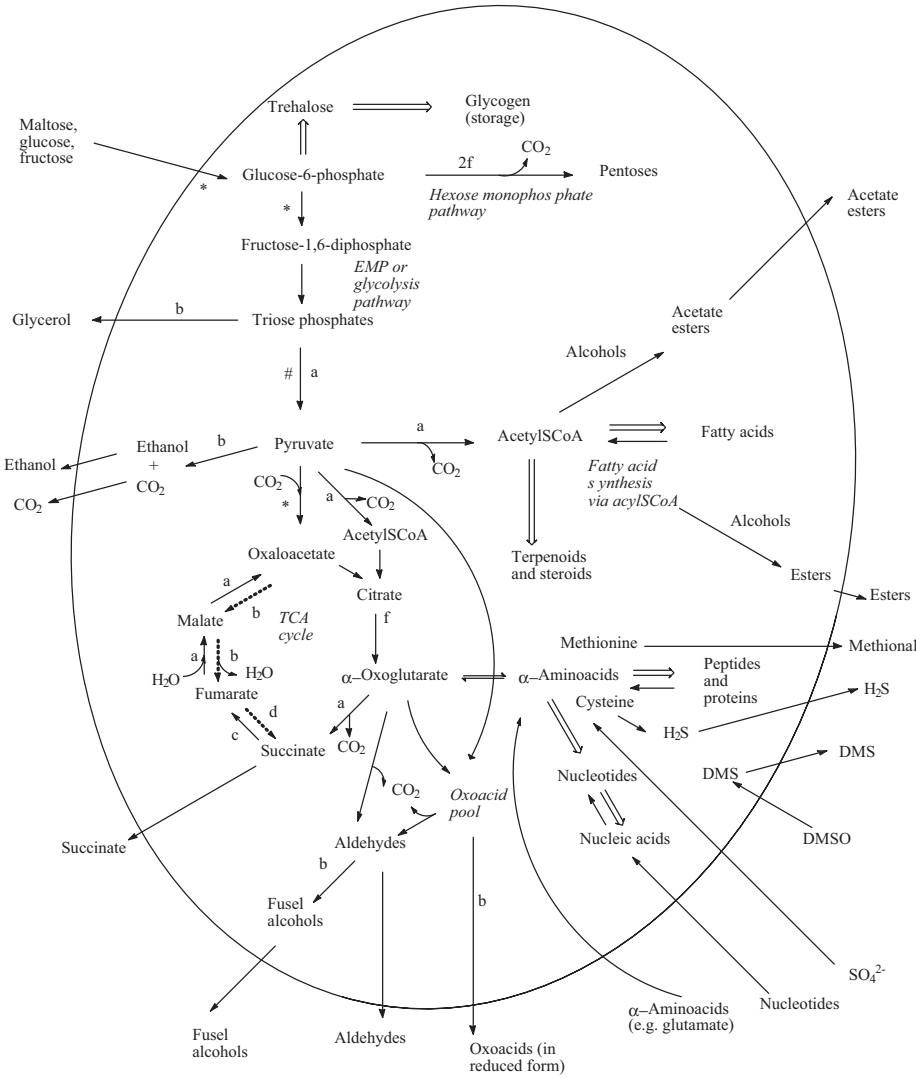


Figure 2.6.14 *Summary of anaerobic carbohydrate metabolism in yeast cell*

⟶ Degradation or nonsynthetic reaction

⟹ Synthetic reactions

a $NAD^+ \rightarrow NADH + H^+$ c $FAD \rightarrow FADH_2$ * $ATP \rightarrow ADP + P_i$

b $NADH + H^+ \rightarrow NAD^+$ d $FADH_2 \rightarrow FAD$ # $ADP + P_i \rightarrow ATP$

f $NADP^+ \rightarrow NADPH + H^+$

ethanol content compared with clarified worts under similar conditions (Kühlbeck *et al.*, 2007). It appears that the particulate nature of trub improved suspended yeast cell concentrations, allowing optimum fermentation efficiency and at the same time improved the evolution of CO_2 from the fermenting wort, thereby minimizing the inhibitory effect of CO_2 on yeast metabolism. The presence of lipid and mineral nutrients in trub may also play a part.

2.6.4 Fermentation

The classification of yeast and a general discussion of alcoholic fermentation performed by the various *Saccharomyces* species (including *S. cerevisiae* and *S. uvarum*) can be found in Section 2.2.1. Other fermentations – desirable and undesirable – are discussed briefly in Chapter 2.4, along with microorganisms that promote them. Consequently, the discussion here will focus on aspects of fermentation that are directly relevant to the brewer. Good accounts of the fermentation of beer wort can be found in other sources (e.g. Hornsey, 1999).

The two most widely used yeasts, ale and lager, belong to the same genus, but different species. Ale yeasts are classified as *S. cerevisiae* and are related to baker's yeast – indeed some of the indigenous beers of the Baltic Sea countries are still brewed with baker's yeast. Lager yeasts are described as *S. uvarum*, but until recently they were classified as *S. carlsbergensis*, since they were first isolated and characterized by E. C. Hansen of Carlsberg Brewery in the 1880s. Within these broad classifications are many strains (varieties) that have different physiological and biochemical characteristics, often differing in subtle ways. Brewers strive to maintain their own healthy stocks of yeast for consistent brewing and many breweries have colonies of their yeasts propagated and preserved at yeast depositories (yeast banks), such as the National Collection of Yeast Cultures, at Norwich, UK.

Yeast strains easily mutate and the brewer is wise to carry out various tests on yeast stocks and to generally keep a watchful eye on yeast performance (such as attenuation behavior) and on certain physical characteristics (such as flocculation and sedimentation). Flocculation of yeast cells during fermentation of wort is a genetic characteristic that can be used to monitor yeast performance.

Brewing yeasts fall into three broad categories, although some authorities (Hough *et al.*, 1982c) quote no less than seven. The categories are strongly flocculating (sedimentary), powdery and nonflocculating and all three types are used in brewing. These terms describe the yeast's sedimentation tendencies: strongly flocculating yeasts form sediments at an early stage in the alcoholic fermentation whilst nonflocculating types remain largely in suspension, even toward the end of fermentation.

Yeast flocculation occurs via cell surface interactions involving mannans (poly-D-α-mannose), sugar-binding proteins (known as flocculins or lectins, or more specifically zymolectins) and Ca^{2+} ions. The mannans are present on the cell surface at all times, but it is believed that in flocculent yeast strains, zymolectins are expressed by the FLO genes when these are activated by lack of nutrients toward the end of fermentative activity.

The mechanism is not fully understood, but is believed to involve zymolectins binding to mannans on the surface of neighboring cells, leading ultimately to the formation of flocs of aggregated cells. Because of reduced surface to volume ratio, the flocs sediment more rapidly than individual cells. A study of cell surface hydrophobicity, charge density and zymolectin density of flocculent and nonflocculent strains of *S. cerevisiae* showed a positive correlation between cell surface hydrophobicity and flocculation ability (Jin *et al.*, 2001). The role played by Ca^{2+} ions is unclear, though it has been shown that Pb^{2+} competes reversibly with Ca^{2+} and inhibits flocculation. Using a fluorescent avidinfluorescein isothiocyanate (Avidin-FTC) microscopy probe, it was observed that zymolectins were not fixed to the cell walls in the presence of Pb^{2+} (Gouveia and Soares, 2004). This suggests that although the Pb^{2+} ions (much larger than Ca^{2+}) bind to the calcium ion binding sites of the lectin molecules, the resulting protein conformations are unable to bind to the cell surface mannans.

More recently, yeast mitochondrial activity was shown to be higher in flocculent yeast strains than in nonflocculent types (Strauss *et al.*, 2007). The same authors also showed, for the first time, cell surface protuberances to be composed of hydrophobic '3-OH oxylipins,' produced in mitochondria by incomplete β-oxidation of long chain fatty acid lipids. Scanning electron microscopy showed oxylipin binding sites and protruding osmiophilic layers, sometimes extending from one cell wall to another. Thus yeast flocculation may be mediated by oxylipin interactions as well as mannan–lectin–Ca^{2+} interactions.

A change in a brewery's yeast flocculation behavior may be a sign that the strain is mutating or that cropping is not being performed properly. For example, some brewery yeasts are composed of several strains with varying degrees of flocculation behavior. Repeated 'bottom cropping' and repitching can rapidly lead to domination by sedimentary strains and hence to a change in the brewery's yeast characteristics (see also Section 2.6.6). The brewer has unintentionally selected the sedimentary strains. Suspended yeast cells give the best attenuation and the lowest levels of undesirable by-products (Fix, 1999d), so brewers who use strongly flocculating or sedimentary strains regularly rouse the fermenting wort. The subjects of flocculation and sedimentation will be considered once more, in terms of top- and bottom-fermentation (Section 2.6.5 and 2.6.6).

Attenuation is the drop in the wort specific gravity caused by fermentation, as sugars are converted to ethanol, carbon dioxide and other products. The specific gravity (SG) or relative density of wort is the density (in g/ml) of the wort divided by the density of water, which is taken as 1.000 g/ml at 17.5 °C. Thus, wort with a specific gravity of 1.044 is 4.4% heavier than an equal volume of water. The temperature must always be specified when quoting specific gravities and if it is not the same as the reference temperature of 17.5 °C, then a correction must be applied, although this is very small if the temperature difference is less than 5 °C. A convenient and widely used way of expressing this is the 'gravity' scale – the above wort is said to have a gravity of 1044 (relative density $\times 10^3$). Another widely used scale, based upon the correlation of the wort specific gravity with those of sucrose solutions of accurately known % (w:w) concentrations, is known as the °Plato (°P) scale. This scale thus expresses extract as the percentage of sugar in the wort by weight. The corresponding scale for wine is °Brix. Other scales, such as °Beaumé scale exist, but the gravity and Plato scales will be used here. Specific gravity and °Plato can be measured by a hydrometer that is calibrated in both scales: single scale hydrometers are also used, in which case the °Plato instrument is known as a saccharometer. Digital density meters have become popular in recent years; they are robust, inexpensive, easy to use and accurate (Section 4.6.3).

Although fermentable sugars make a big contribution to the specific gravity of wort, a contribution also comes from nonfermentable solutes, such as certain carbohydrates, amino acids, proteins, minerals and others, hence the majority of beers possess final gravities above 1000 (typically 1004–1008). Apparent attenuation depends on the drop in gravity that occurs during fermentation and is given by Equation 2.6.1.

$$\%\ \text{apparent attenuation} = \frac{(\text{OG} - \text{FG})}{(\text{OG} - 1000)} \times 100 \tag{2.6.1}$$

where OG is the original gravity and FG the final gravity.

Bottom fermenting (lager) yeasts are generally high attenuators (~80%), whereas top fermenting (ale) yeasts tend to be moderate attenuators (~77%), although there is some overlap in performance of the two species. If attenuation is below 75%, then the yeast may be considered to be underperforming and some attention may be necessary. More detail on attenuation can be found in Sections 2.6.5 and 2.6.6.

The ethanol content of beers is expressed as % ethanol v:v (≡ % alcohol by volume or %ABV) or less commonly as % ethanol w:w. The apparent concentration of ethanol produced by the fermentation of hexose sugars can be calculated from the stoichiometry of the Gay Lussac equation, $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$. However, the actual concentration of ethanol will be a little less than the calculated concentration because of 'losses'; some of the sugars are used in metabolic pathways that do not produce ethanol (Chapter 2.2). Hence, relating % ethanol content (w:w) (A) of a beer to the original and final gravities must take this into account.

Both the EBC and ASBC provide methods for brewers and customs officials to relate A to both the original extract (OE) and the residual extract (RE), expressed in °Plato. The methods involve distillation of the beer: A is determined from the specific gravity of the distillate and RE from the specific gravity of the residue (Section 4.6.3). Thus OE and RE relate to original and final gravities, respectively. The ASBC formula is

given by Equation 2.6.2.

$$OE = (2A + RE) - F \tag{2.6.2}$$

where F is a correction factor that can be found from tables supplied by the ASBC.

Provided fermentation performance is satisfactory and 'true to type,' a brewer will recover and store substantial quantities of yeast from a current brew for fermentation of future brews. For this, a reliable recovery method and storage system is required, one that collects a representative sample of the yeast colony and maintains it in a viable state for a period of time, without contamination by wild yeasts or bacteria. This important aspect of brewing will be dealt in more detail separately for top and bottom fermentations (Sections 2.6.5 and 2.6.6 respectively). Here, the major characteristics of wort fermentation will be discussed, starting with the mixing of wort and yeast.

Pitching the yeast is the act of adding yeast to the wort, often as a slurry in water or fresh wort. In the early stages of fermentation (but not in later stages), yeast cells need oxygen, so the wort and/or the yeast should be aerated. Pitching should result in wort with about 16×10^6 live yeast cells per ml, after thorough mixing. This corresponds to a pitching rate of about 0.2 kg per hl ($\equiv$100 l). Higher pitching rates may lead to yeast autolysis (Section 2.2.1) and the production of off flavors (Cahill *et al.*, 1999). In some breweries, notably in the USA and Canada, the wort is run off the cold trub into another vessel, some 24 h after pitching. In this case, the initial fermentation vessel is known as a settling tank or starter tank. The majority of breweries, however, carry out the fermentation of a batch of wort in a single vessel.

The early part of fermentation is characterized by a relatively gentle evolution of CO_2 and the formation of a fine, white or cream foam on the surface of the wort. Attenuation is very slow at this stage, as the yeast cells are more involved in various preparatory functions than in alcoholic fermentation itself. Initially, yeast cells synthesize various steroids (such as ergosterol and zymosterol), using their own reserves of glycogen to provide the energy for such syntheses. These sterols increase the permeability of the cell walls and plasma membranes, allowing transport of nutrients from the wort that will be required for alcoholic fermentation. These nutrients include certain sugars, NH_4^+, α-amino acids, phosphates, metal ions and various growth factors, such as biotin. Transport in the opposite direction also occurs and certain permease enzymes can carry specific solutes (sugars, α-amino acids and ionic species, such as NH_4^+, K^+, phosphates and SO_4^{2-}) through the membrane in both directions. This is called active transport. Some permeases are constitutive (they have a permanent presence), whereas others are induced only in the presence of a specific substrate. On the other hand, proteins, sterols and carbohydrates embedded in the plasma membrane aid the diffusion of certain other solutes, such as fatty acids; this is called aided transport. Simple diffusion may account for the transport of other solutes.

The major sugar of wort is maltose, followed by maltotriose and glucose. The presence of maltose induces yeast cells to synthesize a maltose specific permease enzyme, maltopermease and a corresponding hydrolytic enzyme, maltase. When all the maltose is used up, synthesis of both these enzymes ceases and hence they are known as induced enzymes or proteins. If the wort possesses substantial levels of D-glucose and D-fructose (e.g. from sucrose adjunct), as well as maltose (from malt), then synthesis of maltopermease and maltase is repressed or inactivated. Hence glucose and fructose are selectively transported by constitutive (permanent) permeases into the yeast cells to be metabolized in preference to maltose. Induced permeases are synthesized toward the end of a cascade process that starts with interactions between the inducing substrate (e.g. maltose) and molecules on the outer membrane surface. This probably signals the synthesis of more cyclic adenosine-5′-monophosphate (cAMP) and the production of a cAMP-protein complex that interacts with the yeast genome and signals the DNA transcription to produce the enzymes (e.g. maltopermease and maltase).

Yeast cells utilize the glycolysis or Embden–Meyerhof–Parnas (EMP) pathway (Section 2.2.9) to convert hexoses to pyruvate, acetyl-coenzyme A (acetyl-SCoA or $CH_3COSCoA$), CO_2 and other products.

Acetyl-SCoA enters the TCA cycle, where the acetyl unit is oxidized completely to carbon dioxide. Some of the energy released by these catabolic processes is used to constitute ATP (from ADP), NAD^+ (from NADH) and other coenzymes from their reduced forms. Likewise, some energy goes into the synthesis of essential molecules, including proteins, fatty acids and nucleic acids. Thus, the initial fermentation stage is characterized by yeast cell growth and reproduction.

Wort normally contains a plentiful supply of nitrogenous nutrients in the form of small peptides, α-amino acids and ammonium salts, the second of which is known as the free amino nitrogen (FAN) content of the wort. About 40% of the peptides are hydrolyzed to amino acids that are then metabolized, the remainder survive and may contribute to either haze formation or foam stability. Amino acids are transported through the yeast cell membranes by specific permeases that act in a specific order. Amino acids with acidic side chains (ASP, GLU), amide side chains (ASN, GLN), basic side chains (ARG, LYS), hydroxy side chains (SER, THR), plus MET are absorbed first and largely consumed within the first 24 h of fermentation. Amino acids with aromatic side chains (PHE, TRY, TYR) plus ALA, ammonia and GLY are generally absorbed most slowly, and the hydrophobic amino acids (HIS, ILEU, LEU, VAL) are absorbed at intermediate speeds. Proline, one of the most abundant amino acids of wort, is absorbed and secreted during fermentation, with no net absorption.

Both the nature and concentration of amino acids present in the wort can have a strong influence on yeast metabolism, affecting the speed of fermentation and the levels of by-products, such as diacetyl and 2,3-pentanedione (Lekkas *et al.*, 2007). Thus, added methionine prevented absorption of hydrophobic aminoacids, slowing down fermentation. Also, diacetyl and 2,3-pentanedione concentrations were lower than in control fermentations. On the other hand, supplementation of the wort with lysine enhanced the fermentation rate by stimulating absorption of amino acids and ammonia. Higher levels of diacetyl and 2,3-pentanedione were also evident after fermentation, probably because of much less time being available for natural yeast removal of these compounds (see Figure 2.6.15).

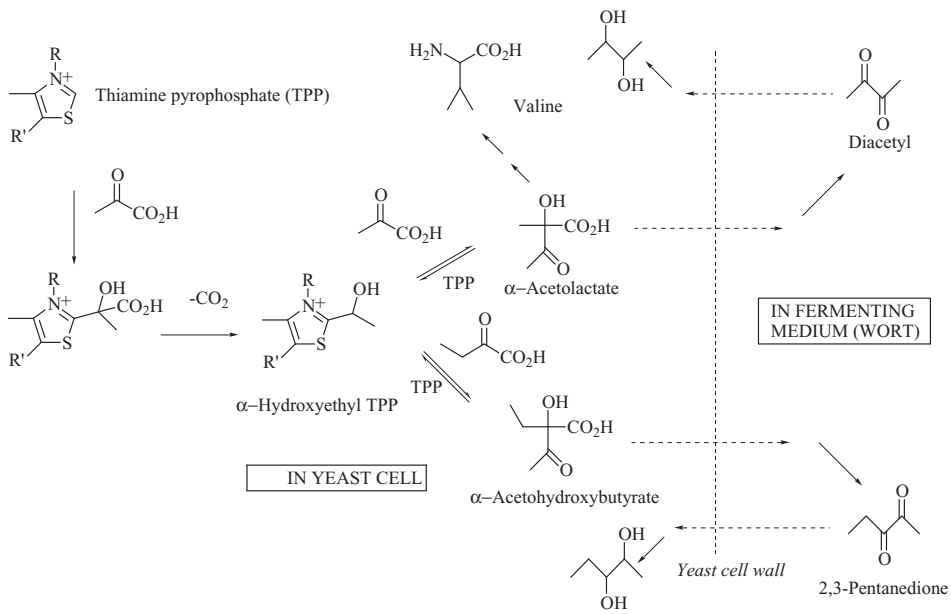


Figure 2.6.15 *Formation of α,β—diketones, competitive valine formation and reabsorption. Reactions are given by →, transport by ⇢*

The second stage of fermentation is the anaerobic carbohydrate metabolism stage (Section 2.2.9), in which sugars are converted to ethanol and carbon dioxide. It is characterized physically by a more rapid attenuation, vigorous evolution of CO_2 and formation of thick creamy white foam on the surface of the wort. The growing mass of yeast cells faced with limited oxygen supplies, switch their main metabolism to the anaerobic alcoholic fermentation pathway. Instead of converting pyruvate to acetyl-SCoA ready for entry into the TCA cycle, the bulk of the pyruvate is now used to produce acetaldehyde and ethanol; a much shorter route that gives a more rapid return of energy when oxygen supplies are low. The TCA cycle works only partially during anaerobic fermentation – as far as succinate and α-ketoglutarate, both of which are fermentation end products, like ethanol. In the earlier part of alcoholic fermentation, when the yeast is still growing and reproducing vigorously, the synthesis of essential biomolecules is still important. The consequent excess of NADH is used up, in part, by the reduction of dihydroxyacetone phosphate to glycerol phosphate. The latter is dephosphorylated to glycerol, which is excreted as another end product. The major features of the anaerobic metabolism of hexoses and other substrates are shown in Figure 2.6.14. For clarity, some steps and intermediates have been omitted. Also, the many relationships that exist between this pathway and others, both degradative and synthetic, are given in outline only. The other fermentation end products, such as aldehydes, carboxylic acids, esters, ketones (including 1,2- or α,β-diketones, like diacetyl) and sulfur compounds are considered next.

All the organic acids of the TCA cycle are excreted by yeast cells, the oxoacids pyruvic acid and α-oxoglutaric acid (from the 'oxoacid pool') being excreted as their reduced forms, lactic acid and 2-hydroxyglutaric acid, respectively. In beers brewed principally with *S. cerevisiae* or *S. uvarum* (the great majority), succinic acid is the major organic acid end product, but others, such as citric acid and malic acid, may contribute to the overall taste and/or flavor. The aforementioned acids are all hydroxy acids; simple carboxylic acids, such as acetic acid and higher fatty acids, probably arise from hydrolysis of acetyl-SCoA and higher analogs of the fatty acid synthesis pathway. Also from the oxoacid pool are the α,β-diketones, diacetyl and 2,3-pentanedione, produced from pyruvate and oxobutyrate, respectively, by reaction with hydroxyethyl thiamine pyrophosphate, in the presence of acetohydroxy acid synthetase. The acetohydroxy acids are excreted into the beer where they are oxidatively (nonenzymatically) decarboxylated to α,β-diketones (Figure 2.6.15).

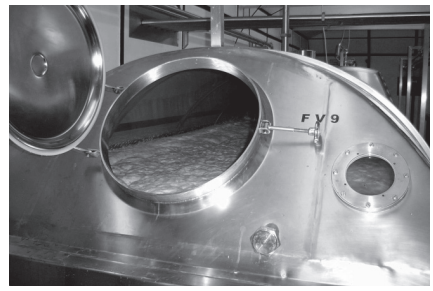
Both these diketones have low OTVs, giving buttery or butterscotch aromas that, like many aroma compounds, can be unpleasant if too concentrated, yet attractive at low levels. Both diketones can be reabsorbed by the yeast, where they are reduced to nonodorous 1,2-diols (glycols). However, yeast strains differ in their abilities to both produce acetohydroxy acids (and hence α,β-diketones) and to reabsorb and reduce the diketones (Fix, 1999e). Also, fermentation conditions are important: rapid fermentation at higher temperatures and overexposure to air favor conversion of acetohydroxy acids to diketones, but a relatively warm resting time after fermentation favors reabsorption and reduction of the diketones (Fix, 1999e).

The oxoacid pool is also the source of many aldehydes (by decarboxylation) and fusel alcohols (by reduction of aldehydes). Esters mostly appear to be derived from the fatty acid synthesis pathway: acetyl-SCoA gives acetate esters with alcohols produced from the oxoacid pool, and other acylSCoA molecules (differing by two CH_2 units) give higher esters.

Yeast cells need sources of sulfur for the synthesis of proteins (i.e. using the sulfur containing amino acids CYS and MET) and certain coenzymes, such as biotin, coenzyme A, glutathione, lipoic acid and thiamine. The preferred source is methionine (MET), but even SO_4^{2-}, sulfate, tends to be metabolized (reduced) to S^{2-} via HSO_3^-, whence it is converted to CYS and MET. Many of the sulfur compounds found in beer are derived from malt and hops rather from alcoholic fermentation. Thus DMS, important in lager brewing, but not in ale brewing, comes mostly from SSM in malt (Section 2.6.2), but a certain amount originates from the enzymatic reduction of dimethyl sulfoxide (DMSO), also from malt sources. Hydrogen sulfide (H_2S) is produced by dehydrosulfuration of cysteine by the enzyme cysteine desulfydrase.

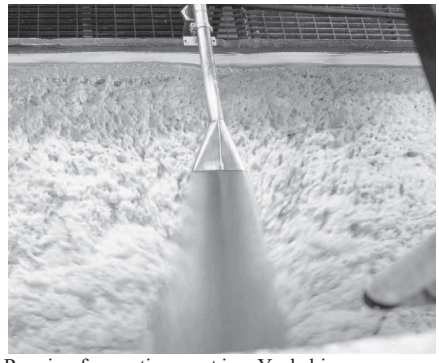
Examples of brewery fermentation and yeast propagation equipment can be seen in Figure 2.6.16.

Yorkshire square at Hardy's and Hanson's Brewery.



Enclosed fermentation vessel at Jennings' Brewery.

Union casks at Firestone Walker Brewery (California, USA). *Photo courtesy of Firestone Walker Brewing Co.*



Rousing fermenting wort in a Yorkshire square vessel at Black Sheep Brewery, Yorkshire, UK. *Photo courtesy of Black Sheep Brewery Ltd.*

Medium yeast propagation vessel at Belhaven Brewery. Smaller and larger vessels form the complete system.

Rousing fermenting wheat beer wort at the Schneider Brewery, Germany. *Photo courtesy of G. Schneider & Sohn GmbH.*

Figure 2.6.16 *Some fermentation vessels and systems. Photo courtesy of Jamie C. Smith from Firestone Beer, CA. Photo courtesy of G. Schneider & Sohn, Nürnberg, Germany*

After the primary fermentation, yeast activity can continue at a subdued level in unpasteurized and unfiltered or aseptically filtered beers being matured in tanks or casks, in preparation for serving on draught. The same applies to maturing bottle-conditioned beers. Metabolism of certain oligosaccharides occurs, increasing the quantity of CO_2 produced and hence increasing the foaming ability. However, yeasts also excrete proteinase A, which degrades some of the foam enhancing beer proteins and thus is thought to compromise foam stability after an extended maturation time (Shengli *et al.*, 2009). At the same time, the presence of β-glucans in the beer can compromise haze stability. Although there is today much consumer resistance towards the use of genetically modified materials in brewing (see Section 1.3.13 for one example), Shengli *et al.* (2009) have produced a recombinant *S. cerevisiae* strain that exhibits reduced proteinase A activity and increased production of β-glucanase. The strain was prepared by insertion of gene bg1S into the PEP4 locus by homologous recombination, and the resulting beer fermented by this strain showed greater foam stability and lower β-glucan levels than beers brewed with control strains.

2.6.5 Top Fermentation

Not so very long ago top fermentation reigned supreme. Today, bottom fermentation dominates brewing, but top fermentation is still used to brew a very wide range of beers in the UK, Ireland, Belgium, Germany, the USA and some other countries (Section 2.6.13). Top fermentation yeasts are often called ale yeasts and the beers that are produced using them are known as ales. Top fermentation is characterized by the substantial crop of yeast that collects at the head as fermentation proceeds.

Well aerated wort at about 15 °C is pitched with yeast, usually as a slurry with wort. If the yeast strain is of the highly flocculating type, further aeration and regular agitation will be necessary during the course of fermentation. This can be done using paddles, or more likely these days by pumping fermenting wort from the bulk and discharging it as a spray over the surface (see Figure 2.6.16 for examples).

During fermentation, at the height of the yeast's metabolic activities, the temperature of the wort may rise to 20 °C or higher. In order to control this temperature rise, fermentations vessels are fitted with internal tubes or coils (attemperator coils) through which cold water is circulated. Toward the end of fermentation, it is common practice to lower the temperature of the wort to 14 °C, or even as low as 11 °C in some breweries, for certain beers. Some ale breweries use a refrigeration unit to lower the temperature of the newly fermented beer to around 5 °C lower temperatures in order to encourage yeast suspension and help clarification.

In ale breweries, removal and preservation of some of the yeast from the head is an important part of the brewer's job. Provided fermentation is proceeding normally, yeast removal may be carried out several times, by skimming, using paddles or 'parachute' devices or by suction. Most brewers use the crop from toward the end of fermentation for repitching, as this tends to have the best attenuation ability. The yeast crop is transferred to a holding vessel (a 'yeast back'), from where it can be used directly in the next brew or it can be pumped and collected on a sheet filter press.

The yeast-entrained ale ('barm ale') – a substantial volume in large scale fermenters – can be added back to the main batch, sometimes after pasteurization, in order to minimize infection. The yeast mass is discharged from the filter into trays which are kept in the refrigerator at 2–4 °C. Repitching can be performed using either the pressed yeast alone or as a slurry with water or wort.

The course of a typical fermentation is shown in Figure 2.6.17(a), for ale wort with an original gravity of 1040. The final gravity will depend on both the attenuation characteristics of the yeast and the ratio of fermentable to nonfermentable sugars, which is determined largely by the mashing regime (Section 2.6.2). For the example in Figure 2.6.17, the fermentation temperature may be controlled from 15 °C at pitching, through 20 °C at the height of activity, to 11–14 °C toward the end of fermentation. Production of esters and

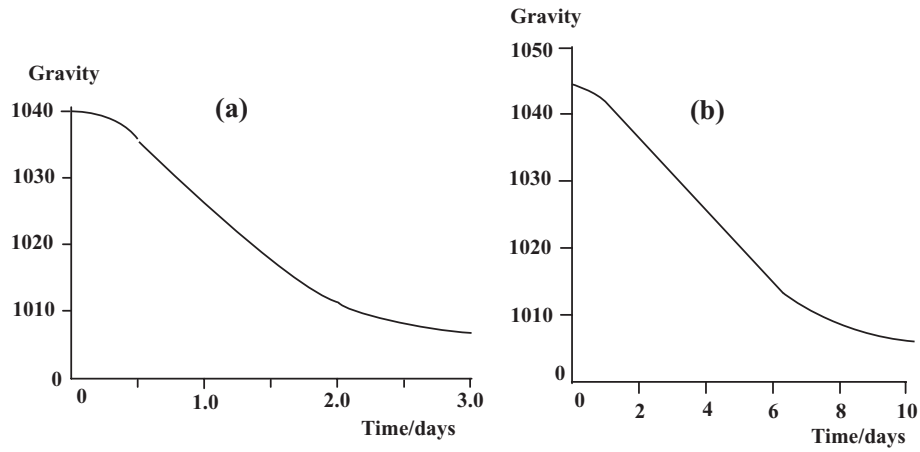


Figure 2.6.17 *Course of typical beer fermentations: (a) top fermentation at 16 °C; (b) bottom fermentation at 9 °C*

fusel alcohols reach maximum values and pH reaches a minimum value after about four days of fermentation (Hough *et al.*, 1982c).

Many top yeasts possess the phenylacrylic acid decarboxylase (Pad 1) enzyme that is able to decarboxylate phenolic acids of the hydrocinnamic acid type (such as *p*-coumaric acid, ferulic acid and sinapic acid) during fermentation (Vanbeneden *et al.*, 2008). These compounds with high odor threshold values (OTV; Section 4.7.3) are thus converted to the much more flavor active phenols (4-vinylphenol, 4-vinylguaiacol and 4-vinylsyringol, respectively), which contribute much to the aroma and flavor of many top-fermented beers, including German wheat beers, Belgian white beers, saison beers and other ales (Section 2.6.13). These volatile phenolic flavor compounds are also produced in much smaller quantities during high temperature brewing operations such as boiling and pasteurization.

Typical final gravity is around 1008 (2 °Plato), of which there will be about three degrees of gravity (∼0.7 °Plato) is still fermentable. Most of the fermentable sugar at this stage is maltotriose, which is only slowly and partially fermented by ale yeasts (Section 2.6.6). Hence, beers destined for cask conditioning are normally racked at this stage, so that a slow secondary fermentation can occur in the cask, concurrently with maturation and clarification. At the point of racking, there will still be about 0.5 million viable yeast cells per ml of beer, quite enough for secondary fermentation. To accelerate clarification, isinglass finings (Section 2.6.9) are added at the time of racking and dry hopping (Section 2.6.3) may also be carried out. The casks are bunged and may be kept at the brewery for a few days before dispatch to the trade, where several more days will pass before tapping and serving. Thus, it is not unusual for cask-conditioned ales (especially those of low OG, say < 1040) to be consumed within three weeks after pitching. However, many cask ales of medium to high OG (say 1045–1085) benefit from much longer maturation/secondary fermentation periods and some breweries perform initial conditioning in tanks before racking into casks. Some larger breweries, especially those that use powdery yeast strains, subject their new or 'green' beer to discharge centrifugation whilst racking into casks. This gives sufficient numbers of viable yeast cells (∼0.5 million per ml) to perform a secondary fermentation in cask, but insufficient to cause haze problems.

Most breweries rack a certain volume (in many breweries the major volume) of new beer into conditioning tanks, where maturation/conditioning occurs at 10 °C (or even lower temperatures) for many days or even weeks, depending on the OG and style of beer. This beer is usually destined for pressure casks (kegs), bottles or cans (Sections 2.6.9 and 2.6.10) and is known as brewery-conditioned beer.

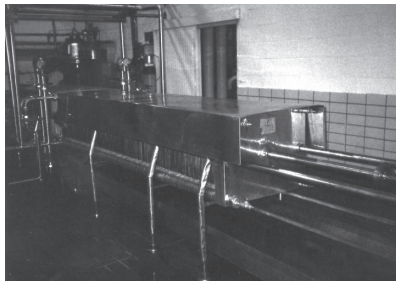
Traditional ale fermentation vessels are usually square and sometimes circular in plan view and, in small to medium breweries, range from 2 to 4 m deep, typical capacities being between 150 and 400 hl (~90–240 barrels or ~3240–8640 gallons). Microbreweries and brewpubs may have vessels of only 4–40 hl capacity and of course larger breweries need much larger vessels. Many ale breweries still have open fermentation vessels, but many others use closed vessels (see the examples in Figure 2.6.16). The main advantages of closed vessels are lower risk of microbiological (and other) contamination and ease of cleaning with in-place cleaning systems. On the other hand, they are more expensive than open vessels and are not so easily cleaned manually. All vessels possess attemperator tubes or outer jackets for control of temperature and some older vessels were fitted with a skimming device known as a 'parachute.'

Modern top fermentation tanks tend to be less shallow than traditional ones and are conical based, rather than flat bottomed. The extremes of these are the cylindroconical vessels, whose heights are typically 3–4 times their diameters. They can be purchased in capacities from 1 hl (or less) to well over 1000 hl – suitable for the smallest and largest breweries. Typically, they are made of cold rolled stainless steel and the medium to large size vessels are lagged and fitted with height adjustable jackets for temperature control. Several advantages are claimed for cylindroconical vessels, including reduced costs, economy of space, increased throughput, excellent drainage, (and hence good yeast separation and minimum beer loss), improved (and more consistent) beer quality, better yeast hygiene and ease of in place cleaning. The largest vessels are located outside the brewery walls, although these are more likely to be used for bottom fermentation (Section 2.6.6); such vessels can be seen at Budweiser plants in the USA, Coors plants in the USA and UK and the Hite plant in South Korea, amongst others (see Figure 2.6.18). The very strong circulatory flow that occurs in fermenting wort when contained in cylindroconical vessels ensures effective yeast suspension, thus maximizing attenuation. Cylindroconical vessels are especially useful for breweries whose yeasts are of either highly flocculant or sedimentary types, although powdery yeasts can be used, especially if centrifuges are available during racking.

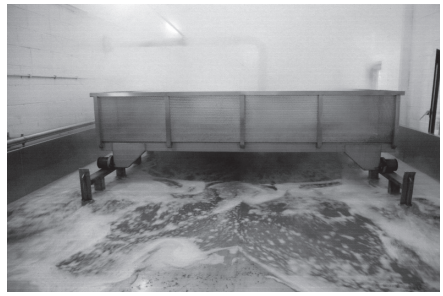
Other modes of top fermentation exist which have highly specialized vessels. Probably the best known of these is the Burton Union system, originally practiced at many breweries in Burton-on-Trent itself and at some others in the English Midlands. At the time of writing (2008) only the Marston, Thompson and Evershed brewery in Burton-on-Trent still operates the Burton Union system, although the Firestone Walker brewery in California also uses an adaptation of this system for some of its beers (Figure 2.6.16), on a smaller scale. The Burton Union system is basically a cask fermentation method with an ingenious way of removing excess yeast. It is especially useful with nonflocculating yeast, which were once the norm with Burton brewers. Two sets of 12 casks are arranged back-to-back on a framework fitted with upper and lower troughs and attemperator tubes. Traditionally, these are oak casks of about 7 hl (5 barrel) capacity, and each of these is connected by a pipe to its two nearest neighbors. Each cask has a swan-necked tube attached to its upper bung hole. Wort is fermented in a conventional vessel for 24–26 h, after which time it is transferred to each cask of the Union. As fermentation continues, excess yeast (with some beer) is discharged through the swan-necked tube into a slightly inclined trough situated above the rows of casks. The trough is held at a lower temperature than the casks and so the yeast tends to sediment in there, whilst the beer runs down the incline into a collecting vessel or 'feeder,' where it is returned to the casks via 'side rods.' At the end of fermentation, nearly all the yeast has been transferred to the top trough and the clear green beer is run via the bottom trough into a 'racking back' vessel, ready for racking into casks.

The Burton Union system maintains excellent yeast stocks and gives clear beer – it probably played a big part in the once unique character and high consistency of Burton beers. Against this, the system is expensive to install and maintain, it uses considerable space and is not easy to clean.

Another specialist vessel for top fermentation is the Yorkshire Square, originally made of stone (Yorkshire Stone Square), but nowadays constructed of slate or stainless steel. Unlike the Burton Union system, the Yorkshire Square works best with highly flocculating strains of yeast. The vessel has a lower and upper



(a) Plate filtration equipment at Belhaven Brewery.



(b) Coolship being filled with freshly boiled wort at Lindemans Brewery, Belgium. *Photo taken by Luc Bohez, courtesy of Lindemans Brewery, Vlezenbeek, Belgium.*

(c) Lambic beer maturation vat at Lindemans Brewery. *Photo taken by Luc Bohez, courtesy of Lindemans Brewery.*

(d) Large cylindrical brewing/maturation vessels at Coors Brewery, Tadcaster, UK.

(e) A bird's eye view of fermentation/maturation tanks at Hite Brewery, Hongcheon. *Photograph by courtesy of Hite Brewery, Seoul, South Korea.*

Figure 2.6.18 *More brewery equipment. Photograph courtesy of Hite Brewery, Seoul, South Korea*

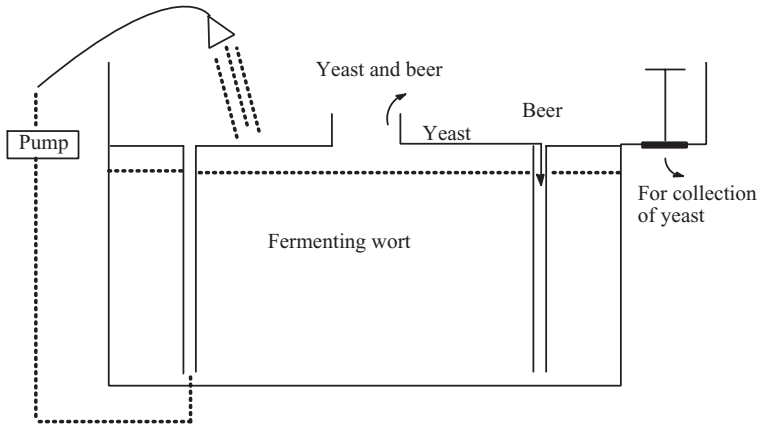


Figure 2.6.19 *Cross section of a typical Yorkshire Square fermenting vessel*

compartment, separated by an overhanging deck (Figures 2.6.16 and 2.6.19). The deck connects the two compartments by a central flanged hole (∼60 cm in diameter) and by off centrally situated thin unflanged pipes ('organ pipes'). The bottom compartment is filled with wort and the yeast is pitched (as a slurry) into the top compartment. As fermentation proceeds, yeast with beer rises through the central hole. The yeast tends to deposit on the deck, whilst the beer drains through the 'organ pipes' into the bottom compartment. Frequent rousing is needed, often by pumping (Figures 2.6.16 and 2.6.19). At the end of fermentation, the yeast can be skimmed off the deck and the beer is racked from the bottom compartment.

2.6.6 Bottom Fermentation

The big majority of the world's beers are produced by bottom fermentation and the family name for the beers thus produced is lager. The greatest differences between top and bottom fermentation are the yeast species, fermentation temperatures and length of maturation/conditioning, although there is much variation in both types of fermentation from brewery to brewery and also between different beer styles produced in the same brewery. Bottom fermentation is carried out by *Saccharomyces uvarum*, which gives much less of a yeast crop in the head and readily forms a sediment, especially toward the end of fermentation and at the low temperatures that are generally used for bottom fermentation.

In the brewing of traditional lagers, well-aerated wort at 7–14 °C is pitched with yeast (as a slurry) at much the same rate as in the brewing of ales. To keep the temperature within the above limits throughout the primary fermentation, an efficient cooling system is required, supplied by a single refrigeration unit serving all, or by several smaller units serving a smaller number of fermentation vessels, or even a single vessel. The brewer of bottom-fermented beers, like his ale counterpart, needs to collect and store viable and representative crops of yeast for repitching. Yeast from bottom fermentation tends to be collected from the middle layers of the sediment. The wort/yeast slurry is subjected to treatment using an oscillating sieve and is then washed with cold water to remove less dense debris and bacteria. The yeast is stored under water at 2 °C in refrigerated rooms, but only a portion is used for repitching. It is usually pitched as a slurry with water or wort.

The course of a typical bottom fermentation is shown in Figure 2.6.17(b), for a lager wort of OG 1045. Compared with a typical top fermentation (Figure 2.6.17(a)), it is of longer duration, with the major activity ('high krausen') occurring around four days after pitching, as opposed to 1–2 days for ale. Ester formation is

about the same as for top fermentation, but fusel alcohol formation is much less. Also, bottom yeasts lack the phenylacrylic acid decarboxylase (Pad 1) enzyme (Section 2.6.5) and so only very small amounts of flavor active phenols, such as 4-vinylphenol, are formed. Indeed, such flavors although highly prized in certain top-fermented beers, are regarded as off flavors when they occur in Pilsner and other pale lagers.

In traditional lager brewing, the beer is racked into lagering tanks whilst some 4 degrees of gravity (1° Plato) of fermentable extract remains and there is in suspension at least 1 million viable yeast cells per ml of beer. Lagering is often carried out for several weeks at 4 °C in traditional breweries, especially if the brewery uses a powdery yeast strain. This is to ensure that the yeast sedimentation is extensive and that secondary fermentation occurs slowly and incompletely, leaving some unfermented extract as residual sweetness. Alternatives to this involve the use of a centrifuge as in top fermentation (Section 2.6.5) or the use of 'krausening' techniques. In the latter, a powdery yeast is allowed to ferment the wort right out and then freshly fermenting wort (at the 'krausen stage' and brewed with flocculating yeast) is added at 5–10% of the volume of the lagering beer. Agitation is used here to make sure that no more than about 1° Plato of fermentable extract remains at the end of the lagering process. Closed fermentation vessels are the norm for lager brewing, but rectangular open vessels are still used in a number of Bavarian, Czech and Slovak breweries. In order to maintain low fermentation temperatures, traditional vessels are fitted with cooling jackets on the walls, or with internal attemperator tubes (or panels), depending on the vessel dimensions. Smaller rectangular vessels of, say, up to 500 hl capacity (~5 m × 5 m × 2.75 m depth) need only cooling jackets. Closed vessels are generally favored because of more efficient refrigeration: about 15% less energy is needed to cool a closed vessel, with respect to an open vessel of the same dimensions.

The largest breweries, such as Budweiser and Coors, use large outdoor vessels that have elaborate cooling, pressure relief and in place cleaning systems (Figure 2.6.18). A single tank, known as a 'Unitank,' is often used for both primary fermentation and postfermentation treatment, whether this is lagering (secondary fermentation/maturation) or 'accelerated ageing' (see next). A common procedure is to allow secondary fermentation in a number of tanks at 4 °C and then when almost complete, the beer is given a coarse filtration before being transferred to a second set of tanks held at 0 °C. Some breweries use tall cylindroconical vessels (Section 2.6.5), which can also be used for lagering, because a strongly sedimenting yeast in the base cone is well compressed and can be run off, leaving comparatively clear beer above. However, many breweries that have powdery yeasts use two such vessels per batch – one for primary fermentation, the other for lagering – with a chiller and centrifuge device connecting the two.

Fermentation characteristics of yeast cropped from the cones of two large (2000 hl) cylindroconical fermentation vessels showed variability according to how the yeast was cropped, indicating extensive heterogeneity within the crop (Powell *et al.*, 2004). Environmental conditions (ethanol, gravity, pH and temperature) within the cone were found to vary widely. It was considered that successive cropping and pitching with a specific portion of the cone may lead to selection and possibly to fermentation inconsistency.

For several decades now, lager brewers worldwide have sought to cut the primary fermentation and particularly the lagering time for batch fermented beers. This, of course, is in response to increasing economic pressures for increased throughput (see also Continuous Fermentation, Section 2.6.8). Indeed, by fermenting the wort completely (e.g. by using a powdery yeast) at the higher end of the bottom fermentation temperature scale (~14 °C), removing the yeast by filtration and then lowering the temperature to 0 °C (either slowly or rapidly), the beer undergoes stabilization and ageing (with no secondary fermentation) over a period of 3–21 days. This is sometimes called 'accelerated ageing.' Lagers can thus be produced on the same time scale as ales.

After lagering or ageing, most lagers are filtered (Section 2.6.9) and some (especially those destined for bottle or can) are pasteurized, either in bulk ('flash pasteurization') or in bottle ('tunnel pasteurization') (Section 2.6.9).

It is important for the brewer to be sure of the identity of the yeast: is it a bottom yeast or is it really a top yeast that has been accidentally supplied to brew the next batch of lager? DNA fingerprinting techniques will give

the answer, but there are many other simpler and more accessible methods that the brewer or brewery analyst can apply to the yeast himself. The simplest method is incubation: ale yeasts will grow on an agar medium at both 25 °C and 37 °C, whereas a lager yeast will grow only at the lower temperature. Similarly, bottom yeasts will ferment a liquid medium containing the disaccharide melibiose (α-Gal(1-4)α-Glc) (as witnessed by evolution of CO_2 and drop in pH, monitored by methyl orange indicator), whereas top yeasts will not.

2.6.7 Other Fermentations

Here, an account is given of the fermentations that are used to produce some specialized, but traditional styles of beer. Although the focus is on features of fermentation, other aspects of brewing, such as the grains used, mashing and boiling will also be considered, in order to give a balanced account.

Lambic is one of the most distinctive styles of beer. It originates in Pajottenland, a small area to the southwest of Brussels – although versions of it are brewed in other countries (Section 2.6.13). Lambic beer is brewed from grist of about 70% malted barley and 30% unmalted wheat. A decoction mashing method (Section 2.6.2) is used to take the wort through a series of temperature rests, ranging from 45 °C to 85 °C. Such a regime gives a cloudy, highly nutritious wort that is suitable for the wide variety of microorganisms that will ferment it. In the boiling stage (which at 6 h is exceptionally long), aged, dried hops (never fresh ones) are used. This is to give low iso-α-acid levels, so that antimicrobial activity is limited and the desired proliferation of a number of yeasts and bacteria can occur. Also, high concentrations of polyphenols from these old hops give the finished beer a certain astringency and the presence of short chain fatty acids (from the degradation of α-acids in old hops – see Section 2.6.3) may give some cheesy notes to the finished beer. Typical ingredients of lambic beer from Lindemans Brewery can be seen Figure 2.6.20.

The 'hopped' wort (pH ~5.3) is transferred to shallow wooden or steel coolships (see Figure 2.6.18) that are open to the atmosphere. Here, sufficient microorganisms are able to grow as the wort cools, so that a 'spontaneous fermentation' begins when the wort is transferred to fermentation vessels, often wooden casks that have been used previously for lambic beer production. The microorganisms are abundant in the whole fabric of the brewery, as well as in the air above the coolships. Fermentation is dominated initially by various *Enterobacter* species – *Escherichia coli*, *Klebsiella aerogenes* and several others (Hornsey, 1999b).

Figure 2.6.20 *Lambic beer ingredients at Lindemans Brewery. Malted barley, unmalted wheat, aged hops and microbes. Photo taken by LucBohez and used with kind permission of Lindemans Brewery*

Here, a slow fermentation of glucose and other sugars to a range of acids (including lactic acid), ethanol and carbon dioxide causes a drop in pH and (after about four weeks) makes the wort more suitable for the growth of various *Saccharomyces* species, especially *S. cerevisiae*, whereupon a slow alcoholic fermentation commences. This lasts for up to 12 weeks and produces a range of products already discussed in Section 2.6.4.

Toward the end of alcoholic fermentation, acetic acid bacteria become active, thus lowering pH further by the production of a small quantity of acetic acid. At roughly the same time, *Lactobacillus* and *Pediococcus* species begin to proliferate, their fermentations producing lactic acid, diacetyl, acetoin and various esters, with further lowering of pH (now less than 4). Following this, fermentation is taken over by *Brettanomyces bruxellensis* (initially) and *B. lambicus* (later) for about 6 months. These fungi produce a range of short chain fatty acids (cheesy notes) and a number of esters. On the other hand, certain unwanted odorous compounds, notably isoamyl acetate, diacetyl and DMS, produced in earlier fermentations, are reduced to lower levels during the *Brettanomyces* period of fermentation.

A 'young' lambic beer is around one year old, whereas pure ('old') lambic beers are three years old and are usually served on draught. They are flat, hazy and often rather tart drinks (pH $\sim$3.3), with ester-like and cheesy aromas. Much lambic beer is used to make a blend (Gueuze) and flavored beers (Faro and fruit lambics). Gueuze is a blend of young lambic and old lambic in a 2:1 ratio. It is normally bottled, whence it undergoes a secondary fermentation (bottle conditioning). Dextrinase enzymes, of microbiological origin, in the old lambic hydrolyze unfermented α-glucans (dextrins, Section 2.6.2) of the young lambic and the resulting sugars are fermented by the *Saccharomyces* species from the young lambic, thus producing CO_2 in the bottle. Faro is old lambic with brown sugar added. The many fruit lambics (lambic beer with fruit pulp or syrup added) are produced as bottle-conditioned beers. After sufficient ageing, the bottle-conditioned lambic beers possess a fine mousse, more like Champagne than typical beer foam.

Steam beer, now sometimes known as Californian common beer, is a hybrid beer, neither ale nor lager. Steam beer wort is fermented with lager yeasts at temperatures more typical of top fermentation ($>$12 °C) in shallow stainless steel vessels that more resemble coolships than conventional fermentation vessels. The large surface to volume ratio encourages dissipation of the heat of fermentation into the air and at the same time facilitates settling of the yeast when fermentation begins to subside. Because the lager yeast does not produce a thick head that protects the beer from airborne contaminants, fermentation is best carried out in rooms that possess a positive air pressure from an input of sterile filtered air at ambient temperature.

Formerly, constant skimming of the surface was needed to remove contaminants, but nowadays in a modern sterile fermentation room, only limited skimming is necessary, mainly to remove cold trub. The bottom-fermenting yeasts for the production of steam beer require special properties: in particular, they need to remain in suspension during the course of fermentation, form a sediment rapidly when fermentation is coming to an end and produce acceptable flavors at temperatures far in excess of those used in normal lager fermentations. Not all lager yeasts are suitable for this, but competent strains can be obtained from companies such as Wyeast, yeast depositories or by cultivation of the yeast deposit in bottle-conditioned steam beer. Largely because of the hybrid nature of the fermentation, modern steam beers display characteristics of both ale and lager: some fruity ester notes, but with a clean finish. For further description and a brief account of the history of steam beer, see Section 2.6.13.

Just as modern steam beer takes its name from a process that died out in the 1950s, German Dampfbier is a revival of a style that was of minor but significant importance in mainly the southeastern part of Bavaria (in a region called der Bayerischer Wald) but which disappeared in the early twentieth century. Unlike Californian steam beer, Dampfbier is a true top-fermented beer, where traditionally, wheat beer (Weizen) yeasts perform the fermentation of the all barley malt wort at high temperatures, even for ale ($>$21 °C). This gives the beers a strong, fruity-spicy, phenolic character, not unlike wheat beers and some Belgian saison ales (Section 2.6.13). The word Dampf (steam, in English) comes from the observation of a continuous plume of mist above the surface of the wort at the height of fermentation, caused by vigorous yeast action at such high temperatures.

2.6.8 Modern Methods of Fermentation

High gravity brewing is practiced in a number of large breweries in the USA and Canada, originally to save space and to brew large volumes of beer using fewer vessels. In theory, dilution to normal gravities ('breaking down') can be carried out anywhere between the end of boiling and packaging, but the most usual time is after fermentation, before lagering or maturation, because this demands least extra space and vessels, except for maturation vessels. Typically, worts of OG 1072 (18 °Plato) are prepared and fermented with special high alcohol tolerant yeasts. The resulting beer will have an alcohol content similar to a Moselle wine (~8% v:v). The beer is then diluted with pH adjusted, deoxygenated, sterile filtered water to the required percentage ethanol level and is then treated in a similar way to standard beers (Sections 2.6.5 and 2.6.6). Apart from more economical use of space and vessels, high gravity brewing has several advantages: it has lower energy consumption, lower liquor, water (for washing) and effluent costs, and gives a more efficient production of ethanol. Additionally, by dint of different dilution ratios, use of brewing colorants, and use of isomerized hop extracts and other flavor agents, it is possible to produce widely differing beer styles from a single batch of high gravity wort.

High gravity brewing also allows a higher proportion of unmalted gelatinized adjuncts from the cereal cooker to be included in the mash, which is an important consideration for the North American beer market. In any case, it has been known for many years that all-malted barley high gravity brewing is difficult because of lautering problems, decreased yeast performance (high initial osmotic pressure and later, high alcohol levels) and poor head retention (Section 2.6.9). With respect to yeast performance, pilot scale fermentations of very high gravity wort have shown that increased yeast inoculum size, increased free rise timing and temperature, and optimized oxygenation reduced fermentation time by 34% (Jones *et al.*, 2007). This improved fermentation profile was consistent over three successive inoculations. A key factor is oxygen supply: 25 ppm of oxygen delivered to the wort 12 h after inoculation reduced fermentation time by 33%.

All the beer production processes discussed so far are of the batch fermentation type: indeed batch fermentation still dominates the brewing industry. However, in the 1950s, Morton Coutts of the (now) DB Breweries in New Zealand developed a continuous fermentation method to produce normal strength beers. It is still in use today (2008) and is probably the only large-scale continuous fermentation process in full operation.

The method, of the 'cascade' type, involves a continuous supply of wort to a series of fermentation vessels, with continuous recycling of yeast and fermented beer back to the fresh wort. A continuous flow of 'green' beer is thus produced at the opposite end of the process (Figure 2.6.21). The boiled wort is chilled to 0 °C and is continuously fed into the holding vessel (HV) where, on its way, it is aerated and warmed to about 10 °C. In the holding vessel, it is mixed with already fermenting wort from fermentation tank 1 (FT1). As fermentation proceeds, some yeast from FT2 and the separating tank (ST) is also recycled to HV. FT1 is the major (larger) fermentation vessel, with a residence time of over 30 h. By the time the beer reaches FT2, fermentation vigor is diminishing and a significant degree of attenuation has been achieved.

The fermenting beer in both tanks is stirred, keeping much of the yeast in suspension and thus maximizing attenuation and minimizing production of off flavors. Residence time in FT2 is at least 12 h. Next, the beer passes to ST, where the yeast forms a sediment in the conical base. The beer is run off the sediment into warm (~14 °C) maturation tanks, where it stands for two days. This is essentially a 'diacetyl rest': at this temperature and pH (~4.2), α-acetolactate is secreted by the yeast cells into the beer, where it is converted to diacetyl (Section 2.6.4). Diacetyl is then absorbed by the yeast cells and converted to the flavorless 2,3-butanediol.

The yeast from ST is partially recycled and partially washed (with recovery of beer washings) and then sold to health companies. As in any closed fermentation system, excess CO_2 can be collected, purified and then either sold to gas suppliers or returned to the matured beer to increase the level of carbonation. The advantages of this continuous fermentation process are related to more efficient use of space and vessels, high

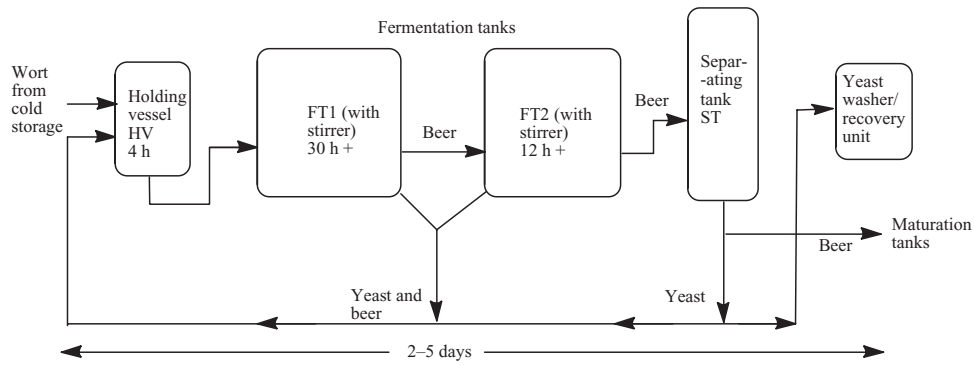


Figure 2.6.21 *Plan of cascade type of continuous fermentation process (based on Dominion Breweries (DB) system, New Zealand). Not drawn to scale*

(but variable) throughput via a rapid fermentation, lower costs and higher beer consistency. Also, it is fully automated and there is reduced wastage due to the lack of stop-start operations. The major disadvantage is that the highest hygiene standards are needed to prevent microbial infection; the CF system can be run for months and during this time it is impossible to carry out any cleaning of vessels.

Continuous fermentation methods using 'bioreactors' that contain colonies of immobilized yeast cells have been adapted to beer production on both pilot and industrial scales (Iserentant, 2003; Brányik *et al.*, 2004). These processes differ from the DB CF system in that a continuous flow of wort is exposed to yeast cells entrained or immobilized on some solid support, instead of continuous recycling of yeast and continuous injection of fresh wort. The solid support ('carrier') includes spent barley grains, sintered glass rings, wooden chips, biopolymers and synthetic polymer gels. Designs of bioreactors vary considerably – from packed bed reactors, through gas lift types to fluidized bed reactors. So far (2008), the application of immobilized cell CF to the regular production of standard beer types has been quite limited, because of a number of problems. These include operational problems (CO_2 removal, excess yeast production, irregular flow and clogging), problems with yeast performance (changed cell physiology, cell ageing and excess diacetyl production) and unexpected additional costs (usually due to unstable operation and breakdown). Also, the lack of a suitable continuous wort production process (Section 2.6.2) has held back this type of CF; wort needs to be supplied in batches, thus increasing the risk of infection. Contamination by *Obescumbacterium proteus* and *Pantoa agglomerans* (both of which produce high levels of DMS) have been the most problematic for immobilized yeast CF. Nevertheless, versions of these processes have been used in continuous beer fermentation, continuous maturation and alcohol-free beer production (Branyik *et al.*, 2004) (see Chapter 2.13). A gas lift type of reactor for continuous fermentation is shown in Figure 2.6.22. Such a reactor would contain yeast immobilized on spent grains, fermenting wort and gases, and is designed to run smoothly with high solid (biocatalyst) loading, with low shear rate and low mass transfer resistance (Brányik *et al.*, 2004; 2005).

Immobilized yeast reactors find greater application to rapid conditioning of beer, especially with respect to yeast mediated reduction of diacetyl to concentrations below its OTV. Green beer, produced in cylindroconical vessels or by a continuous process, is first heated (under anaerobic conditions) to 90 °C for 5–10 minutes to ensure all α-acetolactate is converted to diacetyl. After cooling to ~15 °C, the beer is passed through several reactors, so that during a contact time of around 2 h, all diacetyl is transformed to acetoin and other flavorless products (see Figure 2.6.15). Such a process has been used for nearly 20 years by Synebrychoff (Finland). The yeast is supported on Spezyme GDC® (Cultor, Finland), which is essentially diethylaminoethyl cellulose on a cross linked polystyrene support particles, with TiO_2 incorporated to increase the particle density. It is

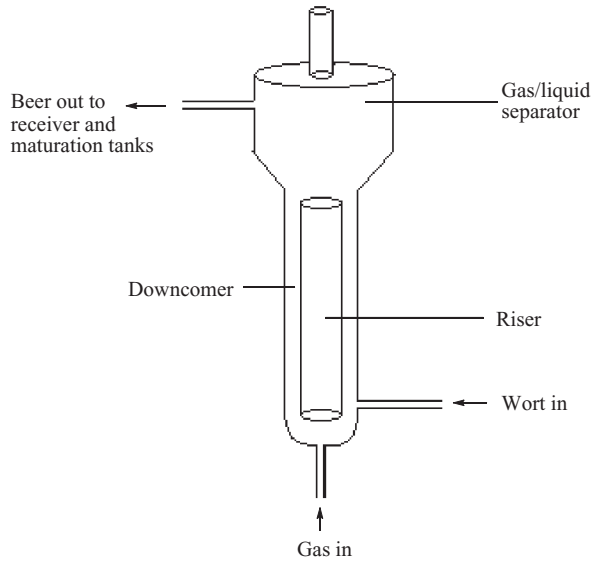


Figure 2.6.22 *Simplified diagram of a gas lift reactor for continuous fermentation of beer using immobilized yeast cells. Not drawn to scale. Based on Brányik* et al. *(2004) and reproduced with permission from the American Society of Brewing Chemists*

claimed that a good quality beer can be produced by this method in just 24 h. The heat treatment confers no flavor or color changes and indeed denatures proteins that might interfere with foaming characteristics of the finished beer (Hornsey, 2003).

It is of interest to note that a combination of a high gravity wort and a continuous ethanol fermentation on a multistage packed column bioreactor system can be used to produce liquors with over 15% ABV for conversion to industrial ethanol, by distillation or other techniques (Bai *et al.*, 2004).

2.6.9 Beer Treatment: Fining, Filtration and Pasteurization

Beer treatment is aimed at achieving and maintaining the perceived commercially attractive characteristics of a beer from the time it leaves the brewery to the point of dispensing and consumption. The main qualities of concern are flavor, clarity, foam quality and lack of microbial contamination, so beer treatments seek to provide flavor stability, haze stability, foam retention stability and microbiological stability. The major reasons for nonbiological beer instability have been discussed by Stewart (2004).

Apart from certain wheat beers (Section 2.6.13) and some other speciality products, like Lambic beers (Sections 2.6.7 and 2.6.13), commercial brewing is heavily orientated toward the production of clear, haze-free beers. There is also a bias toward brewing beers that display a substantial and persistent foam (or 'head') when served. Since proteins are involved in both haze formation and the production of foam, these two topics will be treated together in this section.

There are two basic kinds of beer hazes: temporary and permanent hazes. Beers that have a low tendency to throw hazes are known as 'haze stable' and the processes that are used to minimize hazes are called 'beer haze stabilization.' The various steps that the brewer can take to minimize hazes have been discussed

(Sections 2.6.2 and 2.6.3). At least one authority (Fix, 1999f) maintains that beer brewed properly from the best ingredients should clear spontaneously, given sufficient time, and that permanent beer hazes are caused by brewing faults. Nevertheless, in reality, hazes do occur and the clarification methods of fining and filtration are important aspects of commercial brewing. Haze is expressed in terms of ASBC 'formazin turbidity units' (FTUs), with 0–100 indicating brilliant clarity, 100–200 slightly tarnished clarity, 200–300 translucent haze and >400 turbidity. The corresponding EBC ranges are 0–1.5, 1.5–3.0, 3.0–4.5 and >6, similar to the nephelometric turbidity units (NTU) used to express wine clarity (Section 4.6.3).

Chill haze is the most common type in beer; it is temporary in the sense that the haze appears on cooling the beer, but disappears again on warming. The greater the haze stability, the closer to 0 °C the beer can be brought before chill haze appears. Chill haze is formed by interactions between proteins and polyphenols; the former are derived mostly from malt, whereas the latter come from both malt and hops. Polyphenols are molecules that possess several phenolic OH groups. They can be simple phenolic acids (like gallic acid and ferulic acid) flavonoid phenols (like catechin) monomers (like ferulic acid and catechin) and oligomers (like proanthocyanins) (see Section 5.8.6, Figure 5.8.6). Many monomeric phenols, especially anthocyanins and catechins, polymerize at beer pH to give high molecular weight oligomers (dimers, trimers, etc.) that are variously known as proanthocyanidins and tannins (tannoids), depending on their monomer make up and degree of polymerization. Polyphenols of the catechin family (see Section 5.8.6, Figure 5.8.7), and various oligomers are particularly active in forming colloidal complexes with proteins from malted cereals, especially hordein (barley) and gliadin (wheat).

These proteins are rich in proline (PRO) residues, which are α-helix and β-sheet disruptors and are thus more likely to be found in 'random coils' – relatively flexible polypeptide chains in the proteins. Although these proteins are denatured (unfolded) during the early parts of the brewing process, they will refold to some extent, though probably never retrieving their native structures. The bonding holding the complexes together is probably mostly weak hydrogen bonding (~10–20 kJ/mol per bond) that can be broken at higher temperatures (say, ~25 °C), thus explaining the disappearance of chill haze on raising the temperature.

This kind of model also explains why chill haze formation is maximized when polyphenol and protein concentrations are roughly equal; reducing either polyphenol or protein content (but not both) therefore reduces haze formation. However, chill haze appears to be a frequent precursor of permanent haze, the transformation occurring over a period of time. It is possible that oxidation of catechol (1,2-dihydroxybenzene) units of polyphenols, catalyzed by an enzyme of the polyphenol oxidase family, occurs to give *o*-benzoquinone (1,2-benzoquinone) units which can then react with amino acid residues of proteins to give much stronger covalent bonds, bonds that cannot be broken by raising the temperature by 20 °C. Certainly, 4-methyl-1,2-benzoquinone has been shown to react with proline methyl ester to give 4-(2′-carboxymethyl-1′-pyrrolidinyl)-5-methyl-1,2-benzoquinone (Rizzi, 2006) (Figure 2.6.23). Beers that have been produced by strict low oxygen regimes (Sections 2.6.4 and 2.6.10) suffer least from this problem.

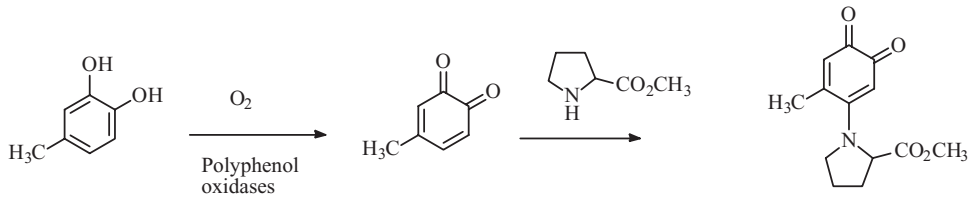


Figure 2.6.23 *Reaction of 4-methyl-1,2-benzoquinone (4-methylcatechol) with proline methyl ester as a model for formation of covalent polyphenol-protein bonds in permanent haze formation. Rizzi (2006)*

Table 2.6.4 *Beer hazes, their causes and some possible remedies*

Haze type	Causes	Remedies and Comments
β-Glucan	Poor malt, unsympathetic mashing and high sparging temperature	Improve malt and mashing/sparging programme. Use of GM malt. Add β-glucanases
Starch	Insufficient mashing and/or oversparging	Modify mashing/sparging regime
Bacterial	Infection	Discard. Find source of infection and improve hygiene
Yeast	Low flocculating yeast strain	Centrifugation, fining, filtration
Protein/polyphenol (including oxidation)	Colloidal complexes of proteins and polyphenols. Made permanent if polyphenols are oxidized by by O_2 and oxidases	Temporary chill haze: fining with PVPP or silica materials, filtration. Permanent haze after oxidation: no remedy. Minimize O_2 uptake at all stages post-fermentation. Lower sparge temperatures minimize extraction of polyphenols. Use of GM modified barley eliminates polyphenols.

Permanent hazes are essentially caused by brewing faults, as outlined in Table 2.6.4. All the haze types displayed here can be avoided in the brewery by using sound, hygienic brewing techniques, along with good quality materials and in the absence of 'market abuse' (rough handling, storage at high temperatures, etc.). In the last case, poor treatment of the beer by the trade can lead to hazes in otherwise sound beer.

Foam is an important attribute of most beers and it plays no small part in consumer acceptance of beers. Many beer drinkers prefer a thick, stable 'head' on their beer that clings to the glass (known as 'lacing') as they drink the beer. Hence, foam production, retention and lacing are all important features by which foams can be characterized. Foam formation indicates the actual quantity of foam (thickness of the head) that is present on the surface of the beer. For beers brewed with high cereal worts, there should be no trouble in producing adequate foam. Retention is a measure of the ability of the foam to take up a stable network of bubbles, thus allowing an extended lifetime. Lacing is a measure of the foam's ability to stick to the walls of a clean glass. Deficiencies in foaming characteristics can be rectified by the use of additives (e.g. hydrolyzed albumen or propylene glycol alginates) in the later stages of brewing.

Large glycoproteins (or protein–carbohydrate complexes) are thought to play a major role in the foaming characteristics of beer. They are composed of hydrophobic high molecular weight proteins, to which are attached long hydrophilic carbohydrated chains. Bubbles are formed as gas is released from solution in the beer. These interact with glycoproteins to form spherical micelles, in which the protein part of the molecules are found at the gas/liquid interface, whereas the carbohydrate chains point outwards and are essentially associated with solvent and solute molecules (Figure 2.6.24). The carbohydrate chains form hydrogen bonds to large numbers of water molecules and other hydrophilic molecules, which are carried to the surface as the bubbles rise. This results in a thick, moist head. If the carbohydrate chains are long enough (just how long has yet to be determined), the viscosity of the liquid between the bubbles is high enough to keep the liquid within the foam matrix, resulting in a moist, stable foam that clings to the surface of the glass.

Other foam enhancing molecules (that is, apart from high molecular weight glycoproteins) include dextrins (α-glucans), β-glucans, iso-α-acids and melanoidins. They probably work by increasing the liquid viscosity or by forming more extensive hydrogen bonded networks amongst the hydrophilic chains.

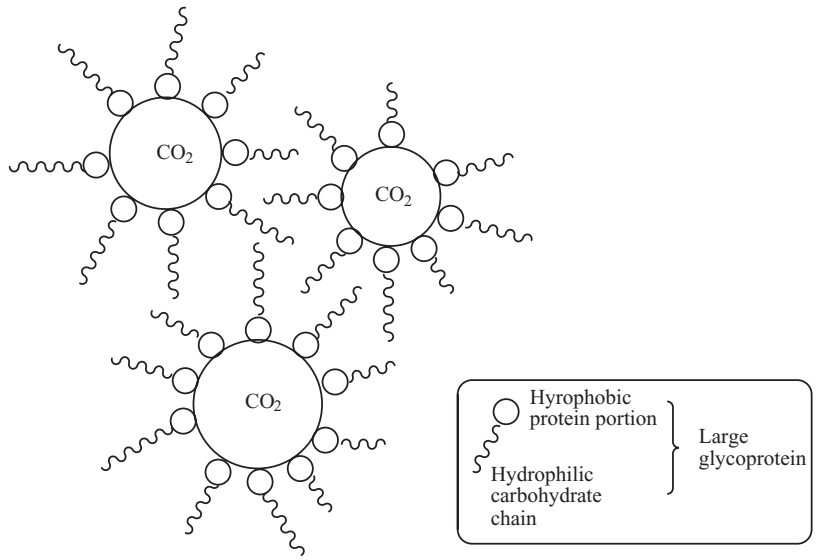


Figure 2.6.24 *Spherical protein micelles at the gas-beer interface (two-dimensional section). Water and other polar molecules are retained in the hydrophilic regions between the carbohydrate chains*

Molecules that compete with large glycoproteins for micelle formation, but which do not have extensive hydrophilic chains, adversely affect foam retention, because lack of hydrogen bonding lowers the viscosity of the entrained liquid between the bubbles. Such molecules include hordeins, lipids (oils and fats), like triglyceride esters, steroids and phospholipids, small glycoproteins and most detergents. This explains why the foam on beer may collapse if the drinker is eating oily or greasy food at the same time. Also, detergent molecules compete for micelle formation, which explains why the use of improperly rinsed glasses (containing detergent residues) leads to poor head retention. Similarly, at the brewery, washing of all equipment needs careful and thorough rinsing. Certain cereals, such as oats, possess relatively large amounts of lipids, explaining why beers brewed with a high oat cereal bill often lack head retention. At the other extreme, wheat contains comparatively high contents of large glycoproteins and β-glucans, which give wheat beers, or beers with a high wheat content, good head retentive properties.

Ethanol has a marked effect on the foaming properties of beers: too little ($< 1\%$ ABV) and too much ($>10\%$ ABV) tend to give beers with low foaming abilities. The optimum positive effect appears to be between 3% and 6% ABV. The role of ethanol here is a complex one and is probably related to hydrogen bonding, viscosity and solubility of CO_2. For example, in beers of ABV $>10\%$, there is enough ethanol to both decrease the viscosity of the liquid entrained between the bubbles and to reduce the solubility of CO_2, thus leading to early release of the gas and drier, low retention foam.

Chill hazes can be eliminated (or at least minimized) by the use of fining agents (discussed next) and /or ice stabilization, whereas yeast hazes can be removed by fining agents, centrifugation or filtration, and often by a combination of these. Since chill hazes are caused by colloidal protein–polyphenol complex formation, the addition of fining agents that selectively adsorb either proteins (protein-active agents) or polyphenols (polyphenol-active agents), will actively restrict the formation of these hazes. Fining agents (often called flocculants) act by adsorbing (chemically bonding) haze forming molecules onto their particle surfaces, thus removing them from the beer (or wort, in the case of kettle finings). Brewers are undecided on whether haze active proteins and foam active proteins are the same or different entities. Hence, it is possible that

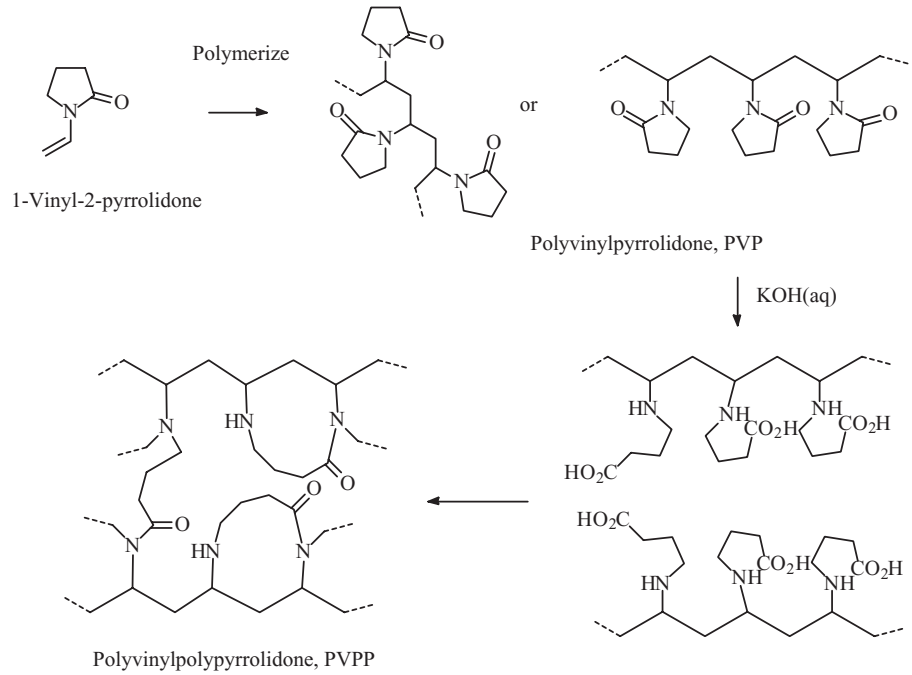


Figure 2.6.25 *Simplified scheme for formation of PVP and PVPP from their monomer*

the use of protein active fining agents (such as bentonite, carageenan and tannin) may reduce the foaming capability of the beer; this would be regarded by many consumers as a serious mark against quality. Certainly, overuse of kettle finings based upon Irish moss is known to adversely affect the foaming characteristics of beer (Fix, 1999g). Hence, a better approach to haze removal may be to use polyphenol active agents. These include polyvinylpolypyrrolidone (PVPP), polyvinylpyrrolidone (PVP) and various silica-based materials, such as hydrogels and xerogels. The consensus of opinion appears to be that removal of polyphenols (whose concentrations are too low to influence flavor) is more desirable than the removal of proteins (which may adversely affect foaming ability) (Mitchell *et al.*, 2005). Two of the most widely used polyphenol active agents are PVPP and silica bound PVP (or PVP–silica composite), typically added at a rate of 50 g/hl. These are more polyphenol specific than silica materials alone, with PVPP being somewhat the more effective of the two (Mitchell *et al.*, 2005). PVPP exists under a large number of trade names or pseudonyms, including Albigen A, Kollidon, Plasdone, Polyclar, PVP-K60, Subtosan and Vinisil. Figure 2.6.25 illustrates representative structures of PVP and PVPP, and their chemical formation from monomers. It can be seen that both polymers (PVP being essentially linear and PVPP being cross linked) possess numerous hydrogen bonding groups: NH (donor) and C=O (acceptor). It appears that these polymers form stronger hydrogen bonds and other attractive interactions with polyphenols rather than with proteins, especially foam active proteins. Silica-based adsorbents predate PVPP and PVP as fining agents by a number of years. They are selective toward haze active proteins, leaving foam active proteins largely unaffected. The surface of silica gel particles is covered with silanol (Si-OH) groups that are sensitive, with regard to ionization, to the pH of the medium. This, in turn, influences the extent (or even type) of silica–haze active protein interaction. Thus, removal of chill haze using silica adsorbents has been found to be greater at pH 3.8 and less at pH 4.4 (Siebert and Lynn, 2007).

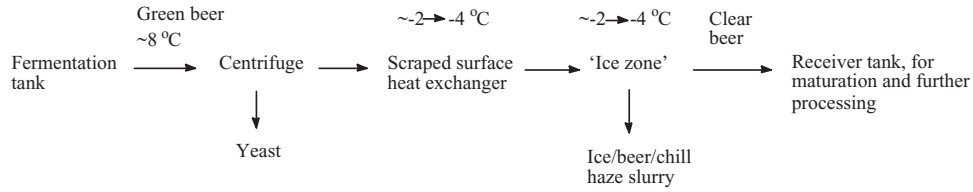


Figure 2.6.26 *Outline of ice stabilization process. Based on the Ice Process of Labatt Breweries, Canada*

Filtration of the beer from the fining residues (the 'floc') is often carried out using silica gel or diatomaceous earth (kieselguhr) cake filters to trap the residue particles.

Ice stabilization has become popular in recent years, as a nonadditive type of beer clarification method, especially when used in conjunction with filtration and/or centrifugation. The ice beer process is typified by the régime used by Labatt Brewing Co. of Canada (Figure 2.6.26). Very small ice crystals (<5% of the total volume) are formed at –2 to –4 °C. The haze active proteins are entrained in and between these crystals and are eventually left behind when, after settling, the cold beer is pumped off the ice/beer slurry in the receiving tank. A similar method can be used to enhance the alcoholic content of beer (or any other alcoholic drink; it is really a form of distillation) as in certain German strong lagers (Eisbock) (Section 2.6.13).

Yeast haze is a natural consequence of the production of traditional unfiltered new ('green') beer. Suspended yeast cells provide a reducing environment for beer, which endows it with high flavor stability, by protecting it against oxidation (Section 2.6.12). Consequently, clarification (especially the total removal of yeast cells) tends to be carried out when the beer is almost ready for packaging. A well made beer should clarify itself of yeast haze during conditioning and maturation, given sufficient time, a method that is used by many European breweries, some American microbreweries and even some British microbreweries, but most brewers in the UK use finings. Isinglass finings are made from the dried swim bladders of certain fishes, nowadays mostly species of catfish or drumfish from subtropical waters. They are shredded, flocked or ribboned and then soaked in dilute solutions of tartaric acid and sulfites for several weeks before use. Isinglass preparations must be kept at low temperatures (~4 °C), as many lose their fining ability above 15 °C and all fail to work if kept above 20 °C. The cloudy viscous liquid contains solubilized collagen, gelatin and insoluble materials, the first of which is largely responsible for the fining action. At beer pH, collagen molecules carry an overall positive charge, so they interact with yeast cells that carry an overall negative charge. It also takes up lipids, antifoaming agents and, to a certain extent, positively charged proteins. A simplified scheme for the action of isinglass finings is shown in Figure 2.6.27. Collagens are supercoiled fibrous proteins with an unusual abundance of the amino acids glycine, hydroxylysine, hydroxyproline, lysine and proline. The collagen in fish swim bladders is of the type I class (Walker *et al.*, 2007) and is composed of triple helical structures of about 300 nm in length and 1.5 nm in diameter, organized into fibrils, which in turn often aggregate into larger structures called fibers. The procedure for the preparation of isinglass finings probably results in the degradation of most of the fibers into fibrils or even triple helix 'monomer' structures. Figure 2.6.27 shows a schematic representation of a triple helix structure, although in reality, much larger collagen structures may account for the fining action. Until recently, the use of isinglass finings was almost entirely limited to the UK, but some brewers in a number of other countries (notably the USA) are realizing its advantages as an aid to filtration and as a foam enhancer. For cask-conditioned ales, isinglass finings are usually added at the time of racking, at 0.4–1.0 l/hl. They can be added to the bulk beer in the racking back or to individual casks, at the brewery, or to the cask at the location of dispensing. Settling of finings and yeast normally takes 2–24 h at 10–16 °C, depending on the yeast strain. More recently, optimum effectiveness of fining with isinglass has been achieved by the use of an inline mixing process (Freeman *et al.*, 2003).

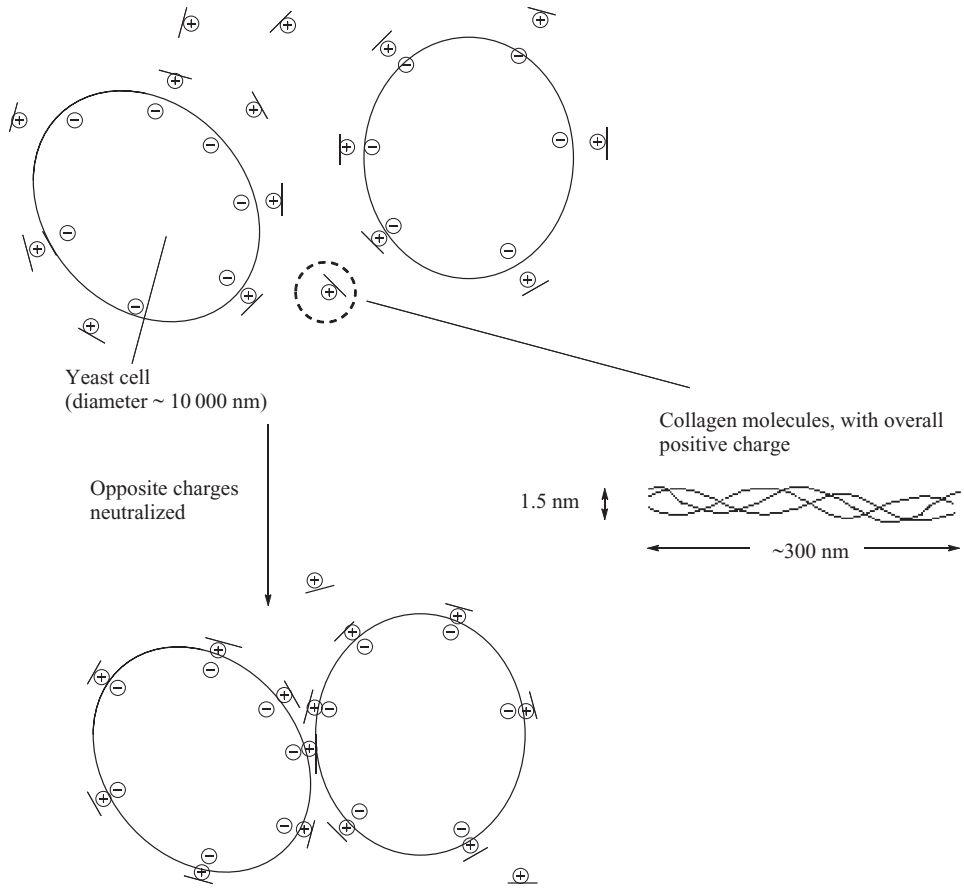


Figure 2.6.27 *Simplified scheme for the action of isinglass finings. Electrostatic (ionic attractions are emphasized here; other attraction types (like hydrogen bonding) will exist. Counterions (Ca^{2+}, SO_4^{2-}, phosphates, etc.) are not shown, for clarity. Not drawn to scale: the collagen molecules maybe much larger polymers or fibrils*

As isinglass is effectively an additive, it is not looked upon favorably by most European brewers, particularly German brewers, whose *Rheinheitsgebot* forbids its use. In the UK and USA, beers produced without the use of isinglass finings (often bottled-conditioned beers) are sometimes labeled 'suitable for vegans' (Section 5.9.5). Alternative collagen containing finings for beer, derived from chicken skins and yellow split peas, have been investigated recently (Walker *et al.*, 2007). On a small scale, both of these worked as well as isinglass and the latter may provide a useful alternative fining agent, particularly as mandatory allergen labeling is required for food by the European Union and the USA. This is despite the fact that isinglass residues in beer are usually very low and the evidence for collagen itself (which is the major organic component of isinglass) causing allergenic reactions is questionable (Baxter *et al.*, 2007) (see also Section 5.9.3). Other agents are used to clear yeast hazes, notably gelatin of animal origin and beechwood chips, both in the USA.

Filtration has been used for decades in the clarification of beer, but nowadays it is frequently applied after fining and/or centrifugation steps, which remove the bulk of insoluble substances that would otherwise quickly clog the filtration system. In this way, it becomes a final 'polishing' step to produce a brilliantly clear and

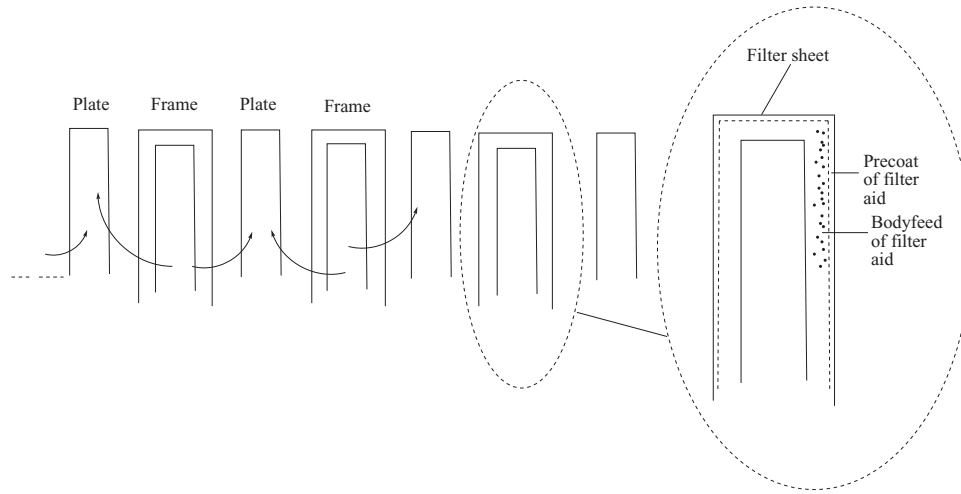


Figure 2.6.28 *Arrangement of plates and frames (vertical section) in a plate and frame filter machine*

microbiologically stable beer. Nevertheless, filtration is still used in varying degrees of severity, depending on the type of beer being produced. See also Section 2.9.4 for filtration of wine. For removal of most yeast cells and other suspended matter, depth filters are used, whereas much finer membrane filters will be needed for absolute filtration – that is, sterilization of the beer by removal of all yeasts and bacteria. Filtration is used to maximum effect on chilled beer (0–2 °C, depending on the beer gravity), where formation of cold trub and chill haze are maximized. Depth filtration is mainly carried out these days using either sheet filter or plate and frame (and related) filter equipment. The former uses sheets of fibrous materials of varying porosities (coarse, fine or sterile), usually made of cellulose and/or kieselguhr, although PVPP can be incorporated for the removal of polyphenols. Filter sheets are obtainable in sizes of up to 1 m^2 (1 m × 1 m) and frame presses are available in sizes that hold up to 200 sheets, thus allowing a throughput of up to 240 hl/h. Plate and frame filtration uses filter frames that are made of fibrous (cellulose) cloth impregnated and layered with the filter aid, usually kieselguhr. For optimum operation of such filters, a slurry of filter aid (called 'body feed') is added to the beer being filtered at intervals of time and in such concentrations that are suggested by the filter manufacturer or that have been found from experience. A typical body feed is 100 g of filter aid per hectolitre of beer and it may also contain PVPP for polyphenol removal. Other filter aids, such as perlite (a material of volcanic origin) and silica hydrogel, are used by some breweries. In a plate and frame filter, the filter frames are held alternately with blank frames in the filter machine (Figure 2.6.28). Variants of this kind of depth filtration include vertical leaf, horizontal leaf and candle (filter stick) systems. In all cases, chilled beer is pumped through the filters under pressure, such that the outflow rate does not exceed 4 or 5 hl/m^2. Pore sizes of filter frames (or leaves or candle elements) vary from a few μm up to about 90 μm, enabling sterile fine to coarse filtration. In all cases of depth filtration, the filter materials not only sieve out particles that are larger than the pore sizes but also adsorb a considerable amount of much smaller particles (see Section 2.9.4, Figure 2.9.26). This is because the particles of filter materials such as kieselguhr carry an overall positive charge, whilst yeast and bacteria cells carry an overall negative charge. Thus, even coarse filters are able to remove some (but not all) of the suspended yeast and bacteria cells. In order to remove all microorganisms, the brewer must resort to sterile fine filtration, for which purpose cellulose acetate membrane filters (pore size ≤ 0.45 μm) probably give the best results. However, it is essential that beer filtered in this way has been previously extensively clarified, for example by a régime consisting of a combination of sedimentation,

centrifugation, fining and filtration. Membrane filtered beer is microbiologically stable and can be sterile packaged (tanked, kegged, bottled or canned) (Section 2.6.10) – suitably chilled and carbonated – immediately, whereas less rigorously filtered beer will require pasteurization (Section 2.6.10) to kill unfiltered microorganisms.

Centrifugation is really an aid to the settling of solids from beer. According to Stokes' Law, the settling rate v is given by Equation 2.6.3, where r_p is the particle radius, s_p the particle density, s_m the medium density, g the gravitational constant and η the medium viscosity constant.

$$v = \frac{2r_p^2(s_p - s_m)g}{9\eta} \tag{2.6.3}$$

Thus by increasing g by rotating the sample, the rate of sedimentation may be increased. Note also that finer particles (smaller r_p) in a high density medium (large s_m), such as powdery yeast in a high gravity beer, will sediment relatively slowly.

Centrifuges in breweries tend to be used in three major ways: as wort clarifiers, as inline clarification devices for beer en route to maturation vessels (or casks) and as devices for the recovery of beer from sediments at the bottom of fermenters or tanks. The last use is important for larger breweries, where considerable losses can arise from beer being entrained in sedimentary material. However, the use of centrifuged yeast for repitching is open to question, since shear stress causes a drop in yeast viability, vitality, foam stability and general beer quality (Chlup and Stewart, 2007). The most widely used centrifuges are of the disk stack, cylindrical bowl, self-clarifying and decanter types. They are hermetically sealed, so that neither oxygen is taken up nor carbon dioxide lost, and they are capable of 5–600 hl/h throughput rate. Although they take up little space, are easy to sterilize (and to keep sterile) and require no filter aids, centrifuges are costly both to purchase and to operate and they tend to be noisy. Also the stresses imposed upon yeast cells under centrifugation can cause cell wall damage, thereby releasing cell wall mannans, extracellular proteinase A and other materials, resulting in hazes and reduced foam stability (Chlup *et al.*, 2008). Proteinase A is known to hydrolyze beer lipid transfer protein 1 (LPT1), one of the proteins that are associated with beer foam formation and stability (Leisengang and Stahl, 2005).

For beers that require a long shelf life (kegged, bottled and canned beers), pasteurization will be required if full sterilization has not been achieved, as noted previously. There are two kinds of pasteurization that are used in breweries: flash pasteurization of bulk beer, mainly destined for tanks or kegs, and tunnel pasteurization, used for bottled and canned beers. Flash pasteurization is carried out in plate heat exchangers, where hot and cold beers are in counter flow. More detail regarding both flash and tunnel pasteurization is given in Section 2.6.10. The degree of pasteurization is given by the pasteurization unit (PU), which is arbitrarily defined as the lethal effect on microorganisms obtained by holding the beer at 60 °C for 1 minute. Increase in lethality caused by increase in temperature (over 60 °C) is given by Equation 2.6.4, where T is the pasteurization temperature in °C.

$$\text{PU}(/\text{min}) = 1.393^{(T-60)} \tag{2.6.4}$$

Typically, bottled beers are given are given between 20 and 30 PUs, but sometimes as little as 5 PUs, when the quality of the filtration is high. The greater the number of PUs applied to a beer, the more pronounced is the cooked flavor. For bottled beers, pasteurization at 20 or more PUs tends to be favored in the UK, whereas in Europe and the USA, a lower degree of pasteurization is often used. Pasteurization can be applied to noncarbonated bulk beer (e.g. in a tank) or already highly carbonated beer (e.g. in bottles or cans). The brewery microbiological contaminants that are most resistant to pasteurization are lactic acid bacteria and *Saccharomyces pastorians*.

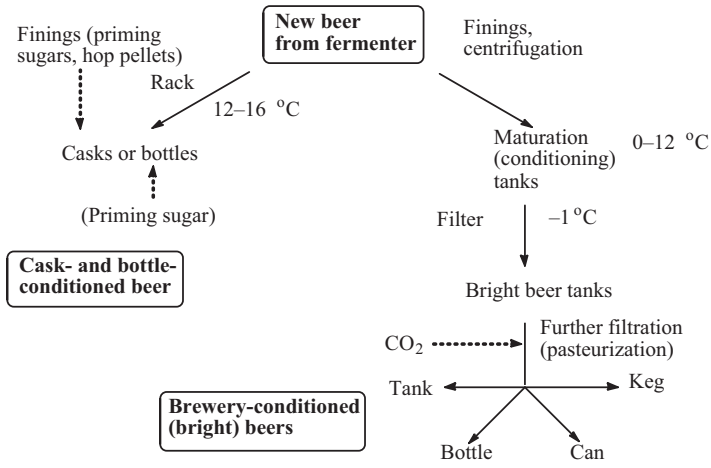


Figure 2.6.29 *Simplified schemes for packaging beer at the brewery*

2.6.10 Packaging of Beer: Casking, Kegging, Bottling and Canning

The beer treatments described in Section 2.6.9 are dependent upon the type of beer being produced, its desired shelf life and how it will be packaged and dispensed. This is summarized in Figure 2.6.29. The simplest, cheapest and most natural mode of packaging beer is bottle or cask conditioning, both most commonly used for top-fermented beers. Here, the beer is served directly from the container in which it has conditioned and matured. Bottle-conditioned beers, although less common than their brewery-conditioned and processed cousins, are available worldwide and include the darkest Irish and UK stouts and porters, through Belgian Gueuze beers, to the palest German Weizenbiere. Like bottle-conditioned beer, beer that is conditioning in cask is a living product: suspended yeast cells are responsible for secondary fermentation, so producing CO_2 that gives the beer its sparkle and foaming head when served. If the number of yeast cells falls below about 3×10^5 per ml, carbonation will be on the low side and the beer may be rather 'flat,' but if much above 4×10^6 per ml, carbonation may be too high and the beer may gush when the cask is tapped, especially at higher temperatures (say >16 °C). Breweries aim for the optimum, but many would argue that overcarbonation is better than undercarbonation, because the excess CO_2 pressure can at least be vented.

When the fermentation progress (see Figure 2.6.17) and level of suspended yeast cells are judged to be correct, casks are filled with beer either directly from the fermenter or from a racking back, using a counter pressure racking equipment to prevent frothing or 'fobbing.' Priming sugars (typically as a 34 °Plato syrup) can be added at 0.4–1.8 l/hl. Many cask beers are not primed, but the big majority are fined with isinglass suspension at 0.4–1.1 l/hl. Many are also dry hopped, often nowadays with pellets rather than cones at rates of 9–50 g/hl. Pellets and isomerized extracts, when used in dry hopping, greatly decrease the risk of clogging beer lines and the cleaning of returned empty casks is far easier at the brewery. The beer in cask may rest at the brewery for a number of days (up to over a week) before being dispatched to the trade, where it may have a week or so rest before being tapped. The maximum shelf life of cask beer is considered to be four weeks, but this depends on a number factors, including alcoholic strength and storage temperatures. Beer casks were once all wooden, but these require skill and care in cleaning and maintenance, especially if they are not in use for some time. They also have a tendency to leak, both through the pores of the wood and at the joints between the staves. Hence, many are lined with brewers' pitch; some lager at the Urquell Brewery

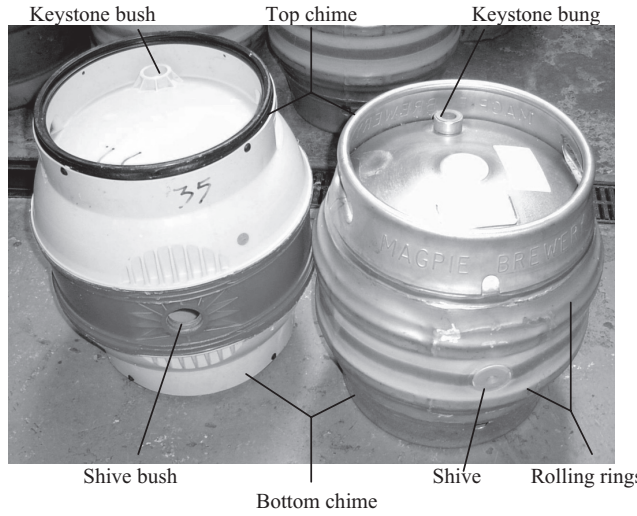


Figure 2.6.30 *Stainless steel and plastic casks at the Magpie Brewery, Nottingham, UK. Note the brewerycolour coding and embossed label*

in Plzeň (Pilsen) was until fairly recently stored for a time in large casks of this type. Even today, a number of UK breweries still supply the trade with some of their beers in wooden casks (e.g. Banks, Theakston and Wadworth), as do some brewers of Altbier and Kölsch in Germany. Wooden maturation vessels and other process vessels are still used for certain beers in a few breweries in the UK (e.g. at Greene King brewery for a strong ale), in Belgian Lambic breweries, such as Lindemans (Figure 2.6.18) (Section 2.6.7) and in a few traditional German breweries. However, the big majority of vats and casks these days are made of stainless steel, aluminium (for casks) or plastic (for casks). Figure 2.6.30 shows a stainless steel and a plastic cask, both of 41 l (9 gallon) capacity; cask sizes in the UK are still nonmetric. Important parts of the cask are also illustrated in Figure 2.6.30 – in particular, casks always have two holes, one for the outlet of beer and the other for the inlet of gas (either air or a blanketing gas: CO_2 and/or N_2). Since casks are regularly returned to the brewery for reuse, the cleaning and washing program is of prime importance. Cask washing systems come in many forms, depending on the size of the brewery and its cask beer output: larger concerns have automated, computer controlled cask washers. Washing cycles vary between breweries, but are normally comprised of an external wash, bung and shive removal, an ullage wash, a hot water rise, one or two detergent washes, one or two hot water rinses, a steam clean and finishing with manual or microbiological inspection (Hornsey, 1999c). Adequate drainage time must be allowed between washing stages and some of the water is recycled between stages to cut down waste. The water washes and rinses may involve water feeds at 6 bar pressure (∼90 l/min) at 90 °C, usually for 1 min each. The detergent wash feed may be at 6 bar at 70 °C and the steam feed is typically at 3 bar (∼80 kh/h). Older casks have often accumulated a deposit of 'beer stone' – basically calcium oxalate, which is derived from calcium salts in the liquor and oxalates from malt. This must be removed with either acidic detergents (based on phosphoric acid) or sequestering detergents.

Brewery-conditioned beer (often called bright or processed beer) is beer that has been matured, conditioned, fined, possibly centrifuged, filtered and possibly pasteurized in the brewery. It is thus microbiologically dead, since all microorganisms, including yeast, have either been removed or killed. Consequently, this type of beer (which represents the big majority of beers worldwide) must be packaged in sterile containers such as

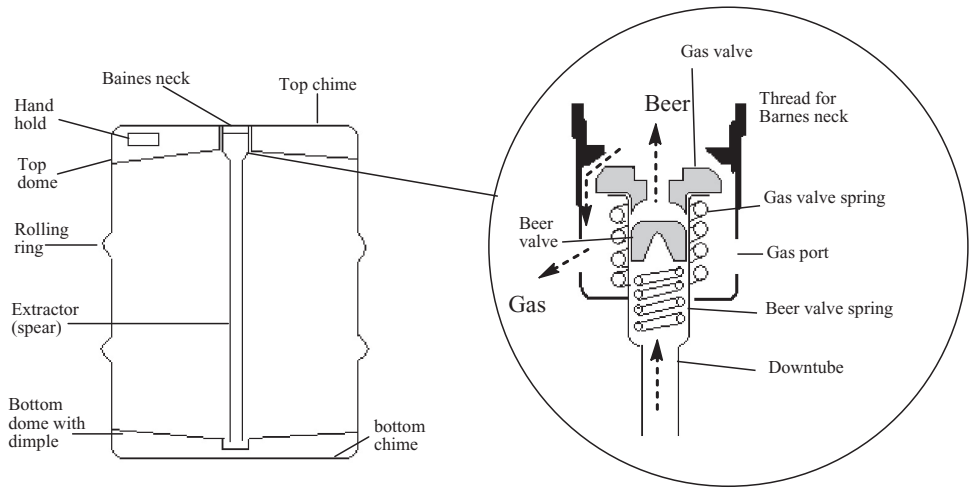


Figure 2.6.31 *Simplified section through a typical beer keg, showingamplification of extractor head, in dispense mode. In filling mode, the kegis inverted; beer enters by the gas ports and excess gas leaves by the downtube*

tanks, or more usually kegs, bottles and cans, where at least some artificial (and often much) gasification (CO_2 and/or N_2) will be used.

High quality traditional lagers, such as some from the Czech Republic, Slovakia and Germany, are likely to have spent many weeks undergoing secondary fermentation and maturation, at gradually diminishing temperatures. At the end of this period, cold break, chill haze and yeast precipitations will be almost complete, the beer will be very nearly bright and will require only a final gentle or polishing filtration before packaging. Some of these lagers are unpasteurized and some are fed (unfiltered) directly from their lagering tanks into casks or kegs for draught dispense, although nearly all are given some artificial carbonation. However, the majority of brewery-conditioned beers are highly filtered and pasteurized and never see the inside of a cask – they are put into kegs for draught dispensing.

Unlike casks, kegs have only a single hole (Figure 2.6.31), which is used for both filling with beer at the brewery and dispensing the beer at the point of sale. Kegs are usually made of aluminium (with oxidized or epoxy resin lined interior surfaces) or stainless steel and contain an extractor system (a 'spear') that screws into the 'Barnes neck.' This extractor system stays in place (more or less) during the keg's lifetime. When filling with beer (and during washing) the keg is inverted, whence beer enters the keg through the ports in the spear head and excess gas is voided through the extractor tube. When dispensing beer, the reverse applies (Figure 2.6.31). Like casks, kegs have to be thoroughly cleaned before refilling with beer, but most breweries carry out both cleaning and filling using a single automated computer controlled machine. These systems consist of two rotating carousels – one for washing and the other for filling – and kegs are passed from the former to the latter via a conveyor. These days, the whole operation uses automated depalletization (for incoming empty kegs) and palletization (for outgoing filled kegs) procedures. At the washing carousels, there are four manifolds: one for deullaging, one for detergent wash (with its attendant rinses and drainage $\sim$1 min per keg), one for steam sterilization (e.g. steam at 120 °C for 1 min) and one for counter pressuring with CO_2 and/or N_2. The last process purges out steam condensate and gives a counter pressure of gas that reduces fobbing when the casks are filled (at the next stage). The kegs are then conveyed to the filling carousel where they are filled with cold, bright carbonated beer (filtered and often pasteurized), slowly at first and then more

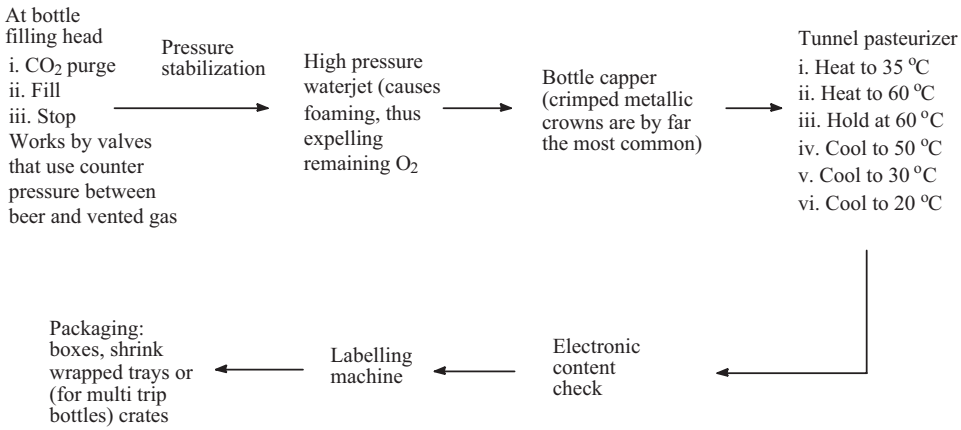


Figure 2.6.32 *Typical bottle filling scheme. This is focused on single trip bottles*

rapidly. Filled kegs are then given an external wash, dried, weighed and labeled. A modern washing/filling machine can deal with 1000 kegs/h.

Like kegging lines, bottling lines tend to be highly automated and computer controlled, but vary according to whether the bottles are returnable ('multitrip'), and hence need cleaning, or nonreturnable ('single trip'), in which case they essentially require filling only, since they arrive at the brewery in virtually sterile condition. A typical bottle filling operation is summarized in Figure 2.6.32 and a typical bottle washing programme is outlined in Figure 2.6.33. It will be noticed from Figure 2.6.33 that a very vigorous washing regime is essential, not only to ensure thorough internal cleansing, but also to remove old labels. It is easy to appreciate that single trip bottling lines are much easier to run than multitrip washing and bottling lines, there being no outlet and inlet end to coordinate.

Canning lines are similar in many ways to single trip bottling lines. Beer cans are constructed of either tin layered low carbon steel or aluminium (actually an aluminium–silicon–magnesium–manganese alloy) for greater strength. They arrive at the brewery (already printed with the brewery logo, etc.) in two parts: the (bottom) body, to be filled with beer, and the top (which contains the opening mechanism, nearly always of

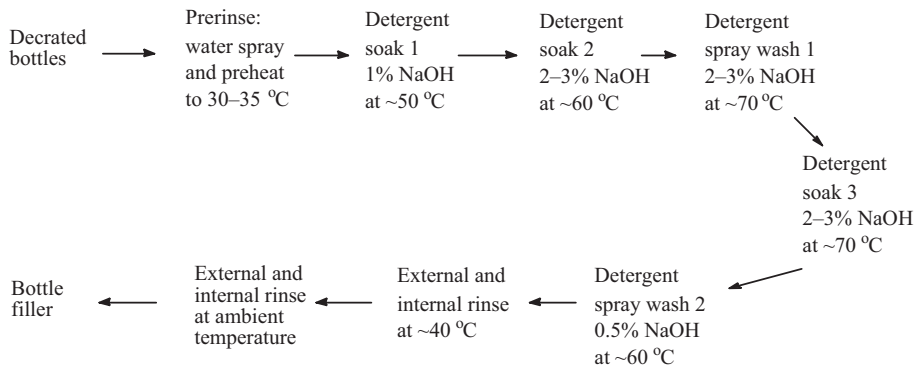


Figure 2.6.33 *Typical multitrip bottle washing regime. Adequate drainage times are allowed between each stage. Washings are often recycled to save water*

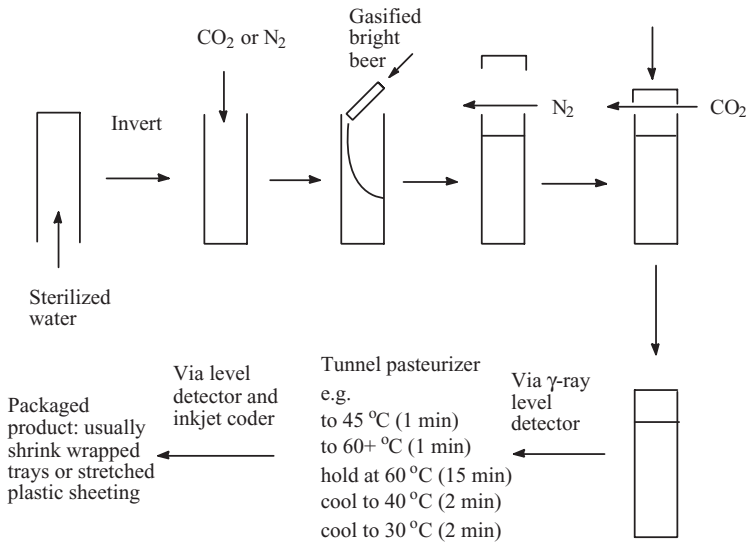


Figure 2.6.34 *Simplified scheme for typical brewery canning program*

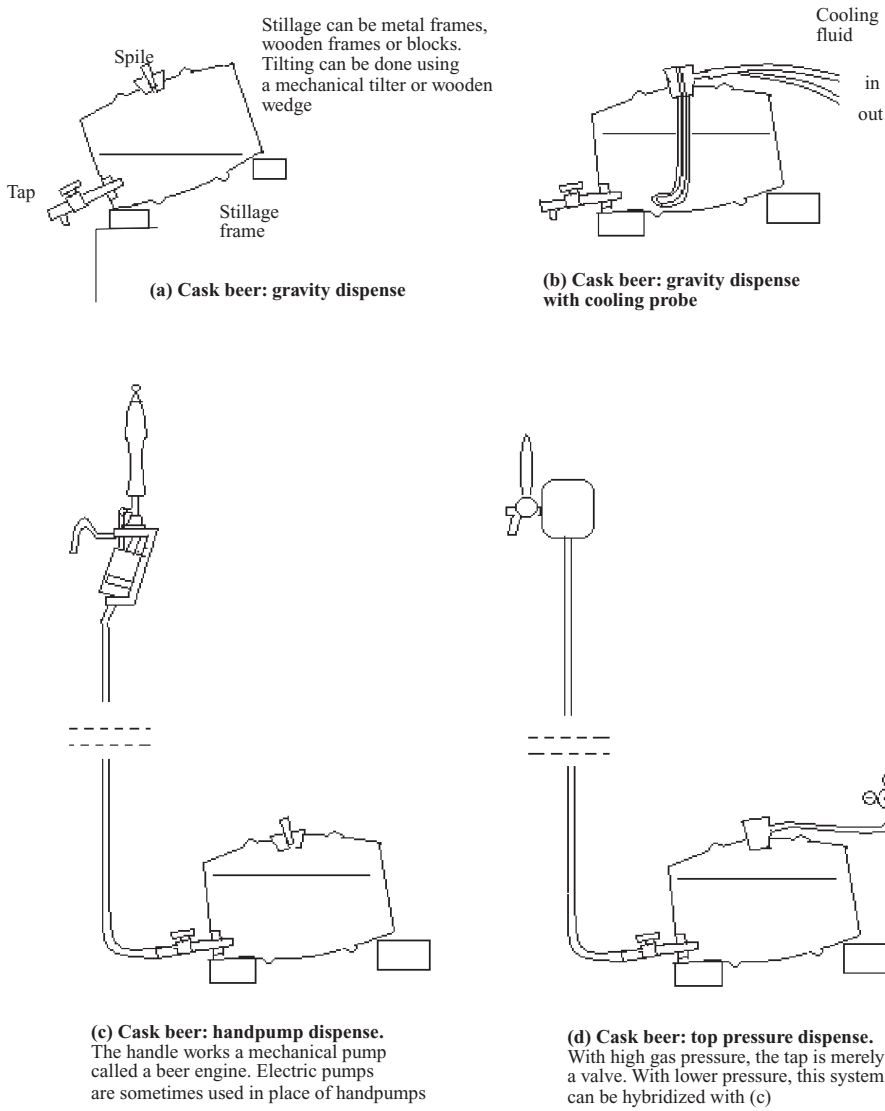
the 'ring pull' type), to be joined to the filled body at the brewery. A typical sequence of events in a brewery canning line is outlined in Figure 2.6.34. Canned beers, like bottled and kegged beers are supersaturated with gas (either CO_2 or a mixture of CO_2 and N_2 – often 60:40); that is, they contain more dissolved gases than is theoretically possible at room temperature and pressure. This is typically equivalent to 4.0–4.5 g/l of dissolved CO_2, or 2.5 volumes of CO_2. In recent years, interest has grown in the use of N_2 as both a gasification agent for canned, bottled and keg beers and as a dispensing gas for cask and keg beers (see Section 2.6.11). Not only is nitrogen much less soluble in beer than carbon dioxide, but it produces smaller bubbles which result in a compact, creamy head that resembles that of well conditioned cask beers, especially if a 'sparkler' is used on the dispensing tap (see Section 2.6.11). As far as can be ascertained, N_2 makes no contribution to beer taste, whereas it is generally agreed that high volumes of dissolved CO_2 give beer an acidic taste and prickly mouthfeel. In some canned beers (and a few bottled beers) the use of a plastic device known as a widget and dissolved N_2 (*ca.* 20 ppm) gives a thick, compact head resembling that of draught beer. Upon opening the can, the rapid shearing action of the widget (due to the suddenly reduced pressure) induces the formation of very small bubbles than are seen in normal canned beers, provided that the temperature is low enough ($< 5\ ^\circ$C), so that the maximum volume of N_2 is released from solution.

2.6.11 Dispensing of Draught Beers

This section deals mainly with the methods of dispensing various forms of draught beer, both of the cask-conditioned type and of the bright (keg or tank) type. Also discussed here is the behavior of gases (CO_2, N_2, O_2) and how they influence tank and bottle headspaces and beer dispensed by a top pressure system.

Figure 2.6.35 shows a numbers of ways in which it is possible to serve cask-conditioned beers, although in practice there are many other methods that are not illustrated here.

There can be no doubt that the simplest and most natural way of dispensing draught beer is by gravity, straight from the cask, without the use of external gas pressure (Figure 2.6.35(a)). Of course, this applies to



(a) Cask beer: gravity dispense

(b) Cask beer: gravity dispense with cooling probe

(c) Cask beer: handpump dispense. The handle works a mechanical pump called a beer engine. Electric pumps are sometimes used in place of handpumps

(d) Cask beer: top pressure dispense. With high gas pressure, the tap is merely a valve. With lower pressure, this system can be hybridized with (c)

Figure 2.6.35 *Methods for the dispense of cask conditioned beer. There are many variations on these themes. Spiles are removed when the beer is being served. Cask breathers, which supply CO_2 at atmospheric pressure, are sometimes used in place of spiles*

cask-conditioned beers, which generate their own CO_2 from secondary fermentation in the cask. As the cask is emptied, air is drawn through the spile or shive hole. Oxygen in the air is a potential enemy of beers, but initially it will be held above the blanket of CO_2 above the beer in cask. Soon, oxygen will either diffuse or circulate to the surface of the beers, where it will dissolve and begin to promote oxidation reactions. Many believe that a short contact with limited quantities of O_2 actually improves the flavor of cask-conditioned beer, and indeed, if the beer is all dispensed in one or two days, no deleterious effects of oxidation will be detected.

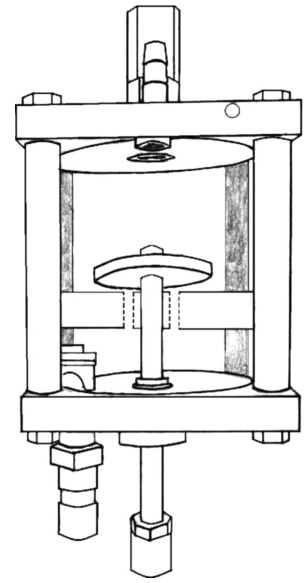


Figure 2.6.36 *Sketch of cylinder part of beer engine, with section through piston showing breather holes and piston shaft. Note floating top is raised as piston moves down, thus allowing beer to fill the cylinder*

The problems arise when turnover of the beer is slow or if the half empty cask has been stored overnight without a hard spile. Beer drawn by gravity is especially impressive if the casks are installed in public view in the bar area, but some kind of cooling system is required, like the probe illustrated in Figure 2.6.35(b). Much more likely, cask-conditioned beer will be drawn from casks that are stored in a cellar beneath the bar and this is a laborious job if done by gravity. For this reason, beer engines operated by (often ornate) hand pumps are a common feature of bars in the UK, especially England (Figure 2.6.36).

A beer engine consists of cylinder of borosilicate glass containing a plunger made of acetal, nylon or other inert polymer that is connected to the pulling handle by connecting rods and a pivot. Figure 2.6.36 shows the basic working parts of a beer engine – in particular the inlet valve, the plunger with 'breather' holes and the floating top. At the commencement of serving beer from a newly tapped cask, the pump handle is upright, the plunger is at the bottom of the cylinder and the inlet valve is closed. When the pump handle is pulled forward, the plunger moves up the cylinder, creating a vacuum below, which causes the inlet valve to open and fill the cylinder with beer. This stroke is often called the priming stroke. The pump handle is now forward and the cylinder is full of beer.

The handle is next returned to the upright position. On doing so, the plunger moves down the cylinder without much resistance because the downward motion raises the floating top, revealing four 'breather' holes, through which the beer easily flows. The plunger is now at the bottom of the cylinder with beer above it. Upon the second forward pull of the handle (and on all subsequent forward pulls, called dispensing strokes) the plunger moves up the cylinder and the force of the beer above keeps the floating top tight against the breather holes, thus pushing the beer out of the cylinder, through the beer tap into a glass. At the same time, a vacuum is once again created in the cylinder below the plunger, opening the inlet valve, through which more beer flows. With the handle now forward and a measure of beer in the glass, the plunger is at the top of the cylinder, which is again full of beer. On returning the handle to the upright position, the sequence of events described above is repeated, ready for the next dispensing stroke.

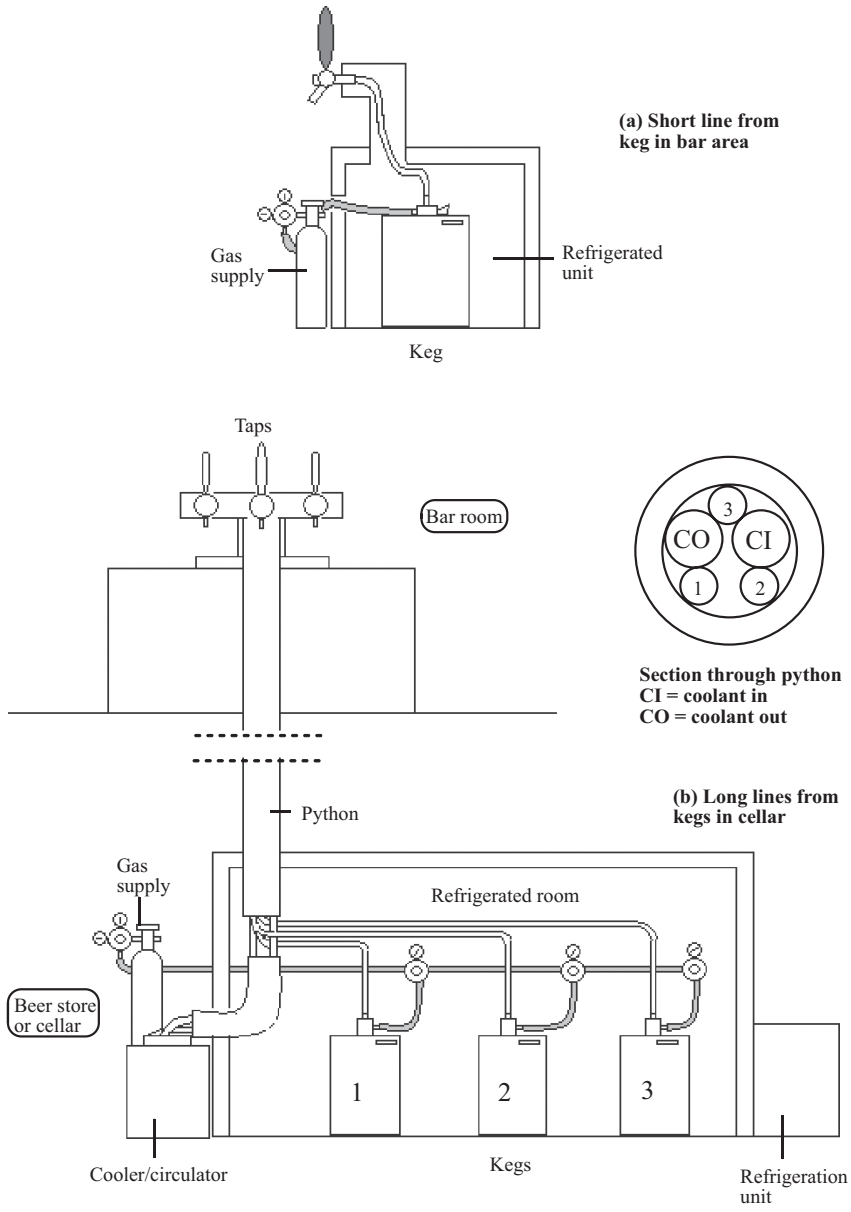


Figure 2.6.37 *Dispensing keg beer. Not drawn to scale*

The measure of dispense from a single pull depends on the cylinder volume, but is usually $^1/_2$ pint. The cellar temperature can be controlled (at, say 10–15 °C) by an air conditioning unit, and if several casks are being used at any one time on a regular basis, the beer lines can be bound up with ethylene glycol chill lines in an insulating plastic sheath (the whole thing is called a 'python'), leading to individual hand pumps. This ensures that the beer is served close to a predetermined 'cellar temperature.'

A similar arrangement (but at lower temperatures) is used for keg beers (see Figure 2.6.37).

Table 2.6.5 *Carbon dioxide headspace pressure (atm) as a function of the concentration of dissolved gas (volume of CO_2 per volume of beer) and temperature*

Temp/°C \ Vol. CO_2	1.4	1.8	2.0	2.5	3.0
4	–	0.25	0.45	0.81	1.17
6	0.08	0.38	0.61	0.96	1.34
10	0.25	0.61	0.81	1.34	1.73
12	0.33	0.67	0.93	1.40	1.96

Beer served from a cask using extraneous CO_2 (or other) gas pressure from a cylinder to push the beer to a tap on the bar counter above the cellar, is known as top pressure beer (Figure 2.6.36(d)). Beer that is conveyed from the cask using a beer engine and gas is used merely to re-establish equilibrium pressure within the cask is known as blanket pressure beer. In both top pressure and blanket pressure, it is difficult to prevent extraneous uptake of gas by the beer, especially if CO_2 is used.

Keg beers are served using CO_2 (or a CO_2/N_2 mixture) from cylinders, as shown in Figure 2.6.37(a) (short lines) and Figure 2.6.37(b) (long lines). Generally these beers are served at 4–6 °C, as opposed to the normal 10–15 °C for cask beers. The amount of dissolved CO_2 in a beer is quoted in units of volume (vol), where/volume = 1 liter of CO_2 per liter of beer. Cask-conditioned beers contain 1.0–1.5 vol, keg beers 2.2–2.8 vol and bottled beers 2.4–3.0 vol of CO_2.

The relationship between quantity of CO_2 in the headspace above a beer (as given by its partial pressure, P) is proportional to the concentration of CO_2 in the beer, provided that equilibrium conditions are in operation. This is Henry's Law and is best expressed by Equation 2.6.5.

$$P(CO_2) = kX(CO_2) \tag{2.6.5}$$

Henry's Law constant, k, is temperature dependent and X is the mole fraction of CO_2 in the beer (= number of moles of CO_2/total number of moles). The relationships between the volume of dissolved CO_2 and the corresponding headspace gas pressure at different temperatures are summarized in Table 2.6.5.

Two other laws that are useful in calculations involving pressure of CO_2 and other gases in both beer and the headspace above beer are Dalton's Law of partial pressures and the ideal gas law (Equations 2.6.6 and 2.6.7, respectively).

$$P(\text{total}) = P(CO_2) + P(N_2) + P(O_2) \tag{2.6.6}$$

$$P(CO_2)V = n(CO_2)RT \tag{2.6.7}$$

Dalton's Law states that the total pressure in a headspace is the sum of the partial pressures of the gases present. Equation 2.6.6 relates, say, to a bottled beer with some air in the headspace. The ideal gas law relates pressure P (in atm), volume V (in liters), temperature T (in K which is defined as °C + 273.15) and molar quantity (n) (= mass/molar mass) of an ideal gas. The constant R is the gas constant, and with the units for P, V, T and n described above, it has the value 0.0802 l atm/mol/K. Under the conditions involved in contained beer (tank, bottle, cask or keg), the gases (CO_2, N_2 mainly) approximate to ideal gases. For carbon

dioxide, where the density at standard temperature and pressure (273 K, 1 atm) is 1.96g/l and molar mass is 44.01 g/mol

$$\begin{aligned}\frac{n(\text{mol})}{V(\text{l})} &= \text{vol}\left(\frac{\text{density(g/l)}}{\text{molarmass(g/mol}}\right)\\ &= \frac{\text{vol}}{22.45(\text{l/mol})}\\ \text{or vol} &= 22.45(\text{l/mol})\left(\frac{n(\text{mol})}{V(\text{l})}\right)\\ &= 22.45(\text{l/mol})\left(\frac{P(\text{atm})}{R(\text{latm/mol/K})T(\text{K})}\right)\end{aligned} \tag{2.6.8}$$

These laws can be used in various calculations involving gases, taking into consideration a number of factors that have been determined as a result of experience. The first example involves carbonation of a storage tank of 1500 l capacity, containing 1200 l of beer so that CO_2 content of the beer is 2.5 vol. Firstly, the 20% headspace in this example is typical. Secondly, it can be assured that 0.2 vol of CO_2 will be lost in processing, so carbonation should be conducted to 2.7 vol. Now, the calculation of the mass of CO_2 to be added can be started.

At equilibrium, 2.5 vol of CO_2
$= 2.5$ (l/l) $\times$ 1.96 g/l
$= 4.9$ g/l
or 5.88 kg in 1200 l

If the tank temperature is 4 °C, then from Table 2.6.5 (based upon Henry's Law), this will require 0.81 atm of headspace CO_2 pressure for equilibium.

Next, the volume of CO_2 in the tank can be calculated using Equation 2.6.8.

$$\begin{aligned}\text{i.e.vol} &= 22.45\left(\frac{P}{RT}\right)\\ &= \frac{22.45 \times 0.81}{0.0821 \times 277}\\ &= 0.80\text{vol}\\ &= 1.57 \text{ g/l or } 1.88 \text{ kg in } 1200 \text{ l}\end{aligned}$$

Hence the total mass of CO_2 needed is $5.88 + 1.88 = 7.76$ kg.

The second example involves the dispensing pressure. Three pressures need to be considered, as shown in Equation 2.6.9.

$$P_{\text{eff}} = P_{\text{E}} - P_{\text{H}} - P_{\text{LR}} \tag{2.6.9}$$

P_{eff} is the effective pressure. When P_{eff} is zero, there will be no beer flow and $P_E = P_H + P_{LR}$. In practice, P_E, the equilibrium pressure, is chosen to be 0.14 atm higher than that calculated from Equation 2.6.9, to give a flow rate of 4.5–-5.5 l/min. P_E is essentially the pressure required (via CO_2 cylinder) to preserve the beer's CO_2 contact during serving. P_H is the gravity head, the pressure needed to push the beer against gravity from tank or keg to tap. A widely used value is 0.11 atm/m (height).

Finally, P_{LR} is the line resistance, the pressure needed to overcome resistance (friction, capillary attraction, etc.) in the beer lin;, it is taken as 0.022 atm for 9.5 mm i.d. (internal diameter) tubing, 0.134 atm for 6.4 mm i.d. tubing and 0.49 atm for 4.8 mm i.d. tubing.

Suppose beer with 2.5 vol of CO_2 is to be dispensed at 4 °C to a tap 5 m above the keg or tank, via a 9.5 mm i.d. tube. From Table 2.6.5, 2.5 vol of CO_2 at 4 °C is equivalent to 0.81 atm of headspace gas (= P_E). $P_H = 0.11 \times 5 = 0.55$ atm. Now, $P_{LR} = P_E - P_H = 0.81 - 0.55 = 0.26$ atm. The length of tubing thus required is given by $0.26/0.022 = 11.8$ m, rather longer than the 5 m of height. Thus, a better choice might be tubing of 8.0 mm i,d. for this particular example. The dispensing pressure would actually be $0.81 + 0.14 = 0.95$ atm, as noted previously.

The use of N_2 or an N_2/CO_2 mixture for top pressure beer dispensing has been gaining popularity for a number of years now. The main aim is to have sufficient CO_2 to balance the equilibrium pressure of the beer, so that the N_2 pressure overcomes the gravity head and line resistance. Typically, a 75% N_2, 25% CO_2 mix may be used with a beer kept at 4 °C, with a total headspace pressure of 1.11 atm (∞ 0.84 atm N_2 and 0.28 atm CO_2, from Dalton's Law). The partial pressures of the gases in the bubble are much closer to atmospheric partial pressures ($\sim$0 for CO_2, $\sim$0.8 atm for N_2), than in a bubble of CO_2 from a 2.5 vol beer (at 4 °C) : 0.81 atm in the bubble and $\sim$0 in the atmosphere. Gas loss (by collapse of the bubble) is thus less for N_2/CO_2 bubbles, leading to a tighter, more stable foam.

2.6.12 Oxidation and the Staling of Beer

Many beers are brewed for distribution to many points of trade (often at significant distances from the brewery) and are required to have long shelf lives – that is, they should be able to survive in peak condition for a considerable time, so that at the point of consumption they are still lively and 'fresh,' with no 'stale' odors or flavors. This applies most strongly to brewery-conditioned beers that are destined for keg, bottle or can. These beers have undergone extensive brewery processing, particularly with regard to filtration and in many cases pasteurization. Generally, keg beers are expected to last about four months in peak condition, whereas bottled and canned beers are often given shelf lives of over a year. However, sooner or later the flavors of these beers gradually change (in a sequence of steps or phases) from typical fresh profiles to what is perceived as stale. The process is called staling and arises largely as the result of oxidation of certain beer constituents by molecular oxygen or by various oxidized species that have been formed at various stages of the brewing procedure. Superimposed on this are various oxygen independent aging processes, such as the formation of heterocyclic compounds (via the Maillard reaction), carbonyl compounds and esters, and other reactions that can lead to undesirable flavors in aged beer (Kuchel *et al.*, 2006; Vanderhaegen *et al.*, 2006). Depending on the beer and stage or phase of staling, the flavors have been described variously as 'currant,' 'metallic,' 'paper' and 'cardboard,' and are often linked to an increase in astringent or bitter taste and also sometimes to permanent haze formation.

Many breweries operate a 'minimum oxygen' regime for the brewing of brewery-conditioned beers. Here, oxygen uptake is minimized in both the prefermentation stage (mashing and boiling/cooling), called 'hot side aeration' and also from fermentation onwards, called 'cold side aeration.' A 'zero oxygen' process is impossible, but a minimization program should at least ensure that the packaged beer undergoes staling later, rather than sooner. The steps that can be taken include gentle 'mashing in' (with minimum

splashing), mashing in an enclosed vessel, mash filtration, proper separation of hot and cold trub and removal of air from the headspace of the packaged beers, To this we can add the choice of malt with low lipid levels.

Classical beer production involves either aeration of wort or yeast preoxygenation, since oxygen is required for yeast biosynthesis of unsaturated fatty acids and ergosterol for initial growth and subsequent good fermentation performance. It is widely believed that wort aeration, although a helpful prefermentation process, may ultimately be detrimental to beer quality, because of more rapid beer staling. However, recent work has demonstrated that normal aeration (8 mg/l) of both ale and lager worts did not determine flavor stability in a direct way (Depraetere *et al.*, 2008). No significant differences were found in either the natural or forced aged beers produced from aerated worts or by use of preoxygenated yeast. On the other hand, it has been found that beers produced in a working brewery from nonaerated wort and yeast that had been mixed with olive oil during storage had greater flavor stability over those brewed by the traditional wort aeration method (Hull, 2007). These beers had similar flavor, but with higher ester (fruity) profiles, and similar foam retention to the traditional beers.

Staling of a bottled beer may occur prematurely, despite all reasonable efforts to minimize oxidation during the brewing and subsequent processes, as described above. This is often caused by oxygen ingress through the stopper of conventional glass bottles or through the fabric of polyethylene terephthalate (PET) bottles. So-called 'crown corks' have been used on bottled beers for decades. They are crimped metal caps that originally had cork sealing liner material, but nowadays this has been replaced synthetic polymer materials, sometimes incorporating a chemical oxygen scavenger. Also, many modern crown stoppers are also designed as screw stoppers, being fixed to bottles that have a few grooves engraved in the glass at the top of the bottle neck. The drinker has the option of levering off the stopper (as per conventional crown stopper), but he or she is much more likely to unscrew it. It is the perceived lack of a reliable barrier to oxygen ingress that has resulted in several brewers in the USA, led by Sierra Nevada Brewing Co. (Chico, California), to switch from the modern kind of crown stopper to a much more dense stopper that has to be levered off. This is sometimes known (rather grandly) as barrier crown technology.

PET, having been used for some time to produce fruit juice, soda and other bottles is becoming an important bottle material for beers, despite earlier problems of premature staling and loss of condition as a result of both oxygen ingress and carbon dioxide egress. Nowadays, PET beer bottles are manufactured with multilayer, coated or monolayer structures, an oxygen absorbing or blocking substance being used in each case. In the former, at least one layer of oxygen barrier/scavenger, often a nylon nanocomposite (e.g. clay or H_2 rich carbon) is sandwiched between inner and outer layers of PET. The second type involves the coating of a super-thin layer of barrier material to one surface of a monolayer PET bottle. In the third option, now being developed, the oxygen absorber or barrier material is incorporated into the body of the single layer of PET material. This method does away with the need for multilayer or coating equipment, but is not yet widely feasible because of the high cost of materials.

The brewing of cask-conditioned beers does not require such elaborate precautions against oxygen absorption, since these beers are normally consumed within a few weeks, and the presence of yeast has a number of stabilizing effects, provided consumption occurs within one or two days once the cask is breached. Even so, the brewer will be careful not to expose the wort and subsequent beers to unnecessary amounts of air. An exception to this can be found in lambic beers (Section 2.6.7), where parts of the brewing and maturation processes deliberately promote a certain amount of oxidation.

Oxidations of wort or beer components can be divided into two broad groups – those that occur in the absence of enzymes (nonenzymic oxidations) and those that require enzyme catalysis (enzymic oxidations). These categories can be further subdivided according to whether molecular oxygen is involved or not. With this in mind, some typical oxidations that cause staling of beer are summarized in Figure 2.6.38. Oxidation of iso-α-acid side chains by headspace oxygen (Figure 2.6.38(a)) leads to a change in hop aroma and is

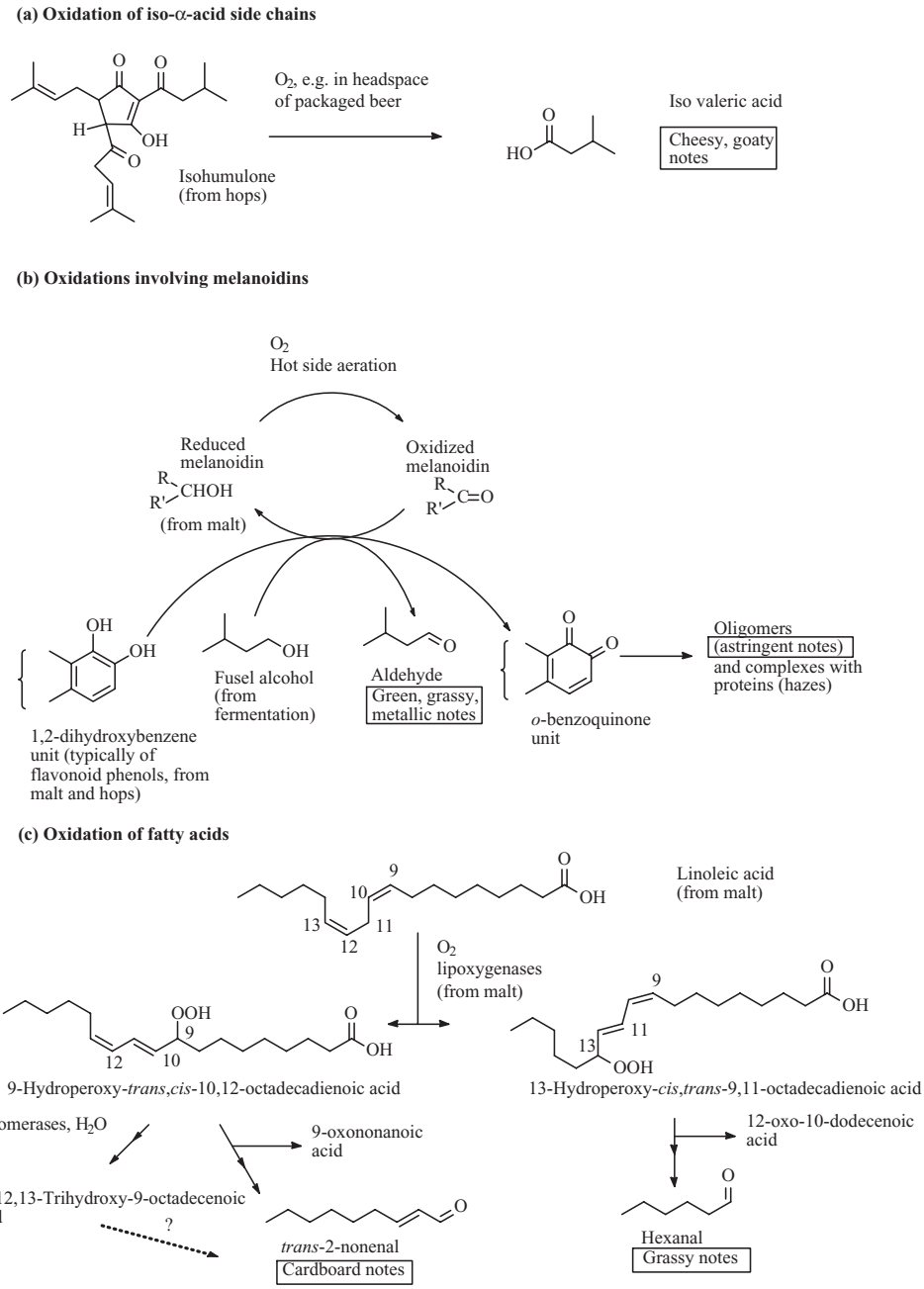


Figure 2.6.38 *Some oxidation reactions that cause staling of packaged beer*

generally taken to herald the onset of staling. Melanoidins appear to play a key role in staling oxidations (Figure 2.6.38(b)). These are produced in parts of the brewing process that involve higher temperatures: kilning, mashing (Section 2.6.2) and boiling (Section 2.6.3). Oxidation of melanoidins seems to require higher temperatures, so it is thought it occurs only during hot side aeration. The oxidized melanoidins are oxidizing agents that are capable of oxidizing fatty acids (not shown), fusel alcohols and polyphenols. In the latter case 1,2-hydroxybenzene units are oxidized to 1,2-benzoquinone units; these products are not only astringent, but cause permanent hazes by forming stable complexes with proteins (see Section 2.6.9 for enzyme catalyzed oxidation of phenols). On the positive side, dark beers with high (reduced) melanoidin content and that have been brewed with low hot side aeration are generally more resistant to staling compared with pale beers, because of the protecting (antioxidant) effect of the melanoidins. Oxidation of unsaturated fatty acids, from malt lipids, can lead to *trans*-2-nonenal (Figure 2.6.38(c)) and other aldehydes (C6 and higher). Excess lipids in the beer may also partly be the cause of the presence of Strecker aldehydes (Section 2.6.2, Figure 2.6.6) in staling beer. It has been shown that lipid oxidation products, like 4,5-epoxy-2-alkenols are able to form Strecker aldehydes (such as phenylacetaldehyde) from amino acids (such as phenylalanine) in model systems (Hidalgo *et al.*, 2005). *Trans*-2-nonenal is regarded by many as the most serious staling agent of all, because of its low odor threshold value (OTV; see Section 4.7.3) of 0.1 mg/l. This means it will make a marked organoleptic impact (cardboard notes) even though it is produced in very small quantities. Probably the best way of minimizing formation of *trans*-2-nonenal is to curtail the amount of lipids and fatty acid oxidizing enzymes (lipoxygenases) that get into the wort. Choice of low lipid malts, use of more highly kilned malts (with less lipoxygenase content), mash filtration, and good hot and cold trub separation are all reckoned to be beneficial in this respect, especially if coupled with a minimum oxygen program, because it is thought that *trans*-2-nonenal may also be formed by the aldol condensation of smaller aldehydes that probably originate from oxidation of alcohols formed during fermentation.

Recently, Saison *et al.* (2010) have shown that it is possible to remove aged aromas (e.g. black-currant (Ribes), cardboard, Madeira, Maillard notes) by refermenting the aged beer with fresh brewing yeast. During the new fermentation, certain molecules responsible for the offending aromas (diacetyl, 5-hydroxymethylfurfural, (*E*)-2-nonenal and Strecker aldehydes) are reduced to odorless products by yeast metabolic processes. Although some malodorous compounds, such as 2-furfuryl ethyl ether (and other ethers) are not affected and aldehydes are not entirely reduced by fermenting yeast (and indeed, other aldehydes are formed during fermentation), several aged aromas disappeared entirely as a result of refermentation. There may be a parallel here with bottle-conditioned beers, where the beer is in contact with its yeast for the whole of its 'shelf life.' The reducing ability of yeast may offer the beer some protection against oxygen or other species that promote beer aging. Certainly, it is common for bottle-conditioned beers to have a considerably longer shelf life than filtered (and maybe pasteurized) carbonated beers.

It has long been known that the presence of metal ions (especially Fe^{2+}/Fe^{3+} and Cu^{2+}) in beer promotes staling via the production of hydroxyl radicals from the Fenton and Haber–Weiss reactions (Figure 2.6.39), the hydrogen peroxide having been formed from molecular oxygen in packaged beer by a variety of reactions (Uchida and Ono, 1996). It is thought that hydroxyl radicals are the most potent producers of flavor changes in staling beer (Bamforth and Parsons, 1985). The origins of these metal ions in beer include raw materials, brewing equipment and filtration aids, such as diatomaceous earth, although it is known that their general levels fall during the brewing process, so that maturing beers often have only 50% of the wort concentrations of these ions (Zufall and Tyrell, 2008). More recently, it has been demonstrated that Mn^{2+} contributes significantly to beer staling both by forming hydroxyl radicals from H_2O_2 and by forming unsaturated fatty acid radicals directly (Figure 2.6.39) (Zufall and Tyrell, 2008). The Mn^{2+} ions emanate from brewing cereals and persist to a greater extent than either Fe^{2+} or Cu^{2+} throughout the brewing process, so that maturing beer has concentrations of Mn^{2+} typically three and five times those of Fe^{2+} and Cu^{2+}, respectively.

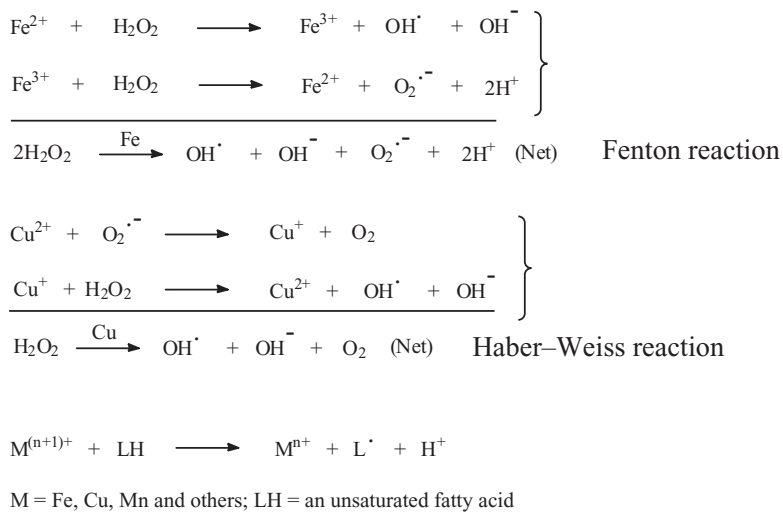


Figure 2.6.39 *Metal ions in the generation of radical intermediates involved in beer staling. Based on Zufall and Tyrell (2008)*

2.6.13 Beer styles

This section gives a description of both the more common and the more specialist styles of beer, in terms of original gravities, % ABV, color, flavor and other characteristics that are related to technical aspects of their production. It should be noted that in several cases there is overlap between styles, and divisions between certain beer types can be rather arbitrary. For example, where do strong ales end and barley wines begin, with respect to, say, % ABV? For some styles a short history is given and in most cases, notable examples are quoted. The styles are discussed in alphabetical order. Light (or lite) and diet (or diät) beers are discussed in Section 5.7.2.

Abbey Beers

This is a name that is reserved for beers brewed in abbeys, or by outside breweries by arrangement with the abbeys, principally in Belgium, but also in Holland and northern France. They are nearly all top fermented (ales), but the best known (and considered by many to be the best) are those brewed in Cistercian Trappist monasteries. According to EU law, only the five Trappist abbeys that still brew on the premises are allowed to use the description 'Trappiste' for their beers. These are Chimay (the original), Orval, Rochefort, Westvleteren (all near the French border) and Westmalle (near the Dutch border). Other abbeys, of the same or different religious orders, have licensing arrangements with outside breweries to brew beers bearing their names. Examples of these are Leffe (probably the best known and owned by the giant Anheuser-Busch InBev group), Floreffe, St. Sixtus and St. Feuillon (all Belgian). Dutch and northern French examples include Raaf and Rimaux, respectively. The Trappiste beers of Belgium are commonly regarded as masterpieces of the brewing art. They vary in alcoholic strength from 6 % to10 % ABV. Likewise the color varies between full gold/amber (∼6/10 °SRM) through copper to full brown (∼20 °SRM). The color is no guide to alcoholic strength (e.g. Westmalle's 6 % ABV beer is darker than its 8% ABV beer), instead alcoholic strength is indicated by the use of colored bottle caps (e.g. Chimay red (6 % ABV), white (7% ABV) and blue (8% ABV)) or by the

words 'Dubbel' and 'Triple' (e.g. for the two stronger Westmalle beers). Although certain Abbey beers are available on draught at or near the abbey, they are best known as their bottle-conditioned versions.

Altbier

This top-fermented beer is found on draught or in bottle in certain cities and towns of the Niederrhein/Westfalen area of Germany. The principle centres are Düsseldorf, Issum, Mönchengladbach and Münster. The term 'Alt' refers to the style of beer that existed everywhere in Germany before beers of the Pilsener type became almost ubiquitous. They are normally copper or pale brown beers (color: 13–20 °SRM) with a creamy white head and are brewed from pale malts, with caramel and other roast malts (but see Pinkus Müller Alt, below). The hop rate is usually rather light (~25 IBU) and alcoholic strengths are around 4.5% by volume. Notable examples include the beers of Düsseldorf old city (Altstadt) home breweries of Schumacher and Uerige. The larger producers include Diebels (Issum) and Hannen (Mönchengladbach). Pinkus Müller Alt (Münster) is much paler than the norm, being produced from Pilsener malt and, until recently, wheat malts. Altbier is given at least four weeks cold maturation (3–5°C) after about a week's primary fermentation. They are usually given a light filtration, although Diebels Alt is also pasteurized.

Barley Wine

This style is basically English in origin and is probably derived from the special strength March and October beers of the eighteenth and earlier centuries. They are top-fermented beers of between 8 % and 12 % ABV, the original gravities of the worts often being over 1100 (24 °P). In color, they range from full gold (Whitbread Gold Label and Harvey's Elizabethan Ale) to mid brown (Robinson's Old Tom). They are often highly hopped (~70/75 IBU) to balance the maltiness and high alcohol content. Many microbreweries and smaller breweries outside the UK have taken to brewing very good versions of the style: Nils Oscar Barley Wine (Sweden), van Steenberg Dragon (Belgium), Monster Ale (Booklyn Brewery, USA), Bigfoot Ale (Sierra Nevada Brewing Co, USA), Old Crustacean (Rogue Brewing Co, USA), St-Ambroise Vintage Ale (McAuslan Brewing Co., Canada) and Old Foghorn (Anchor Brewing Co, USA) are just a few examples.

Bière de Garde

A translation of this phrase is beer for keeping (or laying down). It should refer to bottle-conditioned (= sur lie) top-fermented beer of 4.5–7.5 % ABV from small breweries in northern France. Unfortunately, the same name is sometimes given to a number of pasteurized, artificially carbonated bottom-fermented beers. Color varies from pale gold (blonde: ~6 °SRM) through full gold (amber: ~9 °SRM) to brown (brune: >15 °SRM). Prime examples are produced by the breweries of d'Annoeullin, Castelaine, La Choulette (who give their beers rousing revolutionary names, like 'Sans Culotte' and 'Robespierre'), Duyck (famous for Jenlain beer), Monceau St.-Waast, St. Arnold, St. Leonard and Terken.

Bitter

This is Great Britain's basic draught beer (although there are bottled versions); it is top-fermented and many regard it as the draught version of pale ale. Indeed, some are known as pale ale or India pale ale (IPA) (often erroneously – see Pale Ale and India Pale Ale). Nowadays, bitter beers vary in color from mid gold/light amber (e.g. Boddington's Bitter, Holden's Golden Glow, Magpie Early Bird and Young's Bitter of color ~6–10 °SRM) to mid brown (e.g. Jennings Bitter and Nethergate Suffolk of color ~15 °SRM). However, the most common color range is from light amber to copper (e.g. Fuller's London Pride, Jennings Cumbria,

Blacksheep Bitter, Belhaven Best, Belhaven 80/- and Holden's Bitter). Alcoholic strength varies from just over 3.5% to well over 5% by volume. Likewise, malt character and hop bitterness show a wide variation, the latter generally being anywhere between 20 and 40 IBUs. Dry hopped versions have fine, spicy aromas. As far as alcoholic strength and drinkability (i.e. quantity) go, bitter beers can be divided into three categories: session (ordinary) bitter ($<$ 4% ABV), premium (best) bitter ($\sim$4.5% ABV) and strong (special) ($>$5% ABV). Outside Great Britain and Ireland, there are fine beers of this style, most notably in Australia (Cooper's Sparking Ale), New Zealand (Galbraith's Bitter and Twisted) and Belgium (de Koninck of Antwerp). An outstanding example from the USA (where the style is becoming popular with some microbreweries) is Firestone Walker's Double Barrel, which is brewed using an oak cask union system, similar but not identical to the old Burton Union system (Section 2.6.5). It should be mentioned that bitter as a style of draught beer has its origins in the early twentieth century; previously mild ale and old ale were the main draught styles in England.

Bock, Doppelbock and Related Styles

These are strong bottom-fermented beers that were originally a German speciality, but are now much imitated throughout the world. Bock and Doppelbock beers must have in excess of 6.3% ABV and 7.5% ABV, respectively. Bock beer colors range from pale gold ('blonder Bock') through full gold/amber ('heller Bock') to brown ('dunkler Bock'). The prefix Ur-, or the word 'Urtyp'are often used in the names of Bock beers, to signify purity and originality.

Some noteworthy Bock beers from Germany include Einbecker Urbock Dunkel, Forschungs Blonder Bock and Spaten Franziskus Heller Bock. It is possible that the word 'Bock' in derived from Einbeck, a town in Lower Saxony (Niedersachsen), where the Einbecker brewery is thought to have first brewed this style of beer. Bock style beers are brewed all over the world, some good examples being those from Lindeboom brewery (Holland), Faxe Brewery (Denmark) and Great Lakes Brewing Co. (USA).

Maibockbier is basically a pale Bockbier brewed in the spring. Well respected examples come from Ayinger Brauerei, Einbecker Braühaus and the Hofbraühaus (München). Doppelbock is an even more revered style of beer than Bock. The names of German Doppelbock beers usually end in -ator, as in Celebrator (Ayinger Brauerei), Delicator (Hofbraühaus), Kulminator (EKU) and Salvator (Paulaner Brauerei). Strictly, Kulminator of EKU belongs to a smaller subgroup of Doppelbock, called Eisbock, because of the freezing process that is used to increase its alcoholic content. At 13.5% ABV, it is the second strongest beer in the world. The accolade of strongest beer goes to the Utopia beers (21–25% ABV), produced by the Samuel Adams Brewing Co. (USA).

Brown Ale

Although the color of many beers may be described as 'brown,' only top-fermented beers that use the word 'brown' (in whatever language) in their names should be included in this style. In Britain, brown ale can be considered as the bottled version of mild ale, with generally low alcohol content (typically $<$ 4.0% ABV), mild hop bitterness ($<$ 24 IBUs), relatively high residual sugar ($>$3.0 $^{\circ}$P) and color in excess of 15 SRM. This was not always the case and there are exceptions today: Newcastle Brown Ale is a dry, copper-colored beer of 4.7% ABV. The Brown Ales of Belgium (Bruinnen) are classics of the style: many are over 6.0% ABV and are bottle conditioned. Probably the best known (and very highly regarded) are the Oudenaarde brown ales of the Liefmans and Roman breweries, and also that of the Crombé brewery in Zottegem. The 'brune' versions of bière de garde from across the border in northern France, are not dissimilar in style. There are

notable American brown ales, from such breweries as Brooklyn Brewing Co. and Great Lakes Brewing Co. (Cleveland Brown Ale). These tend to be stronger and hoppier than their UK equivalents.

Cream Ale

This is an American style of beer that was introduced by ale brewers as an attempt to counter the growing popularity of Pilsener style beers in the late nineteenth and early twentieth centuries. Modern cream ales are still mostly top fermented, but like Kölsch, they undergo cold maturation after primary fermentation. Alcohol content is around 5% by volume, color pale-medium gold (3–5 °SRM), with light hoppiness (<20 IBU). Examples of the style include Genesee (High Falls Brewing), Dave's Original (Molson) and Whitetail (Wisconsin Brewing).

Flavored Beers

This rather broad title covers a wide range of very different beers that have been flavored at the brewery with ingredients other than or as well as hops. Flavoring can be carried out at the boiling stage, but is more likely performed during the fermentation or maturation/conditioning period. Ingredients include whole fruits (or concentrates), beans and pods, spices, leaves, needles and oak. Probably the best known flavored beers are the fruit beers of Belgium, especially cherry ales ('krieken') from breweries such as Liefmans in Oudenaarde. Brown ale from the previous year is infused with fresh whole cherries and undergoes a gentle fermentation for a year, whence it is bottled and maturated further before sale. See also Lambic Beers for description of krieken-lambic. Other fruits, such as raspberries (framboise) or peaches are sometimes used as flavorings. On a bigger scale, Coors Brewing Co. (USA) produces Blue Moon Pumpkin Ale in the autumn, for consumption during the winter months. Citrus peel (zest), especially of orange, is a common beer flavoring ingredient, but is often used alongside spices, such as coriander, cardamom and cloves. A prime example is Hoegaardse wit bier (see Wheat Beers). Coriander is a frequently used spice for flavoring beer, as in the Umbel Ale of Nethergate Brewery (UK). A range of strongly flavored spices is used in beers such as Birrificio Troll's Shangrila Ale (8.5% ABV) and in the extreme, Brasserie des Franches-Montagnes' La Dragonne (Switzerland) is uncarbonated and is intended to be drunk while hot. During the past few years, new oak has featured as a beer flavoring; this is not to be confused with normal oak cask-conditioned beers, where the casks are not new and their internal surfaces are often lined with brewer's tar, so that little or no oak flavor is extracted by the beer. Anheuser Busch's Winter's Bourbon Limited Edition Seasonal Brew is not only aged in new Bourbon casks, but over whole vanilla beans as well. Innis and Gunn's IPA is an ale, brewed at the Glenfiddich Distillery in Scotland. It is matured in new oak casks for one month, blended and matured further before being bottled. The emptied casks are never again used for brewing – instead they are used to mature whisky. This was the original intention of the distillery, but the beer brewed in the new casks proved to be so good it was matured as a new style.

Chocolate beers have gained note in the USA during the past few years. Michelob (part of Anheuser Busch) produce Celebrate Chocolate Beer of 8.5% ABV, that is matured on cocoa beans. There are several 'chocolate stouts' such as Brooklyn Black Chocolate Stout (Brooklyn Brewery) and Rogue Brewery's (Newport, Oregon) Chocolate Stout, the latter being brewed with natural chocolate flavor.

Herbs and berries are also used to flavor beers; many of these beers are based on ancient recipes, since herbal beers predate hopped beers. Gruit (gruyt or grut) ale was brewed in Northern Europe using mixtures of herbs, but usually including juniper, heather, may wort, sweet gale and yarrow. Some modern microbreweries have recreated this style of beer in recent years, some using only one or two herb flavorings, along with hops. Included here are Spruce Goose (Steamwork Brewing Co., Colorado), Alaskan Brewery Co.'s Winter

Ale (some spruce) Fraoch (Williams Brothers, Scotland), containing heather flowers, sweet gale and ginger, Rogue Brewery Co.'s Juniper Ale and Birrificio Troll's Febbre Alta. Buds, needles, leaves or twigs tend to be added at the boiling stage, as with spruce, but more delicate flowers (e.g. heather, sweet gale) and berries (e.g. juniper) may be added at the maturation stage. Essence can be added at this stage, too. See also Sahti for use of juniper in brewing.

Nuts also have a place as beer flavorings, as witnessed by Birrificcio Troll's Palanfrina (9% ABV, with chestnuts) and Rogue Brewing Co's Hazlenut Brown Nectar (made with hazlenut extract). Many of the flavorings mentioned here are important ingredients of liqueurs and related beverages – they are discussed in more detail in Chapter 3.8.

Honey Beers

These are mainly top fermented, honey usually being added at the 'rocky head' ('high krausen') stage of fermentation. The honey supplies extra sugars (fructose and glucose mainly) and also aroma compounds; more delicate honeys (those with more subdued aroma profiles) tend to be used in pale beers (the majority of honey beers) for better balance. A well known example of honey ale in the UK is ex-Vaux brewery's Waggledance (now – 2009 – brewed by Charles Wells-Young, Bedford), named after the communication dance of honey bees. Other examples include several from American craft breweries, such as Rogue Brewing Company's Honey Cream Ale and Milwaukee Ale House's Downtown Lites Honey Ale. In Europe, the Brasserie Lefebvre (Belgium) provides Barbar Honey Ale.

Kölsch

In the cities and environs of Köln (Cologne) and Bonn, a pale, refreshing top-fermented beer is brewed at several breweries. It is called Kölsch and, by law, can only be brewed in Köln and Bonn. It is invariably pale (color: 4–5 °SRM), fairly well hopped (20–30 IBUs) and has around 4.5% ABV. Kölsch is brewed at typical ale temperatures, but is then matured for several weeks at low temperatures (3–5 °C), like Altbier. Also, like Altbier, most are filtered and are often served from small wooden casks. Probably the best known examples of Kölsch are those of Sion and P.J. Fruh (Köln). A similar style is brewed by Goose Island Brewing Co. ('Summertime'), in the USA.

Lambic Beer

This is the 'wild beer' of Belgium and is unlike almost all other brewing styles. It is brewed from pale malted barley and unmalted wheat, with old hops. No yeast is added, instead the fermentation takes place in lengthy stages that involve a number of wild yeasts and bacteria that are present in the air and in the very fabric of the brewery. See Section 2.6.7 for a more detailed description of lambic brewing. Lambic styles include young ($\sim$1 year old), old ($>$3 years old), Faro (sweetened old), Gueuze (a young-old blend) and fruit Lambics. Blending is an important aspect of many Lambic beers: Gueuze is a blend of young Lambic refermented with old Lambic (ratio $\sim$2:1); it is found mainly as a bottle-conditioned beer. Likewise, fruit lambics (blended lambics matured and conditioned with added fruit pulp or syrup) are found in bottle-conditioned form. Lambic beers all have a refreshing degree of sourness (even tartness) and some have cheesy or resinous overtones. The sourness is mostly attributed to the presence of lactic acid, arising from the brewing process (Section 2.6.7). Its concentration can be anywhere between 0.1% and 1%, depending on the brewer. They are brewed in Payottenland, to the west of Brussels. The best known breweries are those of, Belle Vue, Boon (famous for its 'Marriage Parfait'), Cantillon (famous for its 'Grand Cru'), De Neve, De Troch, Girardin, Lindemans, Timmermans and Vandevelden (especially for its 'Oud Bearsel').

Malzbier

This is a rich, dark, but low alcohol (<1% ABV) beer that is brewed in Germany, originally as a nutritious beverage for sailors about to embark on a long journey and later for people recovering from illness. Traditionally, it is often the first beer children drink as they grow up. Roast malts give it the dark color and the low alcohol content comes from the high initial gravity and high mashing temperatures and/or very low fermentation temperatures. In recent years, the style has gained some extra popularity as result of increasing health awareness (Chapter 2.13), probably the best known example is Henninger (Frankfurt) 'Karamalz.'

Märzenbier

Traditionally, in the days before the refrigerator, brewing in southern Germany had to be concentrated in autumn to spring, since the warm summers caused fermentation problems. The last brew was in March and this was given a somewhat higher alcohol content to preserve it through the summer months until the autumn. This became known as Märzenbier (March beer) and was served ceremoniously in the autumn, then later at the Oktoberfest in München. The tradition is continued to this day, despite the fact that refrigeration has long made summer brewing a facile process. Modern Märzenbier is pale (color ~5 SRM) and has about 6% ABV; the best-known are those of the Munich breweries – Hacker-Pschorr, Hofbrauhaus, Paulaner and Spaten.

Mild

Modern mild ale is a British style of top-fermented beer, generally of low gravity (OG <40), lightly hopped (<20 IBUs) and of low alcohol content (<4% by volume). Residual sweetness varies, but all in all draught mild ales, especially cask conditioned, are ideal session beers. The best are a living demonstration that volume of alcohol does not necessarily reflect volume of flavor, as anyone who has tasted Bateman's Dark Mild (Wainfleet) or Jennings Dark Mild (both ~3% ABV) will realize. Most mild ales are dark (color 15–25 °SRM), but there are some pale versions (e.g. Mc Mullen's AK (Hertford) and Taylor's Golden Best (Keighley)). The popularity of mild beers in the UK has declined tragically since the 1960s in the favor of bitter beers and lagers, but the past few years have seen something of a comeback, or at least a stabilization, thanks largely to the consumer group CAMRA (Campaign for Real Ale). A number of breweries still produce copybook dark mild ales, Banks (Wolverhampton), Brains (Cardiff), Elgood (Wisbech), Holden (Woodsetton) and Holt (Manchester) being just five examples, apart from those named above. The Dark Ruby Mild of the Sarah Hughes Brewery (Dudley, UK) is probably the best known example of a Strong Mild (6% ABV). The mild style is uncommon in Scotland, although Scotch Ales possess many of the mild ale characteristics, apart from being much stronger in alcohol. In pre-nineteenth century England, 'mild' was used to describe fresh (unaged) beers, all dark and with a fair range of alcoholic strengths. They had an X-rated naming system: X for the weakest (~4–5% ABV) and XXXX for the strongest (>7% ABV). These beers were often blended with aged beers (old ales) in proportions depending on requirements and taste (see Old Ale, Strong Ale and Stock Ale, and also Porter).

Münchner (or Münchener)

This name originally referred to the dark, malty bottom-fermented beers of the city of Munich (München), and as such, this style is imitated in other countries and the moderately roasted malts that are used in these and other brews are still known as Münchner malts. The modern beer equivalent is simply known as Dunkel and is produced by all the Munich breweries, along with their more popular Helles (Pale) and Pilsener beers. Its color varies between dark copper and dark brown (14–28 SRM), alcohol content is around 5% by

volume and it is light to moderately hopped (18–28 IBUs). Notable examples from Munich include Paulanes Dunkel, Augustiner Dunkel Volbier and Spaten Export Dunkel. Good examples of the style are produced by other German breweries (e.g. Kaltenberg, Kulmbacher and Tucher breweries) and in other parts of the world (e.g. Capital Munich Dark (USA) and Harpoon Munich-type Dark Beer (USA)).

Old Ale, Strong Ale and Stock Ale

These are similar styles of English top-fermented beers of moderate to high alcoholic strength. In pre-nineteenth century England, old ales were known as keeping ales, referring to the fact that they were matured in wooden casks or vats for at least a year, and often for considerably longer. These beers varied in color from copper to dark brown and alcohol content was usually in excess of 6% by volume and could be as high as 12%; they were named X to XXXX to signify alcoholic strength. Because of their extended maturation, most old ales had significant sour notes due to the production of acids by *B. lambicus*, as in Lambic beers (Section 2.6.7). At the turn of the nineteenth and twentieth centuries, some strong ales possessed ∼0.3% lactic acid or higher (lambic beer typically has ∼1% lactic acid). Consequently the tendency was to blend them with young (mild) beers. Today, a classic and very agreeable example is provided by Greene King's Old Suffolk Vintage Ale, produced by blending an XXXX Old Ale (12% ABV) with a fruity, fresh beer (BPA).

Nowadays, stock ales are uncommon, but the Samuel Adams Brewing Co. (USA) describes its Boston Ale as a stock ale. Old ales and strong ales, on the other hand, are well represented in England and others countries, notably the USA and Canada, and with good strong ales coming from Belgium. Perhaps the best known strong ale is Thomas Hardy's Ale, which at ∼11.5%ABV is probably the world's strongest beer that is produced without freeze distillation. It was originally brewed by Eldridge Pope of Dorchester, but following the demise of that brewery, it is now brewed at O'Hanlon's Brewing in Devon. Also of high repute are Gale's Prize Old Ale (now brewed at Fuller's brewery in London), Fuller's 1845 Celebration Ale, Theakston's Old Peculier and Usher's 1824 Particular. It is not easy to make a distinction between old ale, strong ale and barley wine, especially as their alcoholic strengths overlap. In some old ales and strong ales there is perhaps more of an emphasis on roasted flavors, coming from the inclusion of modest amounts of roasted malts is their grain bills; dark malts are not usually included in the brewing of barley wines. Outside England, other classics include Scotland's Traquair House and McEwan's Champion Ale, Belgium's Duvel (Moortgat Brewery), Bush (Dubisson Brewery), Goude Carolus (Anker Brewery) and Piraat (van Steenberge Brewery).

Pale Ale

This style of top-fermented beer was essentially developed in Burton-upon-Trent and surrounding areas during the first half of the nineteenth century, when darker beers were much more common. They were heavily hopped, but malty beers of 5–6% ABV and typically of medium gold to amber color (6–10 °SRM). A subvariety, rather stronger and hoppier was India Pale Ale (IPA), first brewed in quantity by Allsopp's Burton Brewery for shipping (in cask) to India. To survive the long sea and land journey, the beer had to be of a very strong constitution. Modern pale ale is often regarded as the bottled version of bitter and tends to have the same range of colors (6–10 °SRM), alcoholic strengths (4–6% ABV) and they are usually well hopped (25–45 IBUs). However, there are draught pale ales (Nottingham Brewery's Extra Pale Ale is a notable example) and draught IPAs, although the latter (usually of 3.5–4.5% ABV) often bear little resemblance to the original style (6–8% ABV). The best known bottled English pale ale is probably Worthington White Shield (∼5.6% ABV), brewed at the White Shield Brewery on the brewing museum site (now owned by Coors Brewing Co.) in Burton-upon-Trent. Also of high reputation, and also bottle conditioned (some have draught versions) are Hop Back Summer Lightning, Freeminer Trafalgar IPA, Kelham Island Pale Rider, Hampshire Brewery 1066 SPA and Young's London Ale. The craft brewers of Canada and the USA (especially) have gone for pale ale

and IPA in a big way and produce some excellent examples, many bottle conditioned, as witnessed by Liberty Ale (Anchor Brewery Co.), Pale Ale (Firestone Walker Brewing Co.), and others produced by Bridge Port Brewing Co. (Oregon), Sierra Nevada Brewing Co., Tankhouse (Toronto) and Keith (Nova Scotia).

Pilsener

This style is without doubt the most emulated and the most widely available of all styles. The first beers of this type (pale, bottom fermented) were brewed in the Bohemian town of Plzeň (Pilsen in German) in 1842, in response to the continued unsatisfactory quality of the town's top-fermented beers at that time. Although the first lager beers (i.e. bottom fermented) were probably brewed in Germany or Austria, the pale, malty and delicately hoppy Pilsen lager style quickly began to compete with (and even oust) the established regional styles (whether top or bottom fermented) in larger cities throughout Europe and (later) in the 'New World.' Nowadays, there are hundreds of pale lager brands (∼2–4 °SRM) throughout the world that have been inspired by the Pilsener style, even though many of the brand names do not include the words 'Pilsener' or 'Pils.' Many that do call themselves by these names bear only a passing resemblance to the real thing, although others are its equal in quality. Many other similar styles emerged at about the same time or shortly after Pilsener, the most noteworthy of which are probably those of Českе Budějovice and Dortmund. The German name for the former is Budweis – Budweiser, the major product of Anheuser Busch, is named after that town. The most famous brewery of Pilsen is that of Plzeňský Prazdroj (Urquell). Like the somewhat newer Pilsen brewery, Gambrinus, it became famous for its beer produced only from Pilsener malt, Saaz hops, bottom-fermenting yeast and ultra-soft Pilsen water. These factors, combined with traditional bottom-fermenting practices (e.g. double decoction mashing and long, cool lagering) resulted in a finely balanced brew. Elsewhere, the Pilsener style can be maltier as in some of the Dortmund beers or hoppier, as in Jever Pils (Embden, Germany) and some of the Belgian Pils brands. Kellerbier, Zoigelbier and Zwickerbier are all cask-conditioned forms of lager, often served cloudy, directly from the cask, although tank, keg and bottled versions exist. They are a speciality of Bavaria and Franconia, Germany. Probably the best known is the bottled Kellerbier of the St. Georgen brewery in Franconia.

Porter

This style originated in London in the early eighteenth century, acquiring its name from its popularity amongst the river and street porters. It was very dark being brewed with 'brown malts' (possibly similar to today's lighter crystal malts) and had an OG of around 1070, with about 6.5% ABV. Over the next 100 years, the style underwent a number of changes, as a result of changing brewing practices that occurred alongside population and brewing expansion during the Industrial Revolution. It became brewed with pale malts and a small portion of patent black malt (instead of all brown malt), in a variety of strengths and ages. The weakest were called single or plain porters, whereas the strongest were known as double or extra stout porters (see Stout). Young ('mild') and old porter (often two years aged) were often blended (e.g. 2:1) at the point of sale. As tastes and economic circumstances changed, porter was increasingly sold in 'mild' from, gradually declining in alcohol content and hop bitterness. Eventually (in the 1930s) the name disappeared, being replaced by 'stout,' until it was revived in England by microbreweries in the 1980s. Modern porter (apart from Baltic Porter) is top-fermented of OG 1040-65, 4-6% ABV, color: 20–35+ SRM and hop bitterness: 20–50+ IBUs. Prime examples from the UK include Flag Porter (Elgood, Wisbech), Old Growler (Nethergate Brewery), and Original Porter (Shepherd Neame) and from the USA there are those of the Anchor, Great Lakes, Yeungling and other brewery companies. The brewing of porter beer was taken up by breweries in countries around the Baltic Sea, as a result of the influence of imported porter and Russian Imperial Stout (see Stout) from England. Porter is still produced there today, although usually bottom-fermented and of 7–9% ABV, color:

17–30 SRM and hop bitterness: 20–40 IBUs. Examples include Sinebrychoff Porter (Finland), Zywiec Porter (Poland), Alderis Porteris (Latvia) and Stepan Razin Porter (Russia).

Rauchbier

These beers, a speciality of Franconia (Germany), are characterized by smoky aromas and flavors derived from the use of smoked malts. Before the advent of hot air kilns, much malted barley was kilned or roasted using wood fires, so that a degree of smoky character would be present in all beers. Nowadays, it is a speciality style, with beech wood being the major fuel. Smoked beers are usually copper colored, the most famous examples being Bürgerbräu Kaiserdom and Schlenkerla Spezial (both from Bamberg). Other breweries producing smoked beers include Budels (Holland), Poretti (Italy) and Alaskan (USA).

Red Beer

There are many beers that can be described as 'red,' but the term here refers only to those beers that have acquired the name or were originally called red beers. On that basis there are two main types. Firstly, the Irish Red Ales are standard top-fermented beers, the most famous of which is Killian's, brewed under license from Lett's (Enniscorthy, Wexford) by Pelforth in France (Bière Rousse). The second style, much more distinctive, comes from Western Belgium, produced by breweries such as Rodenbach. These beers are based on blends of two year old matured ale that has acquired some degree of sourness, and younger ales; Rodenbach Grand Cru is pure two year old Ale.

Roggenbier

This term refers to beers that are produced from worts with at least 50% malted rye in their grain bills. This style of beer was revived in Germany in the 1980s. Formerly, rye was grown exclusively to bake bread, but before the Rheinheitsgebot, rye malt was a common ingredient of beer (in Germany, up to the end of the fifteenth century). Most modern versions (e.g. Paulaner – ex-Thurn und Taxis/Schierlinger) are top fermented. Malted rye gives a fruity, spicy character and modern Roggenbier is bottle conditioned and turbid. Hop bitterness is lowish ($<$ 20 IBU), color is 15–20 SRM and alcohol content around 5.5% by vol.

Sahti and Koduölu

These are strong traditional beers from the Baltic countries of Finland (Sahti), Estonia (Saaremaa Island – Koduölu) and Sweden (Gotland –Gotlandsdricke). They are brewed using baker's yeast and worts made with both malted and unmalted grains, including barley, oats, rye and wheat. The mash is filtered though a thin bed of juniper twigs. Juniper berries, as well as, or in place of hops are used for flavoring. Dried hop cones are sometimes used, along with juniper twigs, as the filter bed. The wort is not usually boiled, but the mash, when contained in wooden vessels, is sometimes heated by hot stones and some sahti wort is given a brisk, short boil. Both ramped and isothermal mashes are used. The dominant flavors are estery, banana and phenolic notes, derived mainly from yeast action. There are also juniper notes in the flavor. There may be some hop aroma, if hops were used, but no hop bitterness. Alcoholic strength varies between 7% and 11% by volume and color is around 15 SRM, due to use of crystal or Münchner type malts. There are several sahti breweries in Finland, some of them microbreweries. Finlandia, Huvila, Joutsen and Lammia are just four examples.

Saison

This is the name given to pale gold to copper top-fermented beers brewed in southern Belgium. They were once seasonal beers (hence the name), brewed in autumn or winter for drinking at the next harvest. Now, they are brewed all the year around by small breweries and are mostly bottle conditioned, having an alcohol content of 5–8% by volume, color of 5–12 SRM and hop bitterness of 25–45 IBU. Saison wort is fermented at high temperatures (>30 °C), which gives the beer peppery, spicy and floral notes. Notable Belgian examples include those of Brasseries Dupont, Frantôme, Lefèbvre, de Silly and à Vapeur. The style is known in the USA; examples include Samuel Adams Saison and Dans Saison (Dogfish Head Brewing Co.)

Scotch Ales

Strictly, this name is reserved for Scottish strong ales, of at least 6.5% ABV. Amber (rather than pale) malts form the basis of the grain bill, which can also include brown malts, or even roasted barley, and hence Scotch Ales vary in color from amber to dark brown. Compared with English strong ales, the hop character of Scotch ales is muted: maltiness (and sometimes smokiness) being more to the fore. The yeasts, though top fermenting, are often sedimentary in character and are low attenuators, thus leaving significant residual sweetness. Typical examples include Belhaven Wee Heavy, McEwan's Scotch Ale and Skullsplitter Ale (Orkney Brewery). Fine beers in this style are also produced in Belgium (e.g. Scotch de Silly and Achouffe McChouffe) and the USA (e.g. Kilt Lifter from Moylan's Brewing, California).

Sourish Shchi

This is a traditional Russian unhopped 'wild' beer, brewed with six ingredients (Shchi is the old Russian for 'six'): malted barley, rye and wheat; wheat or rye flour; unmalted buckwheat and honey. No hops are used and the wort is unboiled (like old English ales), being fermented by the yeasts and bacteria on the ingredients and brewing equipment, followed by sediment from the previous brew. Traditionally bottle conditioned in Champagne bottles, thus give a sourish, winey, spicy effervescent drink – unlike Kvas (brewed from rye bread), which is served 'flat' (Dankovtsev *et al.*, 2002).

Steam Beer

This is a hybrid beer brewed using lager (bottom-fermenting) yeasts to ferment the wort at ale temperatures (Section 2.6.8). The style has become associated specifically with California, but was quite widely spread throughout the USA in the ninteenth century. Steam beer was rescued from extinction in the 1960s with the revitalization of the Anchor Steam Brewery in San Fransisco. The original style probably arose as an attempt to brew cheaper lager beer without refrigeration. Its name probably comes from the high carbonation of the original cask-conditioned beer. Modern steam beers tend to be based on the Anchor revived prototype; they are amber beers (color: 10–15 SRM) of moderate bitterness (∼35 IBU). They have a malty/fruity palate, but with a clean finish. Apart from the Anchor Brewing Co.'s definitive steam beer, examples of the style include Old Dominium Victory Amber, Southampton West Coast Steam Beer and Sleeman Steam Beer (Canada). In Germany, there is Dampfbier (= steam beer), a top-fermented brew named after the steamy mist above the vigorously fermenting wort at temperatures in excess of 21 °C. It was a speciality of southeastern Bavaria (der Bayerischer Wald, near the Czech border), dying out in the early twentieth century, to be revived by Erste Dampfbierbrauerei (Zwiesel) in 1989. Other examples are those of Maisel (Bayreuth) and Borbeck (Essen). Like the original Dampfbier, the modern version is brewed with wheat beer yeast (even though the grain bill is exclusively malted barley), so it has spicy, clove notes over the malt and hop character. High temperature fermentation also gives it phenolic notes.

Steinbier

This ancient style was revived by the Rauchenfels brewery in Germany a few years ago. In the days before large metal kettles, big stones were baked in wood fires and then used to boil the wort, then contained in a wooden vessel. The intense heat of the stones caused localized caramelization of the wort sugars, some of which stuck to the stones' surfaces. After cooling and fermentation, the stones were added back to the beer so that the caramelized sugars could infuse the beer. Modern Steinbier (= stone beer) is a bottom-fermented, copper-colored brew with a smoky, caramelized aftertaste.

Stout

The history and development of stout is intricately connected with that of porter.

Porter's home base was London, but imports of the brew into Ireland caused Arthur Guinness and other Irish brewers to brew their own versions of porter, alongside their other ales. The style rapidly displaced the paler styles. The stronger types of porter were called stout porter and extra stout porter (etc.) and eventually, the term porter was dropped, but Guinness continued to brew porter until the 1960s. The same happened in England, except that changes in taste and economic circumstances, resulted in dominance of the stout beer market by the Irish breweries. Still the most prominent form of stout is bitter stout, as typified by those of Guinness, Murphy, and Beamish and Crawford, the last two of which companies are located in Cork. These beers are characterized by a deep (black) color ($\sim$35 °SRM), with thick white (or off white) head and bitter, but smooth taste. Guinness Extra Stout is available in bottle-conditioned form as well as brewery-conditioned forms in cans and bottles. The dark color and a certain bitter flavor are derived largely from the use of roast barley (black roasted unmalted grain). Their alcohol contents tend to be around 4.5% by volume, but export types have higher than 7% ABV. English stouts tend to be both less bitter and sweeter, though nowadays there are many variations – from the sweet Mackeson ($\sim$3% ABV) to Freeminer Deep Shaft (6.2% ABV). Milk stout is brewed with added lactose for sweetness – lactose is not fermented by *S. cerevisiae*. Oatmeal stout is brewed with a small proportion of oats (5–15%) in the grain bill (too much oats give an astringent taste). By the 1970s English breweries had ceased brewing oatmeal stout, but the style was revived a few years ago by Sam Smith's Tadcaster Brewery (5% ABV). Oyster stout probably refers to the long association of eating oysters with a glass of stout, although some stouts have allegedly been matured over oysters. Highly roasted malts such as chocolate malt and black malt often produce beers with chocolate and/or coffee notes in their flavors. This led some brewers to name their stouts as chocolate or coffee stout; some are even brewed with added chocolate (or cocoa beans) or coffee (see Flavored Beer). The most potent subcategory of stout is Imperial Russian Stout, probably the best known of which was that of Scottish Courage (previously Simonds, Barclay and Perkins, and before that, Thrales). Beers of this kind evolved from stout porters that were exported to the Tsarist Court and to the Baltic countries. They are invariably rich and malty with spicy, chocolate notes and are usually around 10% ABV. Modern versions include Sam Smith's Imperial Russian Stout (7% ABV) and Harvey's Imperial Extra Double Stout (9% ABV). Stouts of all types are now brewed all over the world, notable examples (apart from those already mentioned) being St. Amboise Oatmeal Stout (McAuslans Brewing, Canada), Best Extra Stout (Cooper's Brewery, Adelaide), Øl (Ølfabrikken brewery, Denmark), Hook Norton Double Stout (U.K.) and Wilson Mild Stout (van Steenberge brewery, Belgium).

Wheat Beer

After barley, wheat is the most important brewing cereal and many beers are brewed from wort with some malted wheat in the grain bill. However, the term wheat beer is used here to indicate only those beers that have sufficient malted wheat to give recognizable wheat character (say $>$40%). Malted wheat is higher in protein

content than malted barley (13% compared with 10%) and has a different flavor profile: wheat beers are often distinguisted by biscuity, fruity and spicy notes. Germany is the leading exponent of wheat beer, which falls into two major categories: Weizenbier (or Weisse), prominent in Bavaria and Franconia, and Weisse, from the north, especially Berlin. The latter are brewed from about 30% wheat malt and are light in alcohol (~3.5% by volume), whereas the former are usually brewed with over 50% malted wheat and have over 5% ABV. All are top fermented and many are available in bottle-conditioned form, whence they are known as Hefeweizen. Many are cloudy and are meant to be drunk as such. Classic wheat beers from southern Germany include Spaten Franziskaner, Hofbräuhaus Edel Weizen, Schneider (many brands) and Riedenburger Weisse. From the north come Schultheiss Berliner Weisse, Berliner Kindl Weisse and Haake-Beck Bremer Weisse. Apart from those pale brews, there are Dunkel Weizen, Weizenbock and Weizendoppelbock, notable examples being Schneider Dunkel Weiss, Erdinger Pikantus and Schneider Aventinus, respectively. Additionally, there are German wheat beers that are brewed from the genetic forerunners of modern wheat (*Triticum aestivum*), such as Emmer (*T. monococcum*) and Dinkel (*T. spelta*), a cross between Emmer and wild grasses. These wheats have even higher protein contents (up to 17% for Dinkel) and also high phenolic compound contents, which means that they are either dehusked before mashing or are limited to less than 50% of the grain bill. Prominent examples are Riedenburger Emmer Bier and Neumarkter Lammsbräu Dinkel. Belgium is also a producer of fine wheat beers (Wit Bier or Witte), including Hoegaarden, Lefebvre Blanches Bruxelles and Gouden Boom Brugs Tarwebier. Wheat beer has become popular worldwide during the past few years, being brewed in all major brewing countries, in all styles. Examples include Blue Moon (Coors Brewing, USA), Oakham White Dwarf (UK), and Lion Nathion Breweries Mac's Great White (New Zealand).

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2.7

Cereal-based and Other Fermented Drinks of Asia, Africa and Central/South America

The numerous indigenous nondistilled alcoholic beverages of the continents of Africa, Asia and Central/South America are discussed in this chapter. Most of these drinks are based on cereal crops, including barley, maize, sorghum, rice and wheat, although others are derived from tree sap, cassava and other roots, and plantains.

The production of many of these drinks is via natural ('spontaneous') fermentation and with little in the way of refinement, except simple filtration. Others, such as Asian rice wines are brewed using special starters containing colonies of particular microorganisms. They are mostly consumed fresh (often while still fermenting) and cloudy (sometimes of the consistency of porridge) and hence they often have comparatively high levels of protein, carbohydrate, fiber and unfermented sugar, as well as yeast and other microorganisms, such as lactic acid bacteria (LAB). Consequently, they are usually far more nutritious than the highly refined European style beers that are brewed in the same countries, often by breweries owned by multinational brewing or drinks companies. Indeed, in many countries the indigenous alcoholic drinks, such as pulque in Mexico (Section 2.7.2) and the sorghum beers of Africa (Section 2.7.2) make an important contribution to the diet, particularly of lower paid workers. Additionally, because they are consumed 'live' (i.e. containing large populations of microorganisms like yeasts and LAB), they possess a greater prebiotic potential than the microbiologically dead European style beers brewed in the same countries. On the downside, brewery hygiene and quality control is very variable and there have been concerns about safety, particularly relating to the use of pathogenic mold infected materials (Odhav and Naicker, 2002). The majority of these drinks are produced by home brewers (artisanal brewers), for local consumption, but some, such as rice wines, pulque and African sorghum beers, are also brewed and distributed by small-scale industrial outfits and can be obtained in cans (pulque) or bottles (rice wines).

Although many of these beverages have been produced up to the last few years with minimal commercial exploitation and little supervision from governments and other legislative bodies, there is now growing interest in the regulation of some of these drinks, as in the case of Mexican pulque. The main purpose of this is to ensure good production practice, with an acceptable standard of hygiene, so that a safe, nutritious product of consistent quality can be brewed on a regular basis. Concurrent with this are modern programs of research, especially regarding the microbiological profile of the fermentations and its relation to the organoleptic character of the beverage. Research of this kind is important in determining the combination of

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

microorganisms and the production conditions that give the highest quality product, while maintaining its traditional character.

The rice wines of Asia are discussed in Section 2.7.1, whereas the maize and sorghum beers of Africa, chica and pulque of Central/South America, the palm wines of Africa and the other beverages produced from cassava and plantains are discussed in Section 2.7.2.

2.7.1 The Rice 'Wines' of Asia

Rice 'wine' is produced all over eastern Asia. Unlike wine, which is made by fermentation of naturally sweet grapes and (stretching the definition) other fruit, rice 'wine' results from the fermentation of rice starch converted to sugars. This process is more akin to that used to produce beer; however, beer production employs a mashing process to convert starch to mainly fermentable sugars whereas rice wine uses different amylolytic processes. Both nonglutinous rice (*Oryza sativa* L.) and glutinous ('sticky') rice are used and in Vietnam, purple glutinous rice (*Oryza sativa* var. *glutinosa*) makes rice wines that are often described as having a 'Sherry character.'

A characteristic feature of Asian rice wines is the use of a starter that supplies fungi and bacteria that have been grown on a solid medium: wheat grains in Korea (see below), barley, pea or wheat in China (Fan and Qian, 2006), rice and/or cassava powder in Vietnam (Dung *et al.*, 2006) and cooked rice in Japan (see below). Sometimes, a different starter is used to brew wines destined for distillation; for example in Japan, barley shochu is distilled from wine brewed with a koji made from pearled barley (Iwami *et al.*, 2005).

Some major types of rice wine in northeast Asia (Korea, Japan and China) are listed in Table 2.7.1.

The less refined, cloudy beverages are outlined first, followed by the more refined, filtered versions.

Cloudy Rice Wines

Choujiu is a type of Chinese fermented alcoholic beverage brewed from glutinous rice. It is very thick and has a milky white color, which is sometimes compared to jade. Choujiu is an ancient variety of Chinese wine, and is possibly the original Chinese wine. It can be traced back to the Tang Dynasty, where it was praised by the poet Li Bai. In ancient times, choujiu was referred to as yùjiāng. Today, the city of Xi'an is known particularly for its choujiu. Doburoku is the Japanese equivalent of choujiu, and in Korea gamju and makkoli are similar (Belleme and Belleme, 2007).

Table 2.7.1 *Summary of the major rice-based nondistilled alcoholic beverages of China, Japan and Korea*

Crude or cloudy rice wine	Clear (filtered) rice wine
Makkoli - a milky traditional rice wine indigenous to Korea	*Cheongju* - Korean rice wine *Beopju* - a variety of cheongju
Gamju - A milky, sweet rice wine from Korea	
Amazake - low-alcohol Japanese rice drink	*Sake* (*Nihonshu*) - Japanese rice wine
Doburoku - the Japanese equivalent of makkoli	
Choujiu - A milky glutinous rice wine popular in Xi'an, China	*Mijiu* or *Lao-zao* - a clear, sweet Chinese rice wine/liqueur, made from fermented glutinous rice

Figure 2.7.1 *Typical presentation of Korean makkoli*

Makkoli (or makgeolli), also known as takju, is a traditional alcoholic beverage native to Korea and is used during ancestral rites in Korea. It has a milky, off white color, and a cereal winey taste, with some sweetness. It is made by fermenting a mixture of boiled rice and water, and is about 6.5–7% alcohol by volume. It was originally quite popular among farmers, earning it the name nongju, which means 'farmer's liquor.' A regional variant, originally from Gyeonggi-do, is called dongdongju. Another variety, called ihwaju (literally 'pear blossom wine') was so named because it was brewed from rice with rice malt that had fermented during the pear blossom season. Ihwaju is often so thick that it must be eaten with a spoon.

Commercially, makkoli is most commonly available in plastic bottles or aseptic box containers (Figure 2.7.1). As it is an unfiltered beverage, it is generally shaken before consuming, as the cloudy white portion tends to settle to the bottom, leaving a pale yellow clear liquid on top.

Gamju or dansul is a traditional Korean alcoholic beverage, made from rice fermented with yeast cake. Because its fermentation is incomplete, its alcohol content is relatively low. It is made from steamed rice, to which water and yeast cake are added.

Although production of makkoli and similar drinks in Korea is many centuries old, both the production and demand for traditional Korean wines and liquors declined sharply during the Japanese colonial period (1905–1945). In 1986, in an effort to remedy this situation, the Cultural Heritage Administration of South Korea selected 86 varieties of traditionally brewed alcoholic beverages as cultural properties, with 12 types selected as Important Intangible Cultural Properties, each hailing from its own locality (Cho, 2004; Park, 2005).

Refined Rice Wines

The Japanese sake is probably the best known refined rice wine, having undergone a recent impressive worldwide revival, although not in its homeland. It will be seen in later paragraphs, that there are several variants, differing mainly in the type and extent of filtration used. The Chinese and Korean equivalents are mijiu and yakju, respectively, the latter often being known as cheongju in Korea. One popular brand of cheongju is Chung Ha, which is widely available at Korean restaurants. There are various local variations, including beopju, which is brewed in the ancient city of Gyeongju in the southeast of the country.

Beopju is a traditional Korean rice liquor, of the cheongju family of liquors. It is made chiefly from glutinous rice, and has an alcohol content of about 15%. Beopju first appears in historical records in the Goryeo Dynasty (936–1392). The name literally means 'law liquor,' and refers to its being made in accordance with fixed procedures: an early example of production regulation. Today, it is associated with Gyodong in Gyeongju, where the Gyeongju Choe clan brew a particularly famous variety; this Gyeongju Gyodong Beopju is designated by the government of South Korea as Important Intangible Cultural Property No. 86-3.

Daepo, a branded yakju (literally 'medicinal alcohol') is a refined rice wine made from steamed rice that has gone through several fermentation stages. It is also called myeongyakju or beopju and is distinguished from takju by its relative clarity. Other varieties include baekhaju, which is made from glutinous rice and Korean nuruk and heukmeeju (literally 'black rice wine'), which is made from black rice.

The Traditional Korean Rice Wine Production Process

In Korea, the major crop has historically been rice, and thus most Korean traditional alcoholic beverages have been made from rice, of both the glutinous and nonglutinous variety, which are fermented with the aid of yeast and nuruk, a wheat-based source of amylolytic microorganisms (especially *Aspergillus* and *Rhizopus* spp.) and of a variety of fermentative microorganisms, including lactic acid bacteria (LAB) and *S. cerivisiae*. Additionally, Koreans often use fruits, flowers, herbs and other ingredients to flavor these beverages, to a greater extent than equivalent Chinese and Japanese wines (see later).

The Korean traditional cereal-based alcoholic beverages in general are divided into 'takju,' 'yakju' and 'soju'. Among these, yakju, which is made from takju by removing the residue ('gigemi') from it (by filtration), is the oldest one. Traditional soju can be obtained by distilling takju or yakju (Section 3.4.4). A generalized procedure for brewing traditional Korean rice wines is shown in Figure 2.7.2. It can be also regarded as being typical of Asian wine brewing processes, but with differences in various fine details for Chinese and Japanese equivalent beverages.

Normal brewing is conducted using nuruk, a mash of milled and cooked rice (grain) and water, and fermenting for a specific time at a regulated temperature. This fermentation process is repeated by addition of rice and water to give 1st brewed liquor, 2nd brewed liquor, 3rd brewed liquor even up to a 12th brewed liquor. The flavor of the rice wine changes with the number of brews performed: it gains in alcoholic strength, maturity and depth of flavor. The first three brewed liquors are regarded as the heart liquor; most of Korean traditional alcoholic beverages consist of 2nd brewed liquor (Park, 2005). Both the amylase enzymes for saccharification of cooked rice starches and the microorganisms (bacteria and fungi) for fermentation are supplied by nuruk, a cake made of malted and partially fermented wheat grains.

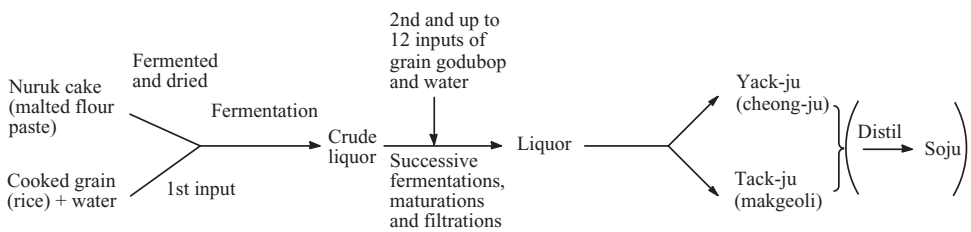
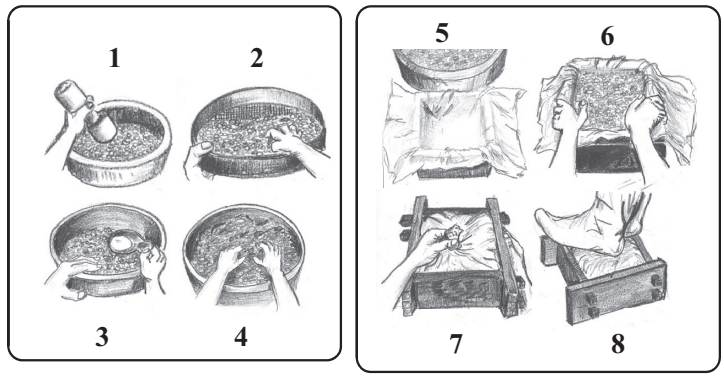
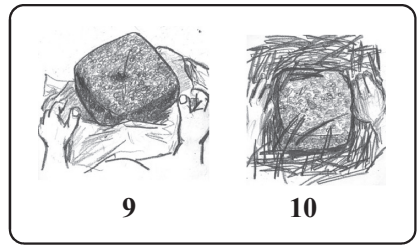


Figure 2.7.2 *Simplified scheme for the production of traditional Korean rice wine*



1. Preparation of rough whole wheat by grinding wheat grain with hand mill or pounding in a mortar.
2. Passing flour through a sieve to remove fine flour (about 30%).
3. Sprinkling water to whole wheat and mixing evenly.
4. Vigorous kneading of flour.
5. Putting wet hemp cloth-wrapper into the nu ruk mold.
6. Filling mold with dough.
7. Twisting the edges of hemp cloth wrapper to seal.
8. Forming the dough into flat cakes (sealed package upside down).



9 and 10. Unwrapping and placing on rice straw in warm, dry place to dry (later in sunny place with good air circulation).

Figure 2.7.3 *Preparation of nuruk*

Preparation of Nuruk

This can be regarded as the first step in brewing rice wine. The various steps in the preparation of nuruk are shown in Figure 2.7.3. Fermentations promoted by lactic acid bacteria (LAB) occur first, followed by alcoholic fermentations conducted by *Saccharomyces* and other species. This is soon superseded by the growth of other fungi, notably *Aspergillus* spp. and *Rhizopus* spp., so that after 30 days the nuruk appears to be covered in mold. After drying in the sun, excess fungal filaments can be shaken off, after which the cake is broken up with a hammer and the pieces are left in an airy and sunny place. The *Aspergillus and Rhizopus* species in nuruk provide glucoamylase (amyloglucosidase) and α-amylase (see Section 2.6.2) enzymes that convert rice starch to fermentable sugars, especially glucose and maltose. Some oligosaccharides (dextrins) are also formed, some of which are not readily fermentable, thus leaving some residual sweetness in the finished wine. Fermentative yeasts and bacteria are also supplied by nuruk, so that in the brewing of rice wines, amylolytic action and fermentation occur side by side. Yeast (*S. cerevisiae*) can also be added separately.

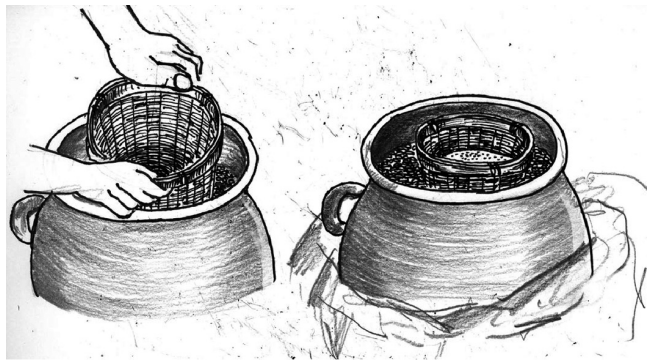


Figure 2.7.4 *Use of a traditional rice wine strainer to make chungju*

Brewing process

Freshly soaked and washed nonglutinous rice (some recipes use glutinous rice) is cooked in water for one hour to form a thin porridge. Small pieces of nuruk powder (∼15% w:w) are added to the cooled porridge in a small pot, which is then closed, wrapped in cloth and kept at 22–25 °C for 24–36 h. It is then unsealed and unwrapped, left in a cool, drafty place for a few hours, and then resealed, rewrapped and left in a cool place for 1–2 days. This first fermentation gives mitsul (밑술), which is essentially the starter; it will be used to ferment subsequent rice liquors, a process known as enrichment (teosul, 덧술 in Korean). Glutinous rice is steamed in an earthenware pot and when cooked, the rice is laid out on straw mats to cool. The cooled rice is mixed with cool water and the liquor from the first brew in a suitably sized pot and is put aside to ferment, as described above. This process can be carried out several times, as outlined in Figure 2.7.2.

Filtration

Chungju is obtained by placing a rice wine strainer in the fermented liquor and drawing out the relatively clear supernatant liquid. Takju, such as makkoli, is obtained by passing the thick porridge through a sieve. Figure 2.7.4 illustrates traditional filtering methods.

Traditional Rice Wine in Japan

Amazake is a traditional sweet, low alcoholic Japanese drink made from fermented rice. Amazake dates from the Kofun period and it is mentioned in the Nihon Shoki. It is part of the family of traditional Japanese foods (including miso, soy sauce and sake), made using inoculation with the fungus *Aspergillus oryzae*).

Sake is the major Japanese alcoholic beverage made from rice. This beverage is called ‘sake’ in English, but in Japanese, sake or o-sake refers to alcoholic drinks in general. The Japanese term for this specific beverage is nihonshu, meaning ‘Japanese sake.’ It is brewed using a similar process to that described for Korean takju and yakju, where the saccharification of rice starch and fermentation of the resulting sugars occur over the same period of time in the same vessel. The major difference is that fermented, malted wheat (nuruk) is not used as a source of saccharification/fermentation microorganisms. Instead cooked rice inoculated with the fungus *Aspergillus oryzae*, known as kōji, is used for this purpose. Like Chungju, most sake is filtered.

Moromi, the main mash sake is produced by the multiple parallel fermentation of rice, that is to say simultaneous saccharification, and alcoholic and other fermentations. The rice is polished to remove the

protein and oils from the exterior of the rice grains, leaving behind starch. A more thorough polishing leads to fewer native congeners and generally a more desirable product. Newly polished rice is allowed to 'rest' until it absorbs enough moisture from the air, so that it does not crack when immersed in water. After this resting period, the rice is washed clean of the rice powder produced during milling and is steeped in water. The length of the soak depends on the degree to which the rice was polished, from several hours or even overnight for an ordinary milling to just minutes for highly polished rice.

After soaking, the rice is either boiled in a large pot or steamed on a conveyor belt. The degree of cooking must be carefully controlled; overcooked rice will ferment too quickly for flavors to develop well and undercooked rice will only ferment on the outside. The steamed rice is then cooled and divided for different uses.

Some of the steamed rice is taken to a culture room and inoculated with kōji mold (麹, *Aspergillus oryzae*). The mold laden rice is itself known as kōji and is cultivated until the growth of the fungus reaches the desired level. This takes about two days.

When the kōji is ready, the next step is to create the starter mash, known as shubo (酒母), or colloquially, moto (酛). Kōji rice, water, and yeast are mixed together, and in the modern method, lactic acid is added to inhibit unwanted bacteria (in the slower traditional methods, formation of lactic acid occurs naturally). Next, freshly steamed rice is added and the yeast is cultivated over 10 to 15 days.

When the starter mash is ready, steamed rice, water, and more kōji are added once a day for three days, doubling the volume of the mash each time. Staggering the process in this manner allows the yeast to keep up with the increased volume. The mixture is now known as the main mash, or moromi (醪, also written 諸味).

The main mash then ferments. This takes two to six weeks. With high grade sake, fermentation is deliberately slowed by lowering the temperature to 10 °C (50 °F) or less.

After fermentation, sake is pressed to separate the liquid from the solids. With some sake, a small amount of distilled alcohol, called brewer's alcohol (醸造アルコール), is added before pressing in order to extract flavors and aromas that would otherwise stay in the solids. With ordinary sake, a large amount of brewer's alcohol might be added to increase the volume of sake produced. Next, the remaining lees (a fine sediment) is removed, and after that most sake is carbon filtered and pasteurized. The sake is allowed to rest and mature and then it is usually diluted with water to lower the alcohol content from around 20% to 15% or so, before being bottled.

Although there are many individual versions of sake (Figure 2.7.5), there are two basic types: futsū-shu (普通酒) and tokutei meishō-shu (特定名称酒). Futsū-shu, 'ordinary sake,' is the equivalent of table wine and accounts for the majority of sake produced. Tokutei meishō-shu, 'special designation sake,' refers to premium sake distinguished by the degree to which the rice is polished and the added percentage of brewer's alcohol or indeed the absence of such an additive. Teiseihaku-shu (低精白酒) is the general name for sake brewed with deliberately highly polished rice, whereas kuroshu (黒酒) is sake made from unpolished rice (i.e. brown rice), more like Chinese rice wine. Generally, more highly polished rice produces fruitier sake, whilst less polished or unpolished rice produces sake with more earthy, grainy flavors of the rice itself.

There are basic categories of special designation sake (Figure 2.7.5). Honjōzō-shu (本醸造酒), has a slight amount of brewer's alcohol added to the sake before pressing, in order to extract extra flavors and aromas from the mash – no more 116 l of pure alcohol for every 1000 kg of rice. Junmai-shu (純米酒), 'pure rice sake,' is brewed with only rice, water and kōji, with no brewer's alcohol or other additives. Ginjō-shu (吟醸酒), brewed with rice polished to 60% or less of its original weight. Sake made from rice polished to 50% or lower is called daiginjō-shu (大吟醸酒). The term junmai can be added to ginjō or daiginjō, resulting in junmai ginjō and junmai daiginjō.

Other types of sake are designated depending on clarity, lack of carbon filtration, lack of pasteurization and extent and type of maturation, as outlined in Figure 2.7.5. Thus, doburoku (濁酒) and nigorizake (濁り酒)

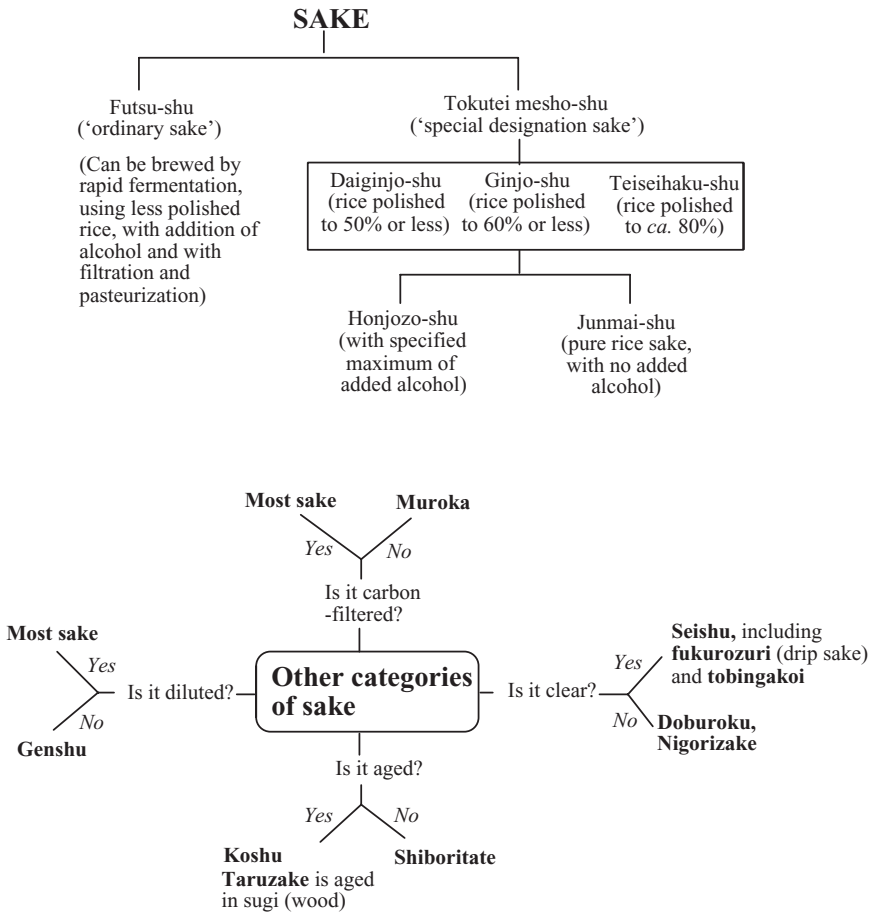


Figure 2.7.5 *Types of Japanese rice wine*

are cloudy versions, muroka (無濾過) is noncarbon filtered, namazake (生酒) is nonpasteurized, genshu (原酒) is undiluted sake (~20% ABV), koshu (古酒) is aged and shiboritate (搾立て) is unaged sake. Finally, taruzake (樽酒) is sake aged in wooden barrels (*Cryptomeria* (杉, sugi), fukurozuri (袋吊り) is made by hanging the mash in bags and allowing the liquid to drip out under its own weight and tobingakoi (斗瓶囲い) is matured in 18 l bottles ('tobin'). Additionally, sake can be distinguished by the way in which way the starter mash is made (Figure 2.7.6): kimoto (生酛) is the traditional method, yamahai (山廃) is a simplified version of the kimoto method, introduced in the early 1900s and sokujō (速醸), 'quick fermentation,' is the modern method of preparing the starter mash.

Sake varies in sweetness, which is indicated by the nihonshu-do (日本酒度) scale, also called the Sake Meter Value, or SMV, based on the specific gravity (Equation 2.7.1).

$$\text{SMV} = 1443\left(\frac{1}{\text{sp.gr.}} - 1\right) \tag{2.7.1}$$

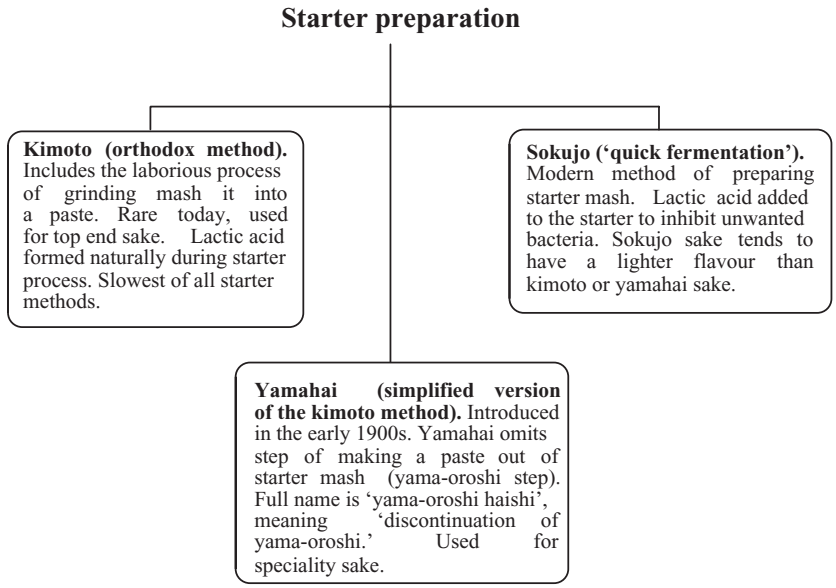


Figure 2.7.6 *Three methods of making a sake starter mash*

Specific gravity (sp. gr.) is measured on a scale weighing the same volume of water at 4 °C and sake at 15 °C. The sweeter the sake is, the lower the SMV. When the SMV was first introduced, 0 was designated the point between sweet sake and dry sake, but now this has been changed to +3.

As in all brewing and distilling processes, there is solid waste material. Kasu (粕) are pressed sake lees, the solids left after pressing and filtering. These are used for making tsukemono pickles, livestock feed and shochu, and as an ingredient in dishes like kasu soup. Rice wine cake is the Korean equivalent. A further product with a high concentration of ethanol (20.7% v:v) can be obtained from rice wine cake by adding crude glucoamylase from *Rhizopus* spp. grown on wheat bran (like nuruk), yeast and various nutrients (Kim and Van Hanh Vu, 2008). Such a wine could be distilled to produce a kind of soju, or grain neutral spirit.

Traditional Chinese Rice Wine

Rice wines are enjoyed all over China, where they are sometimes known as 'yellow wine' and they usually contain 16–20% ABV, having an annual consumption of more than 2 million kl (Luo *et al.*, 2008). Chinese rice wines are generally brewed using glutinous rice with wheat koji and/or red rice koji, which supplies the microorganisms, as with other Asian rice wines. Wheat koji (wheat 'qu') contains a wide range of microorganisms, including *Aspergillus* spp., *Rhizopus* spp., mucor, acetic acid bacteria, lactic acid bacteria and yeasts, while red rice koji contains mainly the purple fungus *Monascus purpureus*. The general brewing method involves cooking a rice–water mixture (1:1, w/w) in a large rice (pressure) cooker at 125 °C for about 1 h. After cooling to room temperature (25 °C), koji is then inoculated into the cooked rice mash in order to provide α-amylase, amyloglucosidase, glucoamylase and β-glucuronidase to break down the starch into oligisaccharides and monosaccharides, and also to liquefy the rice for more complete fermentation. More water is added and after thorough mixing, the liquor is transferred to fired clay containers, where fermentation takes place at 25–33 °C in the closed containers. The rice wine is usually collected after the alcohol content reaches 16–20%, which is after about 14–16 days of fermentation (Zhang and Wu, 1999). There are many

brands or types of Chinese rice wine, which differ mainly in the multiplicity of microbe group used in fermentation. Foshou, Nuomi and Guyuelongshan are fermented with wheat koji, Shousheng with wheat and red rice koji, while Hongqu is fermented with red rice koji. Also in Foshou, bergamot fruit (*Citrus medica* L. var. Sarcodactylis) is infused in the wine following fermentation (fruit:wine ratio ~ 1:10, w:v).

An alternative production method described by Luo *et al.* (2008) as being typical of the main rice wine regions of the south, Jiangsu province, Shanghai city and Shaoxing city (Zhejiang province), is described next. This method involves wheat qu, like the previous method, but also includes the use of a starter with added pure yeast culture and lactic acid. Wheat is milled, mixed with pure water, pressed into molds and then incubated at 28–30 °C for 48 h in a special room, before being dried at 45 °C until the moisture content is less than 12% (w:w). Preparation of the starter ('seed mash') involves mixing wheat qu with previously steamed glutinous rice (weight ratio 1:10) and spring water (weight ratio of steamed rice to water, 1:2). The pH of this mixture is adjusted to ~4 with lactic acid, pure yeast culture (3% w:w) is added and the starter is fermented at 25 °C for 48 h. After this time, the steamed glutinous rice, starter, wheat qu and spring water are mixed in the weight ratio 10:0.6:1:15. Fermentation is carried out over three weeks or so, after which the wine is filtered by pressing and is then transferred to tanks for clarification. The clear wine is colored with caramel, pasteurized at 88–90 °C for 3 min and then transferred to pottery jars, which are sealed and then left to mature for 1–5 or more years. Rice wines are generally blended to yield a consistent product of ethanol content 14–17% (v:v).

Chinese rice wine has many of the aroma/flavor characteristics of its Japanese and Korean counterparts, but with differences that most likely arise from the differences in materials and somewhat different production methods. Carbonyl compounds, carboxylic acids, esters and organosulfur compounds have all been reported to contribute to the aroma of sake, but the characteristic burnt caramel/nutty notes in the aroma of certain sake samples have been attributed to the presence of sotolon (3-hydroxy-4,5-dimethyl-2(5*H*)-furanone) (Isogai *et al.*, 2005). Sotolon is known to make important contributions to the aromas of many fortified wines, such as fino Sherry, tawny Port, vin doux naturel and others (see Chapter 2.10). Indeed, the aroma and flavor of some Asian rice wines, particularly well matured examples, have been compared with those of Sherry, and many western cook books on Chinese cuisine suggest Sherry as a substitute for rice wine in certain recipes.

Luo *et al.* (2008), using headspace SPME (HSPME) (Section 4.2.4) followed by GC-MS analysis were able to identify 97 volatile and semivolatile compounds in 10 typical Chinese rice wine samples, 39 of which were reported for the first time. Included in the headspace were 13 alcohols, 17 aromatic compounds, four carbonyl compounds, eight carboxylic acids, 28 esters, nine furans, three lactones, six nitrogen compounds, six phenols and three sulfur compounds. Of particularly high concentrations were 2- and 3-methylbutanol, benzaldehyde, diethyl butanedioate (succinate), 2-phenylethanol and di(*sec*-butyl) and 2-methylbutanedioate, but the contributions of the components are not known at the present date (2009), since GC-olfactometry and sensory analysis have not yet been carried out.

Traditional Rice Wine of Vietnam

The rice wine of Vietnam, like other Asian rice wines, has several categories and significant amounts of it are distilled. The distilled beverages are consumed per se, but are also used to fortify several of the undistilled rice wines. In the north and south of the country, there are ruou de or ruou nep; these are fermented from rice or glutinous rice, respectively, followed by distillation. In mountainous districts (e.g. Da Lat, Buon Me Thuot, and Dac Lac) Thuong people (an ethnic minority) produce ruou can that is fermented from rice, maize or cassava, with or without distillation. In the Mekong Delta to the south, ruou nep than (purple glutinous rice wine) is made from purple glutinous rice without distillation (Dung *et al.*, 2005).

Like other Asian rice wines, production of traditional Vietnamese rice wine involved the use of a starter to provide the required saccharification and fermentative microorganisms, but the production process favors

the former process first, followed by the latter, although both processes occur in the same vessel, unlike the brewing of European style beers. The traditional brewing method of ruou nep than (purple glutinous rice wine) involves soaking purple glutinous rice in water (mass:volume ratio approximately 5:6) for 4 h at room temperature, followed by steaming at 100 °C for 1 h. The gelatinized rice paste is cooled to about 35 °C and is then inoculated with powdered starter culture ('men') (rice:starter mass ratio ~100:4) and left at 30 °C for three or so days. During this period, most of saccharification processes occur, but a little alcoholic fermentation also occurs. Water (rice:water mass:volume ratio ~50:70) is added, whereupon alcoholic fermentation occurs for ~7 days at 30 °C. The natural ethanol content is about 7% (v:v) and so rice wine spirit is added to bring the content up to about 15% (v:v). The wine can be consumed cloudy or clear after filtration.

Nature of the Rice Wine Starter

The majority of Asian rice wines, including those destined for distillation, are brewed using a starter that contains microorganisms that perform both saccharification and fermentation processes side by side. In Korea, the starter consists of a rice porridge that has been activated by fermentation with nuruk, a dried malted wheat preparation in which *Aspergillus* spp., *Rhizopus* spp. (especially *R. oryzae*), lactic acid bacteria and yeasts (especially *S. cerevisiae*) have been cultivated over several days. In Japan, the starter is rice porridge that has been inoculated with *Aspergillus oryzae*, although pearled barley instead of rice is used for some wines that are to be distilled. Additionally, lactic acid is often added in modern fermentations of sake in order to supress the activities of unwanted microorganisms.

In Vietnam, starters ('men') are made by inoculation of rice and cassava flour dough by molds and yeasts, in which *Amylomyces rouxii* and *S. cerevisae*, respectively, are usually prevalent, although *Amylomyces* aff. *rouxii*, *Rhizopus oligosporus* and *R. oryzae*, and *Saccharomycopsis* (*Endomycopsis*) *fibuligera, Candida* spp. and *Pichia* spp. (respectively) also are normally present (Dung *et al.*, 2006; 2007). New 'men' is prepared by inoculation of the dough with previous (dried) 'men' samples, containing populations of the required microorganisms. The dough is divided into small flattened balls and incubated (e.g. for 1–4 days) on bamboo mats at ambient temperature (~30 °C) often after the addition of herbs or herbal extracts, such as those of 'tieu hoi' (fennel: *Foeniculum vulgare* Miller) and 'dinh huong' (clove: *Syzygium aromaticum* L.) (Dung *et al.*, 2005). The herbal extracts help subdue the activities of unwanted microorganisms and at the same time promote the growth of *Amylomyces* and *Rhizopus* species, as well as *S. cerevisiae* (Dung *et al.*, 2005). After this the dough is quite dry and can be used whole or broken up into granules; it has a 'shelf life' of a few months at ambient temperature.

One of the major problems faced by commercial brewers of rice wine in Vietnam, as with the brewers of other indigenous beverages, is the variable quality of the product. In Vietnam, variability in quality is strongly correlated with the microbiological character of the traditional starter tablets that are commonly used (Dung *et al.*, 2007). In recent years a stable, granulated starter has been developed, containing a defined mixture of mold and yeast cultures (*A. rouxii* and *S. cerevisae*) and defined herbal extracts (those of fennel and clove). This has proven to be shelf stable for more than three months, producing a purple glutinous rice wine of generally superior quality (Dung *et al.*, 2005).

Flavored Korean Rice Wines

Many plants used as herbal remedies are used to flavor Korean rice wines, especially yakju. Flavorings include acacia flowers, fruit of the Chinese matrimony vine, 산수유 (*Cornus officinalis*), ginseng (*Panax ginseng*), chamomile (*Matricaria chamomile*), the root of 'Korean Angelica' (*Ostericum grosseserratum*) (당귀), the root of kudzu (*Pueraria thunbergiana*) (갈근), dandelion (*Taraxacum platycarpum*) and the fruit of the 'Chinese date' *Zizyphus jujuba* var. inermis (대추) (Kim *et al.*, 2000; Lee *et al.*, 2002; Seo *et al.*, 2002). Mushrooms and

fungi, especially the fruiting body of *Ganoderma lucidum* and *Paecilomyces japonica* are also used to flavor yakju (Kim *et al.*, 2004). The triterpenoid ganodermic acid K, was found to be the major antihypertensive agent in *G. lucidum*-infused yakju; it was determined to be an angiotensin I converting enzyme (ACE) inhibitor. ACE regulates blood pressure by converting the inactive decapeptide angiotensin I to the vasoconstrictor octapeptide angiotensin II and at the same time inactivating the vasodilating nonapeptide bradykinin, thus causing high blood pressure. Although rice wines in general (with or without herbal infusions), such as sake, are known to have antihypertensive properties, due to the presence of a number of peptides (Saito *et al.*, 1994), the *G. lucidum* infused yakju exhibited a greater antihypertensive effect than either sake or yakju or a number of other flavored Korean rice wines. Furthermore its superoxide dismutase (SOD)-like activity (an antioxidant activity, see Section 5.8.4) was comparable with other flavored Korean rice wines (Kim *et al.*, 2004).

Geumsan Ginseng Wine is brewed in the Geumsan region of central South Korea from the roots of five year old ginseng shrubs and, rice, malt and clean mineral water by traditional cool fermentation since the fourteenth century. This is a true flavored rice wine and it has its own unique lingering aroma and flavor. It is very different from ordinary ginseng 'wines,' which are simply ginseng flavored soju or distilled liquor, more like liqueurs (Section 3.9.3) than wine. It was originally developed by a local noble family (Kim, Moon-Ki), in the fourteenth century, and has long been brewed in a traditional way, the recipe being handed down from generation to generation.

Ginseng is the most highly respected herb since ancient times by both Chinese and Native American herbalists and the red ginseng from the Geumsan valley is recognized as being one of the very best; it fetches high market prices. As has long been known by these ancient healers, this herb seems to help the mind and body to be in balance. Modern research has demonstrated that there are more than 28 therapeutic constituents in this vitamin rich plant. Regular use of Ginseng is helpful in the treatment of memory loss (Alzheimer's disease), in the balance of blood sugar levels (diabetes), in slowing down the aging process, in supporting the immune system, in regulating blood pressure, in drug abuse rehabilitation, in regulating the digestive system and in increasing stamina. [금산인삼주, http://www.ginsengm.com].

Beksaeju (백세주 or 百歲酒; literally, '100 years wine') is the most popular medicinal rice wine with the Korean younger generation, who sometimes drink it in place of soju (Section 3.4.4). It is a rice wine infused with ginseng and eleven other herbs, including astragalus, cinnamon, ginger, gugija (Chinese wolfberry), liquorice and omija (*Schisandra chinensis*). Sansachun (산사춘) is another commercial Korean rice wine (Bae Sang Myun Brewery), flavored with the red fruits of the sansa, or Chinese hawthorn (*Crataegus pinnatifida*).

2.7.2 Native Fermented Drinks of Africa and South America

In Africa, there are many traditional native beers based on sorghum or maize. They are named according to the language of the local tribes and so in the countries of modern Africa it is often possible to find more than one name for essentially the same beverage. In western states there is shakporo or tchapalo (Benin, Burkino Faso and Côte d'Ivoire), dolo (Burkino Faso and Mali), shukutu (Benin and Togo), pito (Ghana and Nigeria), bil bil (Cameroon) and buruktu (Nigeria). In central, eastern and southern states there is Bantu beer (South Africa), chibuku (South Africa), pombe (Kenya, Tanzania and Uganda) and umqombothi (South Africa). Northeastern Africa has bouza (Egypt and Ethiopia), and merisa (Sudan).

Sorghum provides the main crop for most of these indigenous beverages, although maize is often used, especially in southern Africa and plantains and palm sap are also used to make alcoholic beverages in several central states. The various varieties of sorghum are grown in many parts of the world, in a variety of soils and climatic conditions. The varieties grown in Europe and the USA are mostly of the *Sorghum vulgare* species, often known as broom corn, guinea corn or millet. In Africa, the varieties used for brewing are mostly of

Table 2.7.2 *Top ten producers of sorghum in Africa and Asia (2007)*

Country	Production/$\times 10^6$ tonnes	Country	Production/$\times 10^6$ tonnes
India	10.6	Mali	1.1
Nigeria	7.7	Sudan	0.79
Niger	2.8	Uganda	0.73
P.R. China	2.1	Chad	0.55
Burkina Faso	1.1	Ethiopia	0.50

S. bicolor (L.) Moench; they are well adapted to semiarid climates with less than 600 mm annual rainfall, although they have tolerance to both drought and water logging. Sorghum plants grow extensive root systems and their leaves have a waxy bloom that prevents excessive loss of water, making them suitable crops for dry climates.

The Ethiopians were probably the first people to cultivate sorghum, leading through the years to a gradual selection from wild sorghum between 5000 and 3000 BC. Its cultivation spread throughout Africa and into the Mediterranean countries and Asia, eventually reaching China via the Silk Route. In the seventeenth and eighteenth centuries, seeds were taken to the Americas as part of the slave trade. The USA is today's most important producer, whereas Nigeria and Sudan are the biggest African producers, and India and China are the most important Asian producers (Table 2.7.2). Most of the world crop is used to make flour; it is only in Africa that a substantial part of the crop is used to produce an alcoholic beverage, although sorghum 'wine' is made in China, mostly for distillation (Section 3.4.4).

Like other cereals, sorghum is a rich source of carbohydrate, initially as starch. It can be malted and used to brew Pilsner style beers (Section 2.6.2) or sorghum flour can be used as a beer adjunct (Section 2.6.2) or a substitute for maize or wheat in the production of grain whisky (Section 3.2.2). However, only its role in the brewing of indigenous beverages of Africa and Asia are discussed here.

Although there are many national and local variations in the production of sorghum-based alcoholic beverages throughout Africa, the process described next from western Africa for the brewing of tchapalo (shakporo) can be regarded as being typical. The production of tchapalo is rather complex, involving malting, mashing with cooked cereal and two fermentations, separated by a cooking step (Figure 2.7.7). Sorghum seeds are firstly malted (Section 2.6.2), sun dried and then milled to give malted sorghum flour.

Malting the sorghum is an important step in the process, since it enhances prolamin extractability and concentration, and results in the production of amylases, lipases and proteases, which act upon starch, fats and proteins in the sorghum flour part of the mash. This not only increases the fermentability of the wort, but also enhances the nutritional value of the resulting drink (Correia *et al.*, 2008).

The flour is mixed with water containing a sticky substance to form the mash, which is allowed to separate into a sediment and supernatant. The sediment is cooked for about 2 h at 100 °C, cooled and added back to the supernatant. The latter contains the amylases and limit dextrinase enzymes, as well as the yeasts and bacteria, whereas the cooked sediment contains gelatinized starch. This mixture, called wort, is left for about 12 h, whereupon enzymes hydrolyze some of the gelatinized starch to fermentable sugars (mainly maltose) and a variety of microorganisms perform fermentations ('acidification processes') that give the sour wort.

The major acids produced at this stage are lactic and acetic acids, derived from the fermentation of carbohydrates by species of *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* and others (Vieira-Dalodé *et al.*, 2007), leading to a drop in pH from ~6 to ~4. In the production of dolo and pito in neighboring Burkina Faso and Ghana, *Lactobacillus fermentum* was found to be the dominant LAB

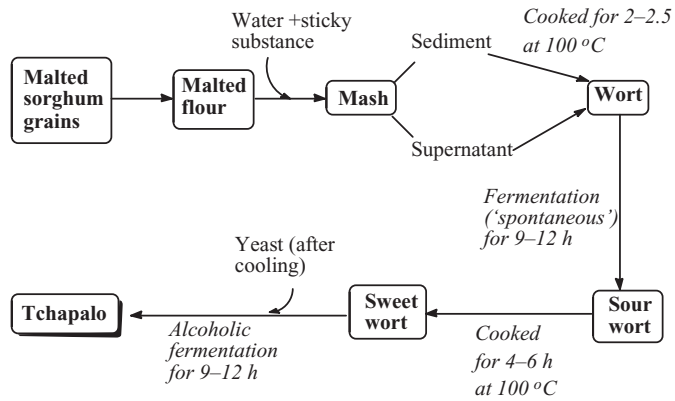


Figure 2.7.7 *Scheme illustrating the production of tchapalo and similar drinks in western Africa*

species (Sawadogo-Lingani *et al.*, 2007). *L. fermentum* was also observed to be a major LAB species for the fermentation of gowé, a sorghum-based drink in Bénin, whose production is like that of tchapa, except that there is no cooking stage (but a milder heating stage) in the process, and the fermented product is cooked before sale and consumption (Vieira-Dalodé *et al.*, 2007).

This first fermentation is 'spontaneous,' since acclimatized bacterial colonies are resident in the brewing equipment, as well as being present in the mash. The equipment is often left unwashed between brewing batches. After fermentation, the sour wort is cooked for 4–6 h at 100 °C to give sweet wort. The cooking procedure not only concentrates the sugars and acids in the wort (Aka *et al.*, 2008), but also kills most of the microorganisms responsible for the sour wort production, thus removing competition for the yeasts and bacteria of the starter used for the alcoholic fermentation (the next stage). After cooling, dried yeast from previous brews is added and alcoholic fermentation (the second fermentation) proceeds for about 12 h, after which time the tchapalo is ready for consumption and is usually drunk within two days from the start of the alcoholic fermentation.

Adding the dried yeast starter to the sweet wort ('back-sloping') helps the fermentation get off to a good start (in European breweries, the yeast slurry does the same job), so that after about 12 h the total soluble solids content has dropped from ∼13 °Brix to ∼ 7 °Brix and alcohol content has reached 3.5–4.5 % (w:v). The rate of inoculation (0.5–2% w:v) does not appear to have a big influence on the fermentation parameters (N'Guessan *et al.*, 2008). During alcoholic fermentation, the pH drops from ∼3.8 to ∼3.6 because the concentrations of citric, fumaric, malic and lactic acids increase somewhat, whereas the levels oxalic and propanoic acids are more or less unchanged (Aka *et al.*, 2008).

The dried yeast starter is prepared from the lees of a previous brew by drying it in the sun. This process may lead to some loss of viability due to UV irradiation or due to contamination resulting from slower drying during the rainy season (Holzapfel, 1997). Examination of yeasts in west African sorghum beers has revealed a varying composition of dominant species, although *S. cerevisiae*, *Candida* spp., *Kloeckera apiculata*, *Torulaspora delbruckii* and *Geotrichium candidum* are prevalent (van der Aa Kühle *et al.*, 2001). Examination of the *Saccharomyces* species found in dolo and pito (of Ghana and Burkina Faso) by chromosome length polymorphism (CLP), intergenic transcribed spacer (ITS), PCR and other techniques showed that alcoholic fermentation is dominated by previously unreported strains of *S. cerevisiae* (van der Aa Kühle *et al.*, 2001).

Several indigenous beers of South Africa and neighboring states are based more on maize than sorghum. Included in these is umqombothi, brewed by the Xhosa people of South Africa from crushed corn meal, crushed corn malt and crushed sorghum malt, and its method of production is similar to other indigenous

African brews. Firstly, the crushed grains are mixed with warm water and left overnight, whence a certain amount of saccharification of starches occurs and fermentation starts. Next, a small portion of the fermenting must is removed and placed on one side (it will be used as a 'starter,' later), while the remainder is boiled to form a thick porridge. The porridge is allowed to cool and poured into a vat, along with the 'starter,' and some additional crushed maize and sorghum malt are added, with vigorous stirring.

The vat is covered and the wort is allowed to ferment for about 24 h, after which it is strained through a large metal sieve, sediment is added to the strained liquor and the beer is served immediately from a communal vat, using cups. The ethanol content of umqombothi is around 3% (v:v) and it is a nutritious drink, with a high carbohydrate and vitamin B content. Like other indigenous beers, however, if maize contaminated with *Aspergillus* spp., *Penicillium* spp. or *Rhizopus* spp. is used, toxins such as ochratoxin A and zearalenone (Section 5.11.4) can get into the beer.

At the time of writing (2009) all the African sorghum- and maize-based drinks described above are produced by home brewers or small-scale industrial outfits, whose in house hygiene regimes differ markedly. The situation regarding the brewing of indigenous African beers is today similar to that of cachaça, pulque and Tequila a few years ago: there is growing interest by governments in regulated processing, to ensure standard and consistent quality, safety and product stability. Such regulated processing will require the use of yeast and LAB starters of typical indigenous microbial compositions in order to preserve the valuable integrity of the beers (van der Aa Kühle *et al.*, 2001; Sawadogo-Lingani *et al.*, 2007).

Chica is the name given to the numerous indigenous nondistilled alcoholic drinks of Central and South America. In many countries the name applies to fermented drinks based upon maize, but it can also refer to beverages brewed from cassava root, fruit or other sources of fermentable carbohydrate. It may even refer to certain nonalcoholic drinks.

Generally, chica de jova is a fermented drink prepared from yellow maize, whereas chica morada is an unfermented beverage made from purple maize and other ingredients. In Central America, the alcoholic beverage brewed from malted maize is known as chica fueste or simply chica, whilst chica batide or chica de maiz are unfermented beverages, often flavored with fruits or vanilla and served very cold. Alcoholic chica is most likely to be made from yellow maize grown at high altitude, but at lower altitudes and in river valleys it is often made form cassava (yucca or manioc) roots or plantains.

Traditionally, the starches in these sources were converted to fermentable sugars by chewing; the amylase enzymes in the saliva catalyzed the hydrolysis of starch to maltose and other sugars. Many of the indigenous fermented drinks of Afica and Asia, such as makkoli of Korea and sake of Japan, were originally prepared using this method (Section 2.7.1). Fermentation then occurred 'spontaneously' by the action of natural yeasts and bacteria in the source materials and in the brewing equipment. In the case of maize, the kernels were ground to flour, which was then moistened in the chica makers' mouths (several female family or community members would be involved with this). The moist flour was rolled into small balls, which were then flattened and left to dry in the sun. Water was added and, after stirring the mixture, fermentation soon commenced. The chica was consumed at celebrations, as a porridge, prickly with the carbon dioxide gas released by fermentation.

A similar process was used to make chica from boiled cassava roots, except after chewing a piece of root, the juicy fibrous pulp was spat into a bowl. When sufficient pulp was obtained, the bowl was placed on a fire and the pulp was cooked, and after cooling, fermentation commenced. Most chica is now made from malted maize, but the traditional methods are still used by some remote indigenous peoples.

A similar beverage named cauim is made from cassava root, or any suitable source of carbohydrate, by certain Amerindian tribes in Brazil. Here the substrate (such as sun dried, peeled cassava tubers and rice flour) is boiled in water to give a smooth paste. After cooling, the paste is inoculated with a mixture of masticated sweet potato and saliva. It is left to ferment for two days or so before being consumed as a porridge. During fermentation, both yeasts and bacteria act upon various constituents, especially carbohydrates, giving a variety

of products, including ethanol and acetate (both minor) and lactate (major). After two days, the pH of the mash has dropped from 5.5 to 3.4 (Almeida *et al.*, 2007). The major yeast at the start of fermentation is *Exophilia dermatidis*, but after two days of fermentation, when the yeast population reaches 6.9×10^7 cfu/ml, the major species were *Candida tropicalis*, *C. intermadia*, *C. parapsilosis*, *Pichia guilliermondii*, *S. cerevisiae* and *Trichosporon asahii* (Schwan *et al.*, 2007). However, bacteria are more abundant than yeasts at all stages of fermentation (by a factor of between 10 and 100). The dominant bacteria were found to be *Lactobacillus* spp. (e.g. *Lb. Pentosus* and *Lb. Plantarum*), *Corynebacerium* spp. and *Bacillus* spp. (Almeida *et al.*, 2007). Many of these microorganisms are able to hydrolyze starch to maltose, glucose and other sugars, which are then metabolized to give lactic acid as a major product.

Pulque is a popular alcoholic beverage of Mexico, but its origins are pre-Hispanic: it was for many centuries an important drink of Mesoamerican civilizations (Bruman, 2000). It is made by fermenting the sap (aguamiel) of various *Agave* species (maguey plants), which are also used to produce the distilled spirits mescal and tequila (Section 3.5.5). The name pulque is probably a Spanish corruption of the Aztec word poliuhquioctli, which means decomposed octli – octli being the Aztec name for this drink.

The maguey plants (wild or cultivated) used to brew pulque are of the species *Agave atrovirens*, *A. mapisaga* and *A. salmiana*, whereas *A. tequilana* var. azul ('blue maguey'), *A. angustifolia*, *A. inaequidans* and others are used to make the distilled beverages mescal and tequila (Section 3.5.5). Many millions of maguey plants are grown in Mexico for the production of these beverages, but only plants of 8–10 years old that are about to produce flowers are harvested for pulque. As a plant approaches flowering time, it makes a sugary sap to provide for the growth and development of the flower, which forms at the end of a tall stem (known as a quiote). This process occurs over 90–120 days, with the production of 200–1000 l per plant over this period.

The farmer first removes the large flower bud, leaving a cavity in the centre of the stem. After an aging period, during which time the cavity heals, the sap sugar content reaches 7–14% (w:v) (average values: 8–10% w:v). The cavity is then scraped, opening the sap vessels so that sap begins to accumulate in the cavity. This is sucked or siphoned out of the plant and the combined sap from many plants is fermented in 700 l open containers. Fermentation is either 'spontaneous' or is induced by adding a sap fermented starter or inoculum of yeasts and bacteria. Lappe-Oliveras *et al.* (2008) give a detailed, illustrated account of pulque production.

The making of traditional pulque is described in Figure 2.7.8. Fermentation starts with type I sap (the cleanest and sweetest) and hence the pulque made by the first fermentation is known as type I (pulque de pic de cuba). Commercial pulque is produced using a fermenting type I starter to ferment type II sap (cloudier and less sweet than type I sap) (Figure 2.7.8).

Agave sap of type I quality contains carbohydrates (fructans, fructose, glucose and sucrose mainly), proteins, amino acid, gums and mineral salts as the most important components (Ortiz-Basurto *et al.*, 2008), with its density being 5–7 °Baumé (∼36–51 °Oe, giving about 6.5 % ABV). Type II sap has a density of around 4.5 °Baumé (∼32 °Oe, giving about 4.5 % ABV). Both kinds of sap are rich in nutrients for the numerous species of yeasts and bacteria that are already present in the sap, but more importantly are present in the winery equipment and starter (pulque de semilla). Intitially (in the sap and during early fermentation), bacteria dominate over yeasts ($8–15 \times 10^8$ cfu/ml and $3–6 \times 10^6$ cfu/ml, respectively), reflecting the relatively high initial pH (7–7.4) of the early substrates. The bacterial population of pulque includes *Lactobacillus* and *Leuconostoc* spp. (LAB), *Acetobacter* spp., *Cellulomonas* spp., *Escherichia* spp., *Flavobacterium johnsoniae*, *Gluconobacter oxydans*, *Hafnia alvei*, *Kokuria* spp., *Macrococcus caseolyticus*, *Micrococcus luteus*, *Sarcina* spp. and *Zymomonas mobilis* (var. mobilis) (Lappe and Ulloa, 1993; Escalante *et al.*, 2004). Yeasts found in pulque include *Candida parapsilosis*, *Clavispora* spp., *Cryptococcus* spp., *Debaryomyces carsonii*, *Kluyveromyces* spp., *Geotrichium candidum*, *Pichia* spp., *Rhodotorula* spp., *Saccharomyces* spp. and *Torulaspora delbrueckii* (Lappe and Ulloa, 1993).

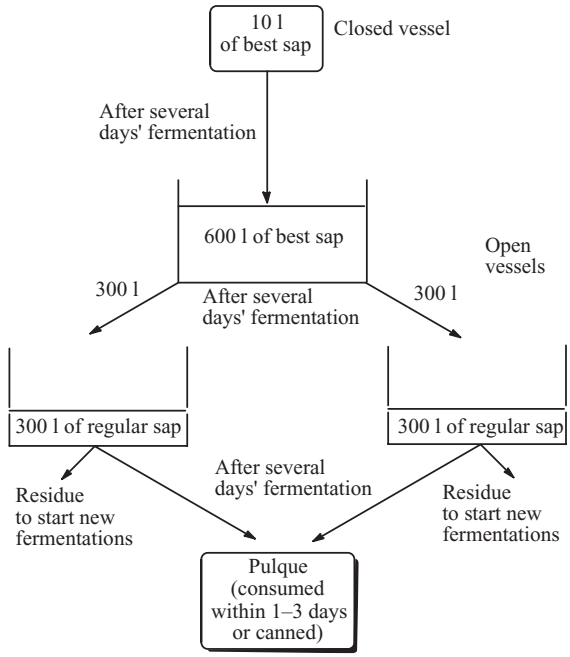


Figure 2.7.8 *Scheme showing traditional production of pulque using a natural (spontaneous) starter. Best sap contains ~10–14% sugar and is clear; regular sap contains ~7–10% sugar and is less clear*

Like other beverages fermented 'spontaneously' (see for example, lambic beer, Section 2.6.7), a succession of fermentations occurs with time, with different species predominating as the chemical and physical characteristics of the medium change. For pulque, the fermentative succession is outlined in Figure 2.7.9, although the beverage will either have been consumed or pasteurized and canned before the final three phases have occurred. *Leuconostoc* spp. are most active to begin with, producing lactic acid, ethanol, dextrans and numerous minor compounds, some of them flavor components. As the pH drops, *Lactobacillus* species become dominant, producing more lactic acid until yeast growth accelerates at pH ~4.5. In the second phase, non-*Saccharomyces* yeasts are most active early in the phase, producing a moderate amount of ethanol, whereupon *Saccharomyces* spp. generally dominate until the end of alcoholic fermentation. The most important *Saccharomyces* species appear to be *S. bayanus*, *S. cerevisiae* and *S. paradoxus* (Lappe-Oliveras *et al.*, 2008). *Zymomonas mobilis* (var. mobilis) also plays an important role in alcoholic fermentation, producing some lactic acid, acetic acid and other substances from fructose and glucose (Ramírez *et al.*, 2004). This species also produces gums and hence may be responsible, along with LAB (which produce dextrans) for increasing the viscosity of pulque.

Although there is very little information on the flavor components of pulque, there is good evidence to suggest that the beverage has significant nutritional value; it is a good supplement to the mainly corn diet of the Mexican low income population (Lappe-Oliveras *et al.*, 2008). It may also have some prebiotic value due to the presence of LAB and yeasts (Steinkraus, 1997) and fructans (fructose oligosaccharides) (Ortiz-Basurto *et al.*, 2008).

Industrialized production of pulque is not extensive at the time of writing (2009), but there are several companies producing a canned version, some of which is exported. Commercial versions are made by adding a starter of LAB, *S. cerevisiae* spp. and *Z. mobilis* spp. to pasteurized sap, the resulting pulque (sometimes

Phase	1 (Lactic) →	2 (Alcoholic) →		3 (Viscous)	4 (Acetic)	5 (Putrid)
Main organisms	*Leuconostroc* spp., then *Lactobacillus* spp.	*Kluyveromyces* spp., *Pichia* spp., then *Saccharomyces* spp.		Dextran producing bacteria	*Acetobacter* spp.	
Populations (cfu/ml)						
Total bacteria	~10^9	~10^8				
Total yeast	~ 3 x 10^6	~3 x 10^8				
pH #	7.5–6.5	~4.5	~3.8			
%ABV #	0	~1	~3–7			
Time*	0		20 h			

Figure 2.7.9 *Phases in the fermentation of pulque. *Time depends on temperature; the above time scale is typical of 12–13 °C and is quoted for a single fermentation. For double fermentation as outlined in Figure 2.7.8, the total time for pulque production is several days at 12–13 °C. #Depends on sap quality*

flavored) being pasteurized prior to canning. It has been suggested that new pulque industrialization processes should focus on hygiene, longer shelf life and nutritional value, thus providing low income consumers with a safe, nutritious drink of uniform quality (Ramírez *et al.*, 2004; Lappe-Oliveras *et al.*, 2008). However, pasteurization would remove some of the prebiotic value.

Palm wine, made from the sap of either the raffia palm (*Raphia raphia*) or oil palm (*Elaensis guineensis*) can be considered as the African equivalent of pulque. It is consumed by over 10 million Africans and is a very popular beverage in countries such as Nigeria (Nwachukwu *et al.*, 2006). Oil palm sap and raffia palm sap contain about 12.5% and 7.5% reducing sugars, respectively, thus giving drinks with potential alcohol content of about 7.5% and 6% (v:v), respectively.

After being tapped from the trees, palm sap undergoes a spontaneous fermentation for over three days, because of the presence of microorganisms in the sap itself and in the winery equipment. Alcoholic fermentation occurs first, when the level of ethanol rises sharply over the first 72 h (Nwachukwu *et al.*, 2006). As with other 'spontaneously' fermented alcoholic beverages, the non-*Saccharomyces* yeasts probably act first, followed by *Saccharomyces* spp. and similar yeasts, when the ethanol concentration rises above 2 or 3% (v:v). Yeasts that have been isolated from Nigerian palm wine include *Hanseniaspora uvarum*, *Hansenula anomola*, *Kluyveromyces marxianus*, *Pichia ohmeri*, *S. cerevisiae*, *S. globosus*, *Schizosaccharomyces pombe* and *Zygosaccharomyces fermentati*.

After 72 h of fermentation (at ~28 °C), the ethanol content drops, with concurrent drop in pH and rise in titratable acidity, suggesting that bacterial fermentations become dominant beyond this point. Bacteria that have been found in Nigerian palm wine include, *Acetobacter* spp., *Bacillus* spp., *Chromobacterium* spp., *Cornybacterium* spp., *Gluconobacterium* spp., *Lactobacillus* spp., *Micrococcus* spp., *Paediococcus* spp., *Peptostreptococcus* spp., *Pseudomonas* spp., *Streptococcus* spp. and *Zymomonas* spp. (Nwachukwu *et al.*, 2006). The drop in ethanol content (down to 2 or 3% v:v) between three and 20 days suggest bacteria that metabolize ethanol are active during this period. This, and the fact that pH drops to 1–2 during the same period, indicates the action of *Acetobacter* species (Chapter 2.4). Certainly, *Acetobacter* are present in the fresh sap and in the wine after 72 h of fermentation (Nwachukwu *et al.*, 2006).

It appears that the optimum time for consumption of palm wine is around three days after the start of fermentation, when the acidity is still reasonable (pH ~4), the ethanol content is at its maximum (5.5–8.3% v:v) and there is still some residual sweetness (~3 mg/ml reducing sugars). As with other indigenous alcoholic beverages (see shakporo, pulque (this section) and neera or toddy (Section 3.5.5)), interest is

growing in industrialized production of the beverage, but in the case of African palm wine, there has been interest expressed in the production of industrial ethanol (bioethanol) via the distillation of palm wine (Nwachukwu *et al.*, 2006). Bioethanol is an important and rapidly growing business on at least two continents, where its production competes with the production of certain alcoholic beverages (e.g. cachaça in Brazil). Bioethanol from palm wine could be realized using local palm sap fermented with a starter made of suitable microorganisms (those giving maximum ethanol production) and distilling the wine at three days into its fermentation. Measurement of palm wine viscosity may help decide optimum consumption or distillation time, since viscosity has been found to drop during active alcoholic fermantation, leveling off at its termination (Osuwa and Mbamara, 2008).

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2.8

Cider and Perry

An apple-mill and press had been erected on the spot, to which some men were bringing fruit from divers points in mawn-baskets, while others were grinding them, and others wringing down the pomace, whose sweet juice gushed forth into tubs and pails.

—Thomas Hardy (The Woodlanders)

2.8.1 Introduction

It is likely that cider and perry have a similar antiquity to beer and wine. There is mention of apple orchards in *The Odyssey* (*ca.* 900 BC) and in other Greek and Roman literature there is ample reference to the production of alcoholic drinks from apples, pears and other fruits. Modern apple varieties are descended from two wild apple species, *Malus silvestris* and *Malus pumila*, which still grow in western Asia. Over the years, favorable varieties (those that give good yields of well flavored fruit, with a good balance of sweetness, sourness and bitterness) became preferentially cultivated and then propagated, mainly by grafting. Later, cross pollination produced newer varieties.

The development of cultivated pears probably followed a similar track; perhaps modern European varieties are descended from hybrids of *Pyrus communis*, *P. heterophylla*, *P. korschinskyi* and others. It is probable that alcoholic drinks made from apples and pears have been produced in almost every country of Europe and western Asia at some time or other.

The Hebrews drank 'Shekhar' and the Greeks drank 'Sikera,' but it is not until the fourth century that Palladius and Saint Gerome gave more detailed accounts of 'Sicera' and 'Piracium' (cider and perry). Nowadays, production of cider and perry is most important in countries that once formed the western boundaries of Celtic culture: Asturias (Spain), western British Isles, Brittany, Euskadi (the Basque country) and Normandy. Both cider and perry are made in other countries all over the world, particularly in those that have historical links with certain of the above-mentioned European countries, such as Canada and the USA (Section 2.8.8). Although at the time of writing (2009), the UK is the world's biggest producer of cider, the world's greatest apple producer is China (well over 2 billion tonnes per annum) (Xu *et al.*, 2007). Much of the apple juice concentrate that (mostly) large-scale cider manufacturers use for their base ciders (for blending) is from China (Section 2.8.6).

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

Traditional cider is the fermented juice of freshly crushed and pressed apples and perry is the fermented juice of freshly crushed and pressed pears. In the USA and some other countries, such as South Korea, the word cider refers to the unfermented juice of crushed apples or to some nonalcoholic drink based (at least in part) on apples. The term 'hard cider' is used in the United States for the alcoholic beverage described in this chapter. Almost any variety of apple or pear can be made into cider or perry, but naturally, some varieties give better results than others, as judged by balance of acidity, sweetness, bitterness, alcoholic strength and aroma/flavor characteristics.

It is generally accepted that the best varieties are those that are categorized as 'cider apples' or 'perry pears.' There are literally hundreds of these (although only a few dozen are used on a regular basis nowadays), many of which are of great antiquity and have survived into the present day simply because they have consistently produced good alcoholic beverages. Other newer varieties have been bred for the specific purpose of cider or perry making. Having stated this, some cider makers in the 'cider apple territory' of the west of England use culinary (e.g. Bramley Seedling) and dessert apples (e.g. Cox's Orange Pippin) for the production of both 'varietal' ciders and blends.

In Sections 2.8.4–2.8.6, it will be seen that blending is important in cider and perry manufacture. Moreover, good ciders can be made from culinary and/or dessert apples alone, as witnessed by the produce of southern and eastern England, the USA, Austria, Canada and Germany. These tend to be lighter and more akin to wine than cider or perry made from cider pears and perry pears, as in western England, northwestern France and northern Spain.

Apart from choice and blending of varieties, the variations in flavor and style arise mainly from the method of manufacture. The use of spontaneous (wild) fermentation, cultured yeasts, the speed of fermentation, use of additives and fruit juice concentrates, the mode and length of maturation, use of finings, filtration and pasteurization, and use of artificial carbonation all have influences on the nature of the finished product.

The distinctions between cider and apple wine, and between perry and pear wine are not clear. Traditional cider and perry tend to have more flavor (including 'bite'), body and color than products labeled as apple or pear wines, due to the greater use of fruit with high polyphenol content, the occurrence of spontaneous fermentation and a certain amount of oxidation. It is known that certain phenolic substances of apples undergo partial oxidation (catalyzed by polyphenoloxidase, PPO). The main reactant is chlorogenic acid (caffeoylquinic acid, see Figure 2.8.2): *o*-quinones are produced initially, which then undergo reaction with caffeoylquinic acid and flavan-3-ols, such as (+)-catechin, but dimeric flavan-3-ols are also produced (Bernillon *et al.*, 2004).

Mass produced cider and perry, as well as wines, tend to be made in a more tightly controlled, less oxidizing manner and are generally lighter in flavor, body and color. Diluted juice concentrate may have been used, along with glucose syrup and a lower proportion of high tannin fruit. Fermentation will have taken place using cultured yeasts and sulfur dioxide will have been used as a sterilant and antioxidant. It is also likely that the product has been filtered and/or pasteurized. Furthermore, apple and pear wines may be made from juice concentrates or from whole fruit by water infusion, along with addition of sugar syrup, acids and even concentrated grape juice.

Research into a range of aspects concerning cider apples, cider and related drinks is or has been conducted at numerous institutes and universities throughout the world. They include the following: Institut National de la Recherche Agronomique (INRA), Le Rheu, France; Université de Caen, France; Servicio Regional de Investigacíon y Desarrollo Agroalimentario (SERIDA), Villaviciosa, Spain; University of Oviedo, Spain; Euskal Herriko Unibertsitatea, Bilboa, Spain; Long Ashton Research Station, University of Bristol, UK; AFRC Institute of Food Research (Norwich and Reading, UK); East Malling Research Station, UK; Cornell University, Ithaca, NY, USA; University of Bath, UK; University of Glasgow, UK; University of Ontario, Canada. Some of the above (e.g. INRA, SERIDA) have departments or sections dedicated to cider, others have research groups whose interests include some aspect of cider.

2.8.2 The Basic Ingredients

Cider apples are classified according to their acidity (which gives sharpness of flavor), phenolic compound ('tannin') content (which gives bitter taste and astringent mouthfeel) and sugar content. The four basic categories are sharp, bittersharp, bittersweet and sweet. EU classification is according to titratable acidity, as malic acid, and phenolic ('tannin') content; it is summarized with examples for each category in Table 2.8.1. Ciders are usually made from a blend of apples from the different categories, the composition of the blend depending on the wish of the producer, the prevailing traditions or commercial demands on the particular area of production (see Section 2.8.8). Despite this, there are examples of single and double variety ciders, especially from smaller, traditional producers. Perry pears are classified in a similar manner to cider apples, but with rather more ambiguity, owing to greater variation in acidity and phenolic compound content (i.e. there is more overlap between categories). Nonetheless, a classification devised originally by Pollard and Beech is widely used in the UK. This is summarized, with examples and typical sugar contents in Table 2.8.2.

Unlike apples, several pear varieties possess appreciable citric acid content. English varieties of this kind include Brown Bess, Gin, Oldfield, Taynton Squash and Yellow Huffcap (all with >0.3% w:v citric acid) and

Table 2.8.1 *Classification of cider apples in terms of titratable acidity (TA), and total phenolic content (TPC), with typical examples*

Class	Titratable acidity range (%w/v)	Total phenolic content range (%w/v)	Examples, with some typical values of OG, TA and TPC			
			Variety*	OG (°Brix)	TA (%w/v)	TPC (%w/v)
Sharp	>0.45	<0.20	Baldwin (U/C)	11.4	0.74	0.06
			Bramley's Seedling (E)	–	0.85	0.08
			Cox's Orange Pippin (E)	–	0.60	0.07
			Golden Russet (U/C)	17.0	0.55	0.04
			Judor (Fr)	–	–	0.11
			Raxao (S)	–	0.6	0.10
			Roxbury Russet (U/C)	15.2	0.71	0.06
Bitter-sharp	>0.45	>0.20	Foxwhelp (E)		1.91	0.22
			Kermerrien (Fr)	–	–	0.38
			Kingston Black (E)	12.6	0.58	0.19
			Meana (S)		0.5	0.3
Bitter-sweet	<0.45	>0.20	Binet Rouge (Fr)	–	0.15	0.24
			Colorodona (S)		0.1	0.2
			Dabinett (E)	14.9	0.18	0.43
			Michelin (Fr/E)	–	0.25	0.23
			Somerset Redstreak (E)	–	0.19	0.28
			Tremletts Bitter (E)	12.4	0.27	0.38
			Yarlington Mill (E)	13.5	0.22	0.46
Sweet	<0.45	<0.20	Bedan (Fr)	–	–	0.34
			Durón Arroes (S)	–	0.3	0.1
			Sweet Alford (E)	–	0.22	0.15

*E = England, Fr = France, S = Spain, U/C = USA/Canada

Table 2.8.2 *Typical original gravities (OG), titratable acidities (TA) and total phenolic contents (TPC) of English perry pears*

Class	Titratable acidity range (%w/v)	Total phenolic content range (%w/v)	Examples, with typical values of OG, TA and TPC			
			Variety	OG	TA (%w/v)	TPC (%w/v)
Bittersharp	>0.45	>0.20	Barland	1058	0.92	0.26
			Butt	1056	0.54	0.54
			Moorcroft	1066	0.50	0.17
			Oldfield	1065	0.73	0.15
			Rock	1068	0.51	0.98
Medium sharp	0.20–0.60	<0.15	Blakeney Red	1056	0.44	0.13
			Brandy	1069	0.44	0.12
			Parsonage	1061	0.47	0.09
			Red Longdon	1059	0.60	0.11
			Taynton Squash	1058	0.45	0.13
			Yellow Huffcap	1064	0.62	0.10
Bittersweet	<0.45	0.20	Flakey Bark	1066	0.43	0.54
			Nailer	1051	0.37	0.23
			Thurston's Red	1069	0.44	0.39
Sweet	~0.2	<0.15	Barnet	1052	0.28	0.09
			Hendre Huffcap	1059	0.37	0.08
			Red pear	1055	0.29	0.09
			White Longdon	1063	0.39	0.14

Barland, Holmer, Parsonage, Pine and Rumbles (all with <0.3%). The major acid of apples and most pears is (*S*)-(–)-malic acid, but minor quantities of shikimic acid, pyruvic acid and others are present in the fruit, along with sugar acids (e.g. gluconic acid), especially in riper fruit. It can be seen from Tables 2.8.1 and 2.8.2 that the total acid content for both cider apples and perry pears is from 0.2 to over 0.6% (w:v).

Almost all the sugars of cider apples are either monosaccharides or disaccharides, nearly all of which are fermentable by yeast, so there is normally very little residual specific gravity left in fully fermented ciders. The major sugars are fructose, sucrose and glucose (*ca.* 74%, 15% and 11% w:v, respectively, on average). The original gravities of most cider apple musts are in the region of 1050–1060, giving about 6–7.5% alcohol by volume when fully fermented.

Perry pears tend to possess rather more sugar than cider apples; many perry musts have original gravities over 1060 (Table 2.8.2). However, pear juice, unlike the juice of most apples, contains significant quantities of nonfermentable sugar alcohols, the most abundant of which is sorbitol. Thus, final gravities of perry may well be over 1005, giving a little residual sweetness, but with alcoholic strengths comparable with those typical of cider. Both apple and pear musts contain starch and soluble pectin (polygalactouronate). The former diminishes significantly upon full ripening, which is the main reason for the traditional process of sweating: storing harvested fruit in a dry place for several days to two weeks before crushing and pressing. Amylases convert the starch to monosaccharides during this period.

The phenolic components (especially the polyphenols) of both apples and pears are important; it is these that give cider and perry their characteristic bitter (even astringent) tastes. The major classes of phenolic

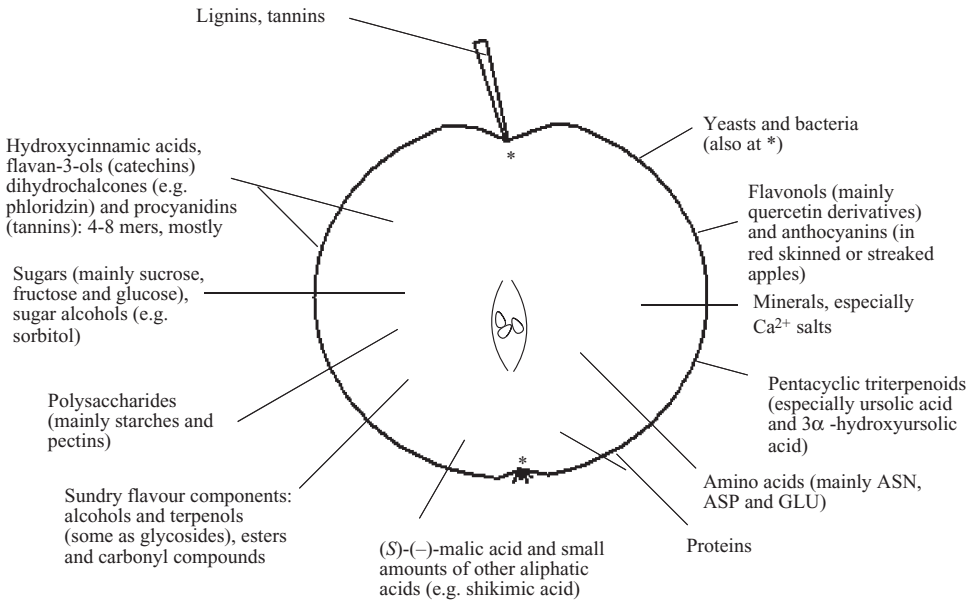


Figure 2.8.1 *Major components of cider apples and their approximate locations*

compounds in cider apples are hydroxycinnamic acids, dihydrochalcones, flavan-3-ols and procyanidins (oligomers and polymers of flavan-3-ols), found in both the skins and flesh (see for example Sanoner *et al.*, 1999; Guyot *et al.*, 2003). To these can be added flavonols and anthocyanins, found mostly in the skins. Figure 2.8.1 illustrates the distribution of important chemical components of apples, including the phenolic substances. Note that, except for anthocyanins and flavonols, phenolic substances are found in both the flesh and skins; they are also found in pips and stalks, but normal crushing and pressing methods should release very little of these.

Typical culinary and dessert apples tend to have lower (sometimes much lower) total phenolic contents (TPC) than typical cider apples. For example, juices made from German cider apples possessed from *ca*. 800–1000 mg/l TPC (Boskoop and Brettacher) to *ca*. 300 mg/l TPC (Kaiser Alexander and Kaiser Wilmhelm) (Kahle *et al.*, 2005). Compare these figures with those in Tables 2.8.3 and 2.8.4.

Table 2.8.3 *Cortex polyphenolic content (mg/kg of fresh fruit) of some French cider apples (with one English cider apple and a popular dessert apple for comparison)*

Variety	Chlorogenic acid	*p*-Coumaryl quinic acid	Phloretin xyloglucoside	Phloridin	(−)-Epicatechin	(+)-Catechin	Pro cyanidins
Bedan	649	147	12	30	473	154	1796
Binet Rouge	601	176	14	37	202	39	1254
Dabinett	390	53	22	102	393	33	2417
Douce Coët Ligné	1195	80	98	68	376	117	1499
Golden Delicious	132	20	11	15	88	14	761
Judor	338	59	10	16	86	20	515
Kermerrien	917	95	35	48	434	45	2932

Source: Data from Sanoner *et al.* (1999).

Table 2.8.4 *Major phenolic components (mg/kg of fresh fruit) of skins and flesh (in parentheses) of selected English cider apples*

Variety	Cyanidin-3-galactoside	(−)-Epicatechin	(+)-Catechin	Procyanidin B2*	Chlorogenic acid	Phloridzin	Quercetin glycosides (total)
Browns Apple	494 (12)	311 (129)	64 (29)	242 (125)	266 (429)	130 (26)	1149 (9)
Broxwood Foxwhelp	236 (12)	294 (nd)	24 (6)	111 (nd)	351 (407)	153 (54)	432 (6)
Bulmers Norman	nd (nd)	833 (684)	55 (37)	812 (571)	959 (745)	1061 (143)	1192 (6)
Dabinett	160 (nd)	868 (543)	52 (36)	567 (412)	198 (455)	528 (159)	1115 (8)
Michelin	nd (nd)	687 (498)	102 (71)	462 (345)	407 (699)	222 (63)	424 (11)
Somerset Redstreak	53 (nd)	424 (399)	23 (18)	211 (297)	273 (447)	350† (60)	396 (6)
Tremletts Bitter	458 (nd)	1285 (1412)	44 (43)	904 (847)	243 (349)	296 (16)	604 (13)
Yarlington Mill	149 (nd)	2095 (748)	265 (408)	1037 (334)	1163 (1766)‡	90 (38)	918 (27)

Source: Data from Marks *et al.* (2007).
*Other procyanidins not included
nd = not determined
†Of these varieties, also contains high levels of phloretin-2′-xyloglucoside
‡Of these varieties, also contains high levels of *p*-coumaroylquinic acid

The most important hydroxycinnamic acids are caffeoylquinic (chlorogenic acid) and *p*-coumaroylquinic acids (Figure 2.8.2). The major dihydrochalcones are phloridzin (phloretin-2-glucoside) (Figure 2.8.2) and phloretin-β-xyloglucoside. Although the most abundant flavan-3-ol is (–)-epicatechin (Figure 2.8.2), (+)-catechin is present in fair amounts in some cider apples. Table 2.8.3 displays some individual polyphenol contents of some French cider apples (Sanoner *et al.*, 1999) and Table 2.8.4 lists similar data for selected English cider apples (Marks *et al.*, 2007). The proanthocyanidins are essentially polymers of flavan-3-ols, mostly (–)-epicatechin, and range from dimers, trimers and tetramers through to polymers with seven or more units, up to 60 or so. An especially common proanthocyanidin is procyanidin B2 (Figure 2.8.2; Table 2.8.4).

The phenolic components of pears are quite different; flavan-3,4-diols (leucoanthocyanins) and their colloidal polymers predominate in the cortex (up to 1% w:v) These polymers may be responsible for the hazes and sediments that are characteristic of perry, rather than of cider.

Both apples and pears contain flavonols in their skins, the most abundant of which are quercetin glycosides (Figure 2.8.2; Table 2.8.4) (Marks *et al.*, 2007). Isorhamnetin-3-glucoside, other glycosides (including acylated ones) and diglycosides of isorhamnetin are present in pear skins, but in apple skins only isorhaminetin-3-glucoside and 3-galactoside have been found, in the German cider variety Brettacher (Schieber *et al.*, 2002). Again, flavonol contents of peel and flesh of cider apples tend to be greater than those of culinary or dessert apples, but some of the latter (e.g. Cox's Orange Pippin and Jonagored) have comparable flavanol contents (Price *et al.*, 1999).

Anthocyanins are present in red or dark skinned fruit and in the cortex of the small minority of red fleshed fruit. However, total anthocyanin content of apple must is rather low, usually less than 50 mg/l, and not much of this survives fermentation through to maturation because of the formation of oligomers with other polyphenols, notably (–)-epicatechin. The major anthocyanins of apple and pear skins are based on cyanidin and are illustrated in Figure 2.8.3. The anthocyanin content of apple and pear skins varies widely (witness the range of skin colors) and although the cortex of most fruit is essentially colorless, there are some varieties with red flesh, either just under the skin or more rarely, throughout (e.g. apple variety Red Field) (Espley *et al.*, 2007). Anthocyanin synthesis in apples, like other fruits, is controlled by an MYB transcription factor, MdMYB10, along with two bHLH proteins (MdbHLH3 and MdbHLH33) that are expressed along with MdMYB10 (Espley *et al.*, 2007).

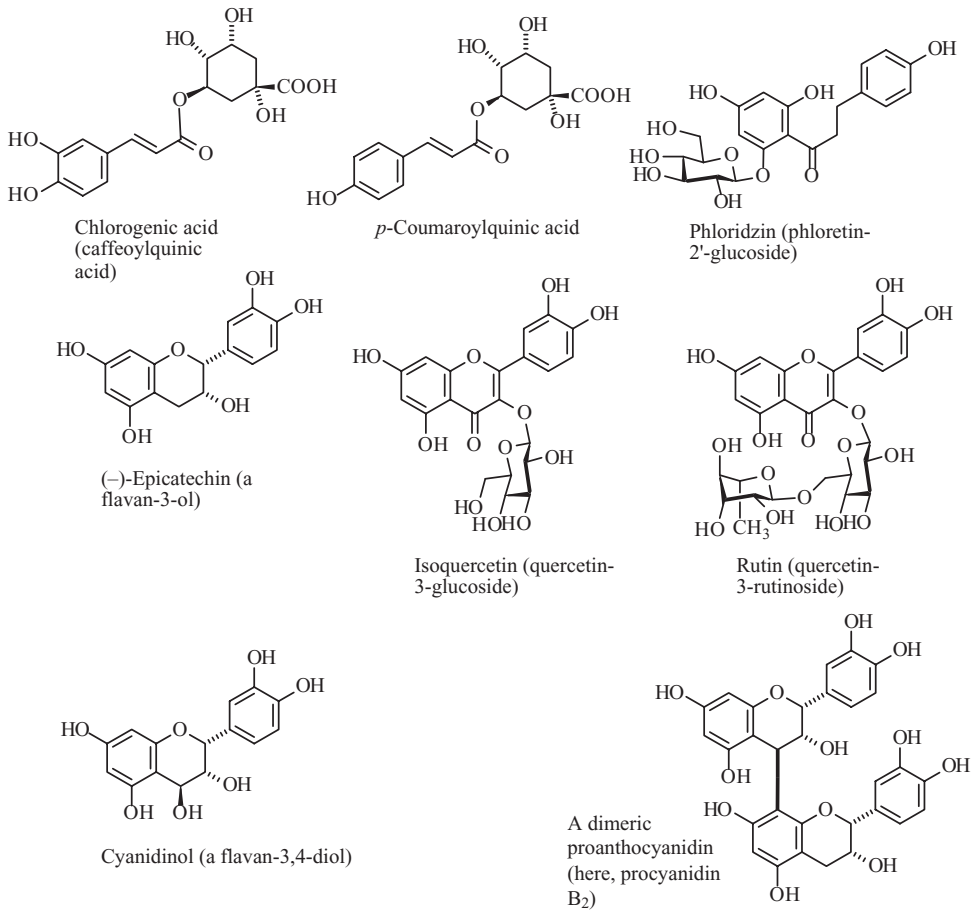
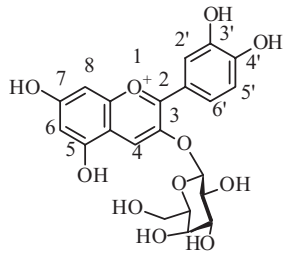


Figure 2.8.2 *Typical phenolic compounds in apples and pears*

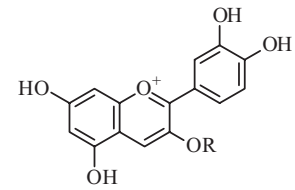
Both apple and pear juices contain rather low levels of amino acids (mainly asparagine, aspartic acid and glutamic acid) and proteins, with pear juice generally being the poorer of the two. Thus, compared with beer wort (Sections 2.6.3 and 2.6.4), apple and pear must is rather poor in yeast nutrients. Proteins are also present in apple must; five major proteins have been found in Asturian apple must, with molecular weights between 16 000 and 110 000 Da, with one of molecular weight 36 400 Da being the most abundant (Blanco-Gomis *et al.*, 2003a). As with beer, some of these proteins contribute to the formation of foam (Section 2.6.9), whilst others are involved in the formation of hazes in media that are generally rich in phenolic substances (Section 2.6.9).

Similarly, apple and pear juices are rather low in mineral content, the most abundant species being Ca^{2+}, K^+, NH_4^+, Cl^-, SO_4^{2-} and phosphates. Calcium ions play an important role in the traditional process of défécation or keeving (Section 2.8.3), in which they form a gel with pectins.

Various pentacyclic triterpenoids, such as ursolic acid and 2α-hydroxyursolic acid, are found in apple skins. These may contribute to the proposed anticancer activity of apples; certainly many have potent antiproliferative activity against cancer cells *in vitro* (He and Lui, 2007) (Section 5.11.2). However, it is unclear how much of these substances are extracted from the skins and survive into the mature cider.

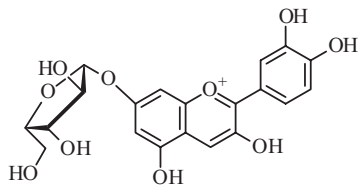


Cyanidin-3-galactoside (most abundant apple anthocyanin

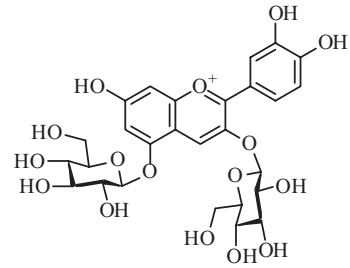


Other cyanidin-3-glycosides

R =arabinosyl, xylosyl, glucosyl, 3"-acetylglucosyl



Cyanidin-7-arabinoside



Cyanidin-3,5-diglucoside

Figure 2.8.3 *Anthocyanins in dark apple skins. Gómez-Cordovés* et al. *(1996).*

The major fungal microorganisms present on the skins of ripe, undamaged apples and pears are *Aureobasidium pullulans*, *Candida* spp., *Hanseniaspora valbyensis*, *Kloeckera apiculata*, *Metschnikowia* spp., *Saccharomyces* spp. (especially var. *cerevisiae* and *uvarum*), *Saccharomycodes ludwigii* and *Torulopsis* spp. In the spontaneous fermentations used to make traditional cider and perry, *S. uvarum* or *cerevisiae* eventually dominates because of its greater ability to live in a sweet alcoholic medium (Section 2.8.5). Bacteria are generally present on the fruit skins in lower numbers than fungal species. These include *Acetomonas* spp. and a variety of malolactic bacteria, such as *Leuconostoc mesenteroides*, *Lactobacillus collinoides*, *Lb. hilgardii*, *Lb. plantarum*, *Oenococcus oeni* and *Pediococcus cerevisiae*.

Various pathogenic bacteria can also be found on the skins and outer flesh of apples and pears, especially on stored fruits that have been bruised or damaged (Basaran *et al.*, 2004). These include *E. coli* O157:H7, *Salmonella* and *Listeria monocytogenes* (Chen *et al.*, 2004). Although the presence of these organisms may have been responsible for outbreaks of disease caused by drinking unfermented juice (called cider in North America) (Basaran *et al.*, 2004), there is no evidence whatever that they survive in the alcoholic medium of the fermented juices. Indeed, in earlier centuries, cider, perry and alcoholic beverages in general were drunk in preference to water because of much greater possibility of contamination by pathogenic bacteria of the latter.

2.8.3 Harvesting, Crushing and Pressing

Different varieties of apples and pears ripen at different times and hence are sometimes harvested separately, made into cider or perry and then mostly blended, although a certain amount may be sold as varietal

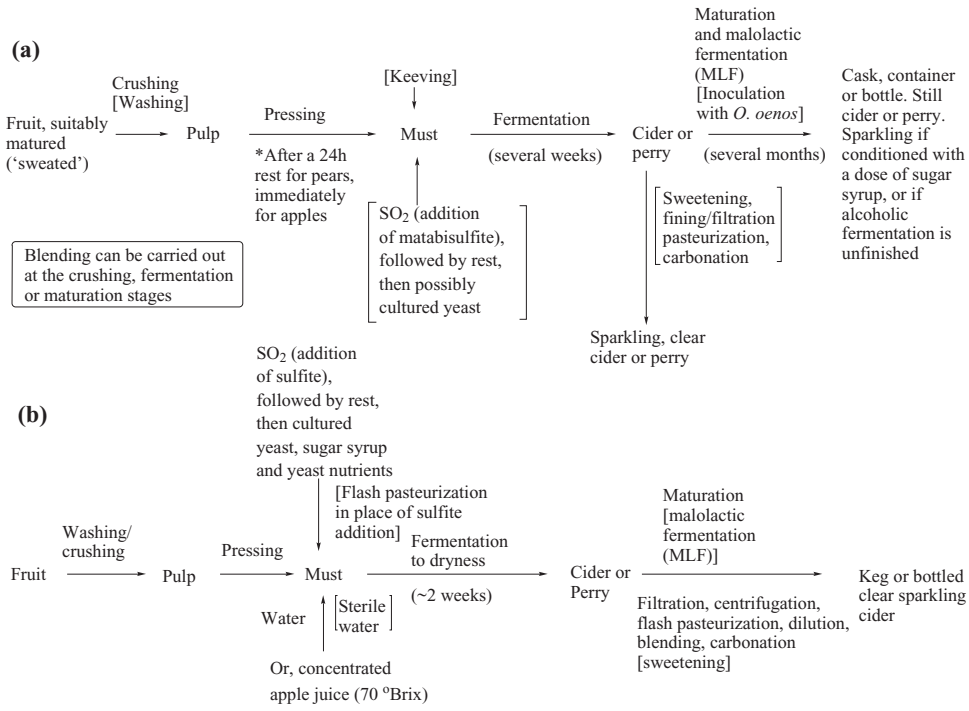


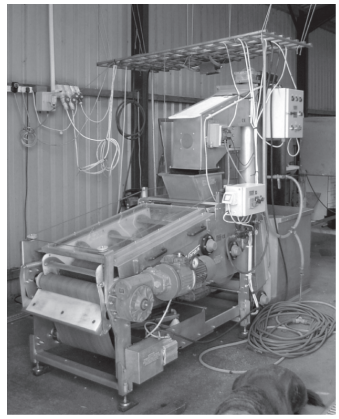
Figure 2.8.4 *Flow diagrams illustrating the production of typical (a) traditional/small-scale and (b) mass produced cider and perry. Optional modifications of the most basic traditional process are shown in square brackets. In practice, combinations of (a) and (b) may be used*

drinks. Traditional, small-scale and mass production methods of cider and perry production are outlined in Figure 2.8.4.

Cider apples are often harvested by shaking the trees, where the 'ripe' apples fall into a plastic sheet or net. Sometimes they are left for a week or so spread out on shelves in a dark room before crushing, in order to gain a bit more maturity. This is sometimes called sweating. Pears have more of a tendency to rot from the core, so are sometimes given a shorter maturation rest, if it all, depending on the ripeness and softness of the fruit. The maturing time for apples allows extra sugar and flavor components to form, and at the same time softens the fruits, making them easier to crush.

Crushing (grinding or milling) is traditionally carried out in a 'scratcher,' a mill consisting of two studded rollers that turn in opposite directions. These mills are rather like grape crushers and can be hand operated or electrically driven. They tend to give rather coarsely crushed fruit, in contrast to the fine pulp produced by more modern centrifugal mills (Figure 2.8.5). Fruit, introduced via the hopper is pulverized by a rotor blade system in the milling chamber, whence the pulp is discharged (sometimes through a perforated mesh screen) into a pomace trough, where it can be conveyed or pumped to the press for the next stage.

Modern cider producers often use a combined washer/crusher/press system (Figure 2.8.5), where the washed fruit is continuously elevated into the mill system, and the resulting pulp pressed by a continuous belt press (see next paragraph). Machines like the ones described above can work at a rate of *ca.* 300–6000 kg of fruit per hour. The combined system has the advantage of minimum exposure to air, as the pulp is pressed immediately. However, this system may not be advantageous in the production of perry, where a 24 h time



Combined crusher/presser at Whin Hill Cider, Wells-Next-The-Sea, UK.

Disused traditional cider press at Whin Hill Cider.

Speidel centrifugal mill for apples, pears and other hard fruit. *Photograph by Courtesy of Vigo Ltd.*

Rack and cloth press. *Photograph by courtesy of Vigo Ltd.*

Figure 2.8.5 *Cider/perry crushing and pressing equipment. Photo courtesy of Vigo Ltd, Devon, UK*

lapse is desirable between crushing and pressing, in order to reduce the quantity of precipitated polymeric phenols.

The traditional cider press, which can also be used for perry, is the rack and cloth press (Figure 2.8.5), where the apple pulp is wrapped completely in strong, open weave cloths which are then placed between two slatted wooden boards (stacks), the whole being known as a 'cheese.' Several cheeses of pulp can be pressed at the same time, depending on the pressure capability and design of the press, and the throughput of fruit pulp to juice depends on the cloth and rack sizes. Pressure is applied gradually, either by a manually operated rack and pinion system or, more likely nowadays, by an electrically operated hydraulic system. In this way, pressures of up to *ca.* 30 tonnes/m^2 may be applied and throughput can be anywhere between 0.5 and 6 tonnes per day.

Belt presses have a much higher turnover than rack and cloth presses. Here, fruit pulp is fed continuously onto a rotating porous belt, where it is pressed against a series of stainless steel rollers (see Figure 2.8.5). Throughput from this type of press can be up to 6 tonnes per hour and pressing efficiency (production of juice from pulp) is comparable to a typical rack and cloth press. Pomace conveyors can be used to move the dry spent pomace from the discharge side of belt presses, thus completing the automation and removing the labor

that is involved with shoveling and shifting spent pomace. When using rack and cloth presses, the pomace is pressed until it is solid and no more juice is produced. The press is then racked up, the cheeses are opened and the pomace mass broken up by hand to be reracked and repressed. Spent pomace can be used as cattle feed, orchard fertilizer, or when dried, it can be used for the manufacture of pectin.

Despite the several outlets mentioned above, spent pomace remains something of an environmental problem, especially in areas such as Asturias, where in excess of 20 000 tons are produced annually. The pomace is high in dietary fiber and polyphenols and is known to have considerable *in vitro* antioxidant activity; the Folin index of pomace from a large number of Asturian cider makers was 2.3–15.1 g gallic acid/kg of dry matter (Diñeiro-García *et al.*, 2009). Hence, there is considerable interest in the use of pomace for human nutritional, pharmaceutical and cosmetic purposes. Diñeiro-García *et al.* (2009) showed that apple pomace had antioxidant capacities, according to the DPPH and FRAP methods (Section 4.4.3), of 4.4–16.0 g ascorbic acid/kg of dry matter. Partial least squares regression analysis gave reliable statistical models, allowing prediction of antioxidant activity (by DPPH, FRAP) as a function of phenolic profile (by HPLC), with phloridzin, procyanidin B2, rutin, isoquercetin, protocatechuic acid and hyperin having the higher modeling power (in that order).

Several treatments to the apple or pear juice can be applied before fermentation begins. The first of these is called 'keeving,' used on apple juice by cider manufacturers in Brittany and Normandy. Keeving (from the French cuvee) or défécation often involves the addition of calcium chloride and pectin methylesterase (PME) to the juice (see Section 2.8.6 for variants of this process and more detail). This causes denaturation and precipitation of proteins, with some hydrolysis of pectins. The proteins are precipitated as a skin on the surface of the must and are subsequently skimmed off. Keeving thus aids the clearing process, but at the same time reduces the nitrogen nutrient content of the juice. This leads to a slow fermentation of the must (typically over several weeks), which is often incomplete, leaving a relatively low alcohol cider (~5% ABV) with residual sugars. This is an important process for French cider manufacturers (although it was developed from smaller-scale producers) because the relatively low alcohol, semi-dry or semi-sweet ciders with complexity of flavor are seen by many as the goal for high quality (see Section 2.8.6).

Other treatments include the addition of sodium or potassium metabisulfite (Chapter 2.5, Section 2.8.4) or application of flash pasteurization, adjustment of acid content, addition of sugar syrup, addition of yeast nutrients and blending of other freshly pressed juices or diluted apple juice concentrate, depending on the style of the producer.

2.8.4 Fermentation and Maturation

This section is closely linked with the following two sections, where specific technical aspects of alcoholic, malolactic fermentation and a number of process aspects (fining, filtration, sulfiting, carbonation and others) can be compared for traditional and small-scale production (Section 2.8.5) and large-scale (factory) production (Section 2.8.6). In the present section, a description is given of alcoholic, malolactic and other fermentations as they relate to cider and perry. A more complete general account of these fermentations (particularly of the organisms, the metabolic pathways and the biochemical end products) can be found in Chapters 2.1–2.4.

The most important microbial agents of alcoholic fermentation in the production of cider and perry are strains of *Saccharomyces cerevisiae*, as in the making of most other alcoholic beverages. However, in the spontaneous fermentations used for traditional cider in northern Spain, northwest France and western England, important contributions may be made from *Kleockera apiculata*, *Hanseniaspora valbyensis* and *Saccharomycodes ludwigii,* as well as other species of the genera *Saccharomyces* and *Kloeckera*. Nevertheless, *S. cerevisiae* dominates and the metabolic pathways associated with this species give rise to similar end products as found in wine (Section 2.2.11) and fruit wine (Section 2.11.3).

Table 2.8.5 *Most aroma active compounds of North American and Chinese cider*[‡]*)*

Aroma compound	Flavour descriptor	Aroma compound	Flavour descriptor
Butanoic acid	Cheesy	4-Vinylphenol[§]	Smoky
2-Methylbutanoic acid	Cheesy	Ethyl butanoate	Pineapple
Octanoic acid	Sweaty	Ethyl 2-methylbutanoate	fruity
2-Phenylethanol	Rose	Ethyl hexanoate	Floral
Eugenol[§]	Clove	Ethyl decanoate[§]	Grape
4-Ethylguaiacol[§]	Clove	2-Phenylethyl acetate[*]	Rose

Source: Data from Xu *et al*. (2007).
[‡]Chinese cider made from Fuji, Gala, Ralls and Starking apples.
[§]Much more pronounced in N. American cider.
[*]Much more pronounced in Chinese cider.

A brief list of the more flavor active compounds in cider is given in Table 2.8.5, most of which are metabolic end products of alcoholic or malolactic fermentation (Xu *et al.*, 2007). The main alcohols (apart from ethanol) produced during alcoholic fermentation of apple must are isoamyl alcohol, 1-butanol, isobutanol, methanol, 2-phenylethanol and 1-propanol (Vidrih and Hribar, 1999). The process of maceration (allowing a period of time – usually 24 h – between crushing and pressing) (Section 2.8.3) results in somewhat increased production of these alcohols during fermentation (Vidrih and Hribar, 1999), presumably because of increased absorption of oxygen. Maceration is carried out by traditional Spanish cider makers (and by some English traditional producers), whereas the related process of défécation (keeving) (Sections 2.8.5 and 2.8.6) is performed by cider manufacturers in Brittany and Normandy. The total quantity of fusel alcohols in cider was found to be inversely related to the nitrogen content of the apple must (Vidrih and Hribar, 1999) and also inversely related to the extent of sulfiting. Thus, traditional ciders tend be rather higher in these alcohols than ciders made from sulfited apple must that has been enriched with yeast nutrients (ammonium salts).

The standard yeast alcoholic fermentation metabolic pathways (Section 2.2.9) are not the only source of alcohols in cider; certain alcohols are also present in apple juice as nonvolatile glycosides. During fermentation, some of these glycosides are hydrolyzed to give free alcohols, which may be flavor active in their own right, or may react with other compounds during fermentation or maturation to produce flavor compounds (Lea, 2004; Lea and Drilleau, 2003). Included here are 2-phenylethanol and octane-1,3-diol. The former is a well known flavor compound (rose, honey notes) found in many fruit juices and wines, whereas the latter may be unique to apples. During fermentation and maturation, octane-1,3-diol and the related (5*Z*)-octene-1,3-diol form cyclic acetals (1,3-dioxolane rings) by reaction with acetaldehyde, a major fermentation metabolite (Figure 2.8.6). The dioxolanes are reckoned to be important contributors to cider flavor (cidery, green notes) (Lea, 2004).

Other aroma and flavor active compounds in cider are esters, principally ethyl butanoate, ethyl hexanoate, ethyl 2-methylbutanoate and 2-phenylethyl acetate (Xu *et al.*, 2007). Mostly the end products of yeast metabolism during alcoholic fermentation, these contribute significant fruity and floral notes, as in other alcoholic beverages (Sections 2.2.11 and 2.6.4). A number of carboxylic acids are produced during alcoholic fermentation, although many at levels that (by themselves) contribute little to aroma and flavor (volatile acids) or taste (nonvolatile acids). The nonvolatile acids produced include fumaric acid, lactic acid (mostly the (*R*)-isomer), malic acid, shikimic acid and succinic acid. Some volatile carboxylic acids contribute cheesy notes, the two most abundant of which being butanoic and octanoic acids, although a number of others are also present (Xu *et al.*, 2007). Amongst the hundreds of other substances present in cider

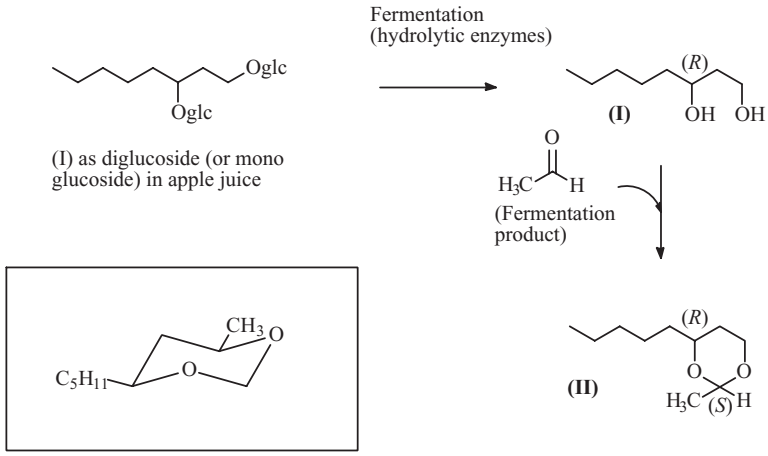


Figure 2.8.6 *Formation of (2S,4R)-4-pentyl-2-methylcyclohexa-1,3-dioxolane (II) from (3R)-octane-1,3-diol (I) and acetaldehyde during cider production. These are the stereochemistries of the major products (I) and (II): the main conformation of (II) is shown in the square. Photo courtesy of Vigo Ltd, Devon, UK.*

(Lea, 2004), various volatile phenolic compounds contribute spicy, clove-like or smoky notes to the overall aroma and flavor of cider. This is especially noticeable in traditional or small-scale produce from cider apples in the UK, France and Spain, but some Canadian and American ciders also possess these flavor characteristics.

Ciders made from culinary or dessert apples, as in China and many other parts of the world, have fewer of these aromas and flavors in their profiles. The phenols contributing most to aroma and flavor include catechol, 4-ethylguiacol, 4-ethylphenol, isoeugenol, eugenol and 4-vinylphenol. These are produced in part by the shikimic acid pathway (via phenylalanine, cinnamic acid and others), during the ripening of fruit or during alcoholic fermentation and in part during the malolactic fermentation, by the decarboxylation, hydrolysis and reduction of various more complex natural phenolic substances in the must (Lea, 2004). Figure 2.8.7 illustrates routes to some of these phenols; thus 4-vinylphenol may be produced by the decarboxylation of *p*-coumaric acid, itself formed from the hydrolysis of *p*-coumaroylquinic acid, a natural phenol of apples. Some of these phenols and others, such as vanillin, may be derived from containment of the cider in wooden fermentation or maturation vessels, or from the effect of added oak chips. However, as with wine, leaching of phenolic (and other) substances from the pores of wooden vessels tends to fall off significantly with continued use of the vessels.

During malolactic fermentation (MLF), the major process is the conversion of (*S*)-(+)-malic acid to (*S*)-(–)-lactic acid and carbon dioxide (Chapter 2.3), leading to a softening of acidic character, as in wine. In traditional English and French cider production, alcoholic fermentation and MLF tend to be sequential, with MLF occurring any time after the finish of the former, or even during its final stages, depending on (amongst other factors) the temperature, acidity and quantity of nutrients present. For traditional British and French cider, the malolactic fermentation can be considered a part of the maturation process, since it frequently starts in the spring following the harvest.

On the other hand, there is good evidence to show that alcoholic and malolactic fermentation occur more or less simultaneously in the production of traditional Spanish cider (Lea and Drilleau, 2003). This may be because of the richer bloom (with higher populations of malolactic bacteria) on apples grown so much further south than those of northern France or western England, but is more probably because of the high

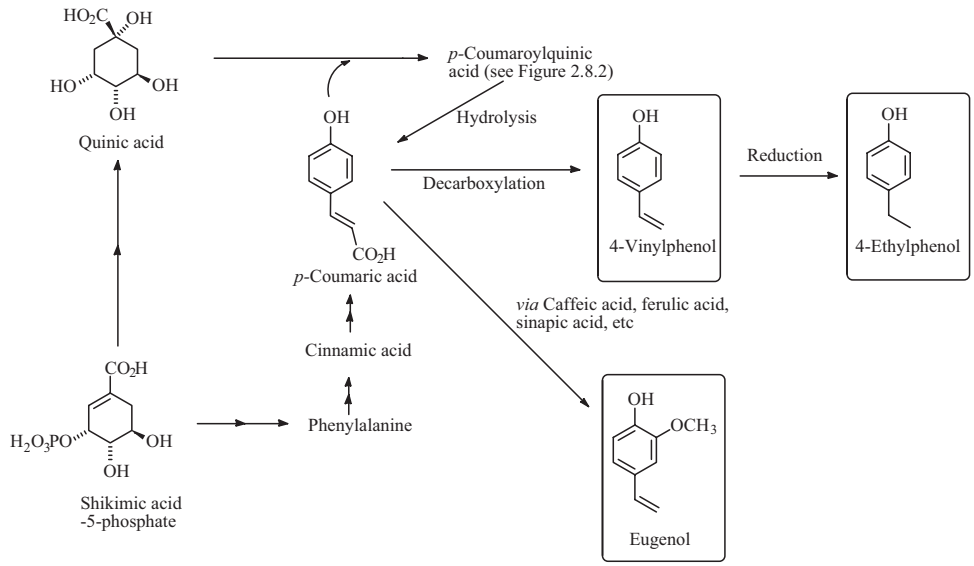


Figure 2.8.7 *Formation of some volatile phenols during alcoholic and malolactic fermentation of apple must. The nonvolatile phenolic precursors may be apple must constituents or fermentation products*

fermentation temperatures (~22 °C) often utilized by Spanish cider makers, and the high populations of malolactic bacteria that exist in the fermentation vats and other equipment.

The simultaneous fermentation by particular bacteria (largely *Oenococcus oeni*, *Leuconostoc* and *Lactobacillus* spp.) under Spanish conditions may explain the somewhat elevated levels of acetic acid in Spanish cider, compared with its English and French counterparts. Far from being a fault, this is much prized by Spanish manufacturers and drinkers alike (Section 2.8.8). However, research on Chardonnay must involving a comparison of sequential and simultaneous alcoholic fermentation/MLF (by inoculation at 19–20 °C) suggests that there is very little difference between the two, and acetic acid levels are only slightly elevated by the simultaneous fermentation procedure at this temperature (Jussier *at al.*, 2006).

As with wine, MLF leads to a large number of bacterial metabolic end products that are considered to give additional complexity of aroma and flavor. Probably the most important of these end products is diacetyl (2,3-butanedione), giving buttery or butterscotch notes below concentrations of *ca.* 5 mg/l. Much above this concentration, diacetyl spoils alcoholic beverages with a powerful rancid butter flavor. Diacetyl is produced in cider and perry by bacterial metabolism of citric acid, as in wine. *Lactobacillus* species produce α-acetolactate as an intermediate (compare with Figure 2.6.15, Section 2.6.4), whereas *Oenococcus oeni* and *Leuconostoc* spp. apparently do not. Otherwise, their metabolic pathways from citrate and pyruvate to diacetyl are similar (Figure 2.8.8).

O. oeni uses a route that involves direct coupling of the thiamine pyrophosphate (TPP) adduct (the 'active acetaldehyde') and acetyl coenzyme A (Figure 2.8.8). Besides diacetyl, acetoin (3-hydroxybutan-2-one) and butylene glycol (butane-2,3-diol) are also produced, but these are flavor inactive. The latter compound, however, when included with glycerol (produced during both alcoholic fermentation and MLF) may contribute to a smoother mouthfeel. Like many yeasts, *O. oeni* exhibits β-glucosidase activity and so nonvolatile glycosides of terpene alcohols and C_{13} norisoprenoids are hydrolyzed during MLF, thus releasing the aglycones to give spicy, floral and honey notes. In red wines, these aglycones include linalool, farnesol and

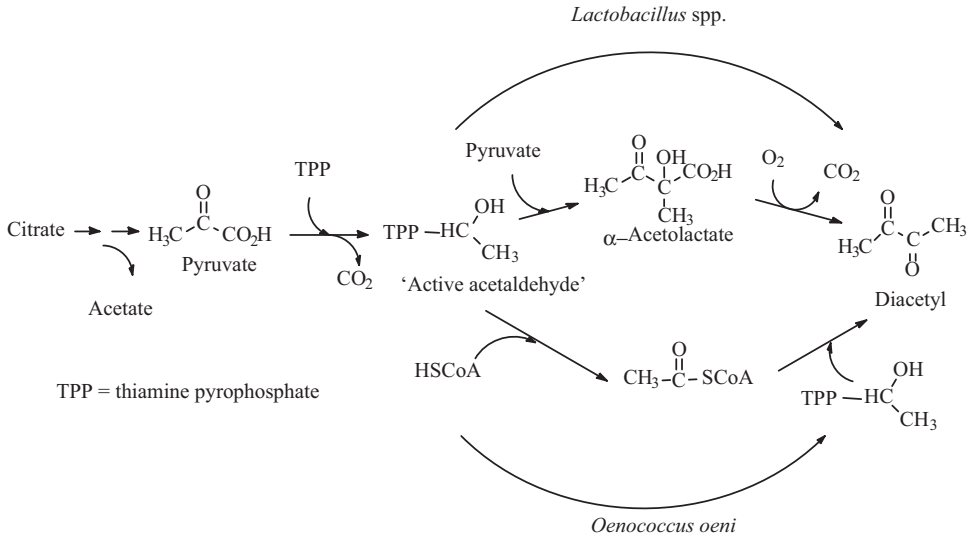


Figure 2.8.8 *Comparison of* Lactobacillus *spp. and* Oenococcus oeni *pathways to diacetyl*

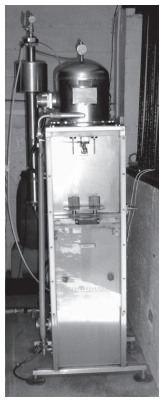
β-damascenone (Ugliano and Moio, 2006). It is probable that such reactions occur in cider that is undergoing MLF, although the levels of terpene alcohols and norisoprenoids are in general considerably lower in cider than in red wine (Xu *et al.*, 2007; Ugliano and Moio, 2006).

Biogenic amines (Section 5.11.3) have been found in cider, as well as in many other alcoholic beverages, particularly wine. Because of the early participation of lactic acid bacteria in the production of traditional Spanish cider, some of the many LAB species were suspected as producers of biogenic amines. Garai *et al.* (2007) were able to isolate 54 LAB strains from Basque country cider. It was demonstrated that six of them (five lactobacilli and *Oenococcus oeni*) were able to produce biogenic amines in culture media. *Lactobacillus diolivorans* was the most intensive histamine producer, whereas this species, as well as *Lb. collinoides* and *O. oeni* produced tyramine. Interestingly, *Pediococcus parvulus* displayed no ability to produce histamine, putrescine or tyramine, even though it is a known biogenic amine producer in beer and wine.

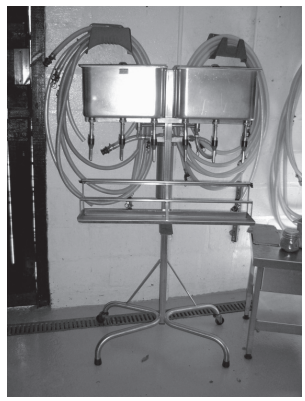
In Europe (but not in Britain), much cider is destined for the pot still or column still to be turned into apple spirit, such as Calvados (Section 3.8.2). In the production of whiskies and rum, distillation is carried out soon after a rapid fermentation, when the maximum ethanol content of the beer has been achieved. Apple spirit could be produced in a similar way, but it has been found that a few months maturation time improves the organoleptic character (particularly the aroma) of the new make spirit (Rodríguez Madrera *et al.*, 2010). The continued action of malolactic bacteria over a longer maturation period gives higher levels of acetic acid, certain alcohols, certain esters and some phenols, which have a notable influence of the aroma of fresh spirit, giving it greater fruity, spicy character.

2.8.5 Traditional and Small-Scale Cider and Perry Production: Fermentation and Beyond

Traditional cider and perry are made only from the juice of freshly crushed and pressed fruit. With the general exception of anthocyanins, the juices contain the range of components described for the fruit in Section 2.8.2 and they contain a wide variety of microorganisms (also described in Section 2.8.2). Alcoholic



Carbonator/bottling machine at Whin Hill Cider.



Standard bottling equipment (for still cider and perry) at Whin Hill Cider.

A flash pasteurizer. *Photograph used by courtesy of Derek Pritchard, Dunkery Vineyard, UK and KTM.*

Earth filter, suitable for filtration of wine and beer, as well as cider and perry. *Photograph by courtesy of Vigo Ltd., Dunkeswell Devon, UK.*

Bottle pasteurizer. *Photograph used by courtesy of Vigo Ltd, UK.*

Figure 2.8.9 *Cider and perry making equipment*

fermentation starts almost immediately, initiated by *Kloeckera apiculata* and others until the alcohol content rises above about 2% by volume. At this stage, most of these pioneering microorganisms cease to function and fermentation is taken over by *Saccharomyces* species (especially *cerevisiae* var. *ellipsoides*) that originate from both fruit skins and the winery equipment with which the juice has made contact. Alcoholic fermentations of this kind are often called 'spontaneous'; they can be slow to become established when using new equipment for the first time, due to lack of sufficient contamination by *Saccharomyces* species. Once established, alcoholic (primary) fermentation can take weeks or even months to complete, depending mainly on the ambient temperature and original gravity of the juice. Primary fermentation of traditional cider is relatively rapid

in northern Spain, presumably because of relatively high ambient autumn daytime temperatures (~20 °C), whereas in England and northwestern France slower fermentations occur at 10–15 °C.

The final gravity of traditionally fermented cider is often around 1000 in the UK, but about 1010 in France, where it is desirable to bottle cider with a certain amount of residual sweetness (Section 2.8.8). English sweet or semi-sweet traditional ciders can be made by racking the liquid off the yeast deposit and debris when the gravity reaches 1015 or 1010. A slow fermentation may well continue after racking, but if the cider is destined for cask (which is most likely), it may be yet consumed while there is a sufficient degree of sweetness. In the UK, the majority of traditional ciders are dry, whether served from cask or bottle (still or naturally sparkling – that is, bottle conditioned).

Traditional cider is often known as scrumpy in the west of England. The name comes from scrump, which means a small apple (in essence a cider apple), but nowadays the term scrumpy is used to describe any hazy cider, whether or not it has been pasteurized (Section 2.8.8). In France, when the cider reaches the above gravities, it is often filtered through kieselguhr into bottles where further alcoholic fermentation and maybe malolactic fermentation take place at *ca.* 10 °C. Traditional French cider is usually bottle conditioned (sparkling or 'bouché') in the style of Champagne, although still ciders also exist (Section 2.8.8). The final gravity of perry is often about 1010 because of the existence of higher concentrations of nonfermentable sugars and sugar alcohols (especially sorbitol) in pear juice (Section 2.8.2).

Cask-conditioned traditional cider and perry (a specialism of the UK) are either flat or lightly carbonated and will probably be hazy, although often only very slightly so. The ciders will be mostly dry, although there are some semi-sweet and sweet cask-conditioned ciders (Section 2.8.8), whereas cask-conditioned perry is most likely to be semi-sweet or semi-dry. Bottle-conditioned cider is made by either bottling whilst primary fermentation is terminating, as in Brittany and Normandy, or by dosing a fully fermented cider or perry with sugar syrup just before bottling, as in much of the UK. Traditional English perry is nearly always served still, from cask or bottle; bottle-conditioned traditional perry is rare (but see Section 2.8.6 for large-scale sparkling perry production). In Asturias, the Basque country, Germany, Luxemburg and some other countries, traditional cider is mostly bottled, either still or semi-sparkling (Section 2.8.8) and is mostly dry.

Malolactic fermentation (MLF) is important in the making of traditional cider and perry, especially when sharp and bittersharp fruit constitute a considerable proportion of the blend, as in northern Spain, particularly. Apart from the overall reduction in acidity (Section 2.8.4; Chapter 2.3), there are subtle beneficial biochemical changes (discussed in Section 2.8.4) that occur during MLF. The general opinion is that MLF makes an important contribution to the perceived extra complexity of flavor of MLF ciders, as opposed to non-MLF ciders. The predominant malolactic microorganism found in English traditional cider vats is *Lactobacillus plantarum* (Swaffield *et al.*, 1997), whereas *Oenococcus oeni* appears to the major organism found in traditional French cider making equipment. In northern Spain, two major species have been isolated from cider or equipment: *Lactobacillus hilgardii* and *Oenococcus oeni* (Herrero *et al.*, 2006).

In England and northwestern France, MLF normally occurs in the spring of the year following primary fermentation, when the ambient temperature is around 15 °C. Under these conditions, MLF may be sluggish and can take several weeks to complete. On the other hand, in Asturias and the Basque country primary fermentation and MLF are reckoned to take place simultaneously at temperatures (~20 °C or higher), which encourage a more rapid biological action. The malolactic conversion, given favorable conditions, is reported to take place when bacteria populations have surpassed 10^6 cells/ml, nutrients for initial growth being provided by both the medium and various yeast metabolites and autolysis products. Populations of lactic acid bacteria in the bloom on apple and pear skins in England and northwestern France are rather low, but it has been shown that bacteria originating from oak vats or casks give rise to MLF in traditional English cider (Swaffield *et al.*, 1997). Large populations of these bacteria (and yeasts) live in the pores of the wood, despite hot wash cleaning between successive cider fermentations.

Traditional wooden cider casks are often bigger than their equivalents in the wine industry (typically 546 l, as opposed to 228 l capacity) and are often several decades old, as opposed to 5–6 years old. It has been shown that in as little as 10 weeks contact between vat or cask wood and cider containing MLF bacteria resulted in penetration of the bacteria into the pores by more than 1 cm (Swaffield *et al.*, 1997).

Nowadays, much traditional cider and perry is fermented and matured in stainless steel, fiber glass or concrete vats, rather than in wooden vats or casks. These smoother surfaces are also colonized by lactic acid bacteria (and other microorganisms), but not to the same extent as wooden vessels. In England, there are still some small-scale and medium-scale cider and perry producers who use oak vats for fermentation and/or maturation. Vat capacities range from 5000 l to 200 000 l, with those of 22 700 l and 45 500 l (5000 and 10 000 gallons, respectively) perhaps being most typical. Cask sizes on the other hand vary from under 200 l to over 600 l; the traditional cider cask size is the 'butt,' of 546 l (120 gallons). Unless the vats or casks are new, they will make little (if any) contribution to the aroma and flavor of the finished product through leeching of oak substances into the beverage. Unlike in some areas of the wine industry, there appears to be no concerted movement in the cider/perry industry for fermentation or maturation in new oak. This may be partly because the prohibitive costs, partly because of the differences in expectations and attitudes between wine drinkers and cider or perry drinkers and partly because of the differences in social function between the beverages. It is possible that this situation will change somewhat (see Section 2.8.7).

In England and Wales, smaller-scale producers often use modified forms of the traditional process described above, for some or all of their products. The major modifications are listed below, but the majority of small-scale cider makers use only a selection of these:

- Purification of must by added sodium metabisulfite
- Fermentation using cultured yeast
- Addition of glucose syrup or invert sugar to must prior to fermentation
- Use of other additives or apple juice concentrate
- Use of fining agents
- Application of filtration and/or pasteurization
- Use of artificial carbonation.

Most small-scale cider makers ('farm cider') employ spontaneous fermentation of the must, with no added metabisulfite, but those that do purify the must normally add enough metabisulfite to give no more than about 60 ppm of SO_2, the active agent (Chapter 2.5). After a 24 h standing period (which also allows a certain amount of clarification), spontaneous fermentation proceeds or an activated yeast culture is added. In this way the most troublesome yeasts and bacteria are eliminated, but sufficient populations of desired yeasts and bacteria survive giving a mixed microflora fermentation, which is thought to produce a greater complexity of flavor. For more discussion on the good and bad points of using metabisulfite and cultured yeasts in cider and perry making, see Section 2.8.6.

It is mostly the larger UK cider and perry producers who use apple or pear juice concentrate, sugar syrups and other additives (Section 2.8.6), although a few medium-sized cider makers use some of their own must for conversion to apple juice concentrate in times of plenty, or buy in concentrate in times of shortage.

It is in the postfermentation treatments that many small-scale producers deviate most from a purely traditional process. Finings are quite widely used, with bentonite being the most popular agent, as it selectively adsorbs proteins, the major cause of haze in cider and perry. It can be added to the must at the same time as pitching the activated yeast culture. Indeed it is possible to purchase mixtures of granulated wine yeast, bentonite and diammonium phosphate (yeast nutrient); suitably activated, an inexpensive 60 g drum of this

mixture will ferment and clarify 200 l of must. However, in the majority of cases, finings are added after fermentation, prior to blending, filtration and bottling. In place of bentonite, isinglass (as for beer – see Section 2.6.9) or albumin (or egg whites, as for wine – see Section 2.9.4) may be used. Other fining agents include gelatin, chitin and kieselguhr.

Filtration in the UK is often carried out by plate and frame or sheet filter systems (Section 2.6.9), although some medium-sized cider makers also use cross flow membrane filtration. Pasteurization normally follows filtration; it can be carried out in bulk before cooling (e.g. by use of a flash pasteurizer – see Figure 2.8.9), carbonation and bottling, or in bottle, after carbonation. Smaller producers tend to use carbonation/bottling machines to bottle the cider or perry under CO_2 pressure at low temperature (Figure 2.8.9). The bottles are then pasteurized by immersing in a hot water bath (e.g. 60 °C for 15 min) (Figure 2.8.9).

Blending can be performed at many points in the cider or perry making process. Some smaller producers who make only blends tend to crush, press and ferment the whole mixture of apple or pear varieties from their orchards. Many others, particularly cider makers who produce single variety ciders as well as blends, prefer to process the varieties separately and formulate the blends at a later stage in the production. Typically, blending is carried out after the maturation period, but before filtration and packaging. Any other adjustments (e.g. acidity, sweetness or color) can also be made at this stage.

In Brittany and Normandy, the ciders produced by small-scale farmers using apples from their own orchards ('vergers') are known as 'cidres fermiers.' The methods of production differ from those used by smaller-scale UK concerns in a number of ways: spontaneous fermentation is almost always utilized, with minimum use of additives. Also, the process of défécation (keeving) (Section 2.8.3) is more or less universal, and filtration tends to be carried out using kieselguhr plate and frame filter systems. Much cidre fermier is bottled, after filtration, in the spring following the harvest, whence further fermentation (by surviving yeast cells) and possibly malolactic fermentation occur, thus producing CO_2 in the bottle. This is known as cidre bouché and is the French equivalent of the English bottle-conditioned cider. In order to promote this bottle fermentation, metabisulfite is never added at the bottling stage. The process of défécation prior to fermentation depletes the apple must of some yeast nutrients, especially nitrogenous compounds, and is reckoned to result in a slow fermentation that is incomplete at the time of bottling. Thus, there may be 30 g/l of unfermented sugars in the newly bottled cider upon which the yeast may feast. If MLF has not already occurred in the bulk cider before filtration and bottling, it may occur in the bottles, which are often stored for several months before sale.

Slow fermentation is of considerable importance to Norman or Breton cider makers. It can be achieved both by juice clarification (keeving) before fermentation, as described above, but also by reducing the biomass at some point during the fermentation via a racking, filtration or centrifugation process. Yeast populations are kept at low levels ($\sim 10^7$ cfu/ml, as opposed to $\sim 10^8$ cfu/ml used for beer or wine production) by application of one or other of these techniques. Obviously, these methods must be applied with care, so that the yeast population does not fall to such a low level that fermentative activity is too low to prevent spoilage, by the action of *Acetobacter*, for example.

Recently, it has been found that a single decimal reduction of biomass (e.g. by filtration) is best carried out when the density of the juice has dropped by 5–10 kg/m^3 (e.g. from an initial gravity of 1055 to 1050 or 1045), corresponding to consumption of 12–24 g/l of sugar (Nogueira *et al.*, 2008). This relates to the growth phase of the yeast. If this biomass reduction is performed at a later stage, the fermentation velocity became too low to reach the desired density.

In northern Spain, small-scale cider makers still use a largely traditional process that involves spontaneous fermentation (with no additives, including metabisulfite) of the naturally clarified must in chestnut casks. As in England, these casks are rarely renewed, and so over the years have acquired large populations of microorganisms. It has been demonstrated that both the alcoholic fermentation and MLF occur more easily

when the crushed apples are pressed in a traditional wooden press and fermented in chestnut casks compared with the use of a modern pneumatic press and fermentation in stainless steel tanks (Blanco Gomis *et al.*, 2003b). This was once again considered to be because of the larger populations of fermentative microbes residing in wooden equipment. Higher levels of fusel alcohols and esters were also apparent in the cider produced by the all wood traditional method, compared with that made by the modern equipment or any combination of wooden and modern equipment (Blanco Gomis *et al.*, 2003b). The all wood process gave cider with low methanol and high 1-propanol contents, which have been shown (along with low levels of acidic polysaccharides) to produce foam with the best sensory characteristics when served in the traditional Spanish way (Section 2.8.8) (Mangas *et al.*, 1999; Lobo *et al.*, 2005).

A molecular genetic study, using mtDNA restriction analysis, of *Saccharomyces* species isolated from Asturian cider fermentations has shown that there was a succession of genetically different strains of *Saccharomyces* during cider production (Suárez Valles *et al.*, 2007). *S. bayanus* dominated the early stages of fermentation, giving way to *S. cerevisiae* in the later stages. Five strains of *S. bayanus* (III, VII, VIII, XV and XVII) were present in significant frequencies in all the fermentation tanks (for two consecutive harvests), while cluster analysis showed higher similarities for patterns in all but the last above-mentioned strains, indicating that these were important microflora of this particular cider plant.

The foaming characteristics of cider are considered very important by the cider makers and drinkers of northern Spain. Good foaming behavior includes good initial foam production, when the cider is poured from a height (the traditional way of serving Spanish cider), rapid collapse of the main body of foam, but leaving of a thin residual layer of foam and tiny bubbles of CO_2 in the cider. Foam quality of a large number of cider samples has been correlated with the presence (or absence) of a number of components, as mentioned above. Additionally, as with beer (Section 2.6.2), high quality of foam has been correlated with the presence of proteins, especially high molecular weight proteins (Blanco Gomis *et al.*, 2007) and fatty acids, especially caprylic acid with regard to foam stability and linolenic acid with respect to foam height (see also Section 4.6.3) (Magolles Cabrales *et al.*, 2003).

It is believed that the most effective proteins regarding foam formation and stabilization are those with relatively little disulfide bridge cross linking and a high proportion of hydrophobic amino acid residues. Both these factors facilitate molecular flexibility, allowing the proteins to unfold and, by interacting with each other (via hydrogen bonding, hydrophobic and ionic attractions), to create a stabilized film around the bubbles. The protein film increases the elasticity and viscosity of the medium at the liquid–gas interface and at the same time decreases interfacial tension.

Good foaming properties of still or 'natural' Asturias cider has been shown to be associated with the presence of low to medium molecular weight proteins from traditional manufacture (Blanco Gomis *et al.*, 2007), whereas the presence of higher molecular weight proteins, more typical of fast press production, was not so good for foam production. For sparkling ciders, foam quality has been found to be more closely related to the yeast species used in the foam production step (Blanco Gomis *et al.*, 2009). More recently, the same group have shown that both still and sparkling cider can be characterized on the basis their HPLC derived hydrophobic protein profile and foam characteristics, using multivariate analysis chemometric methods to process the large amount of data (Blanco Gomis *et al.*, 2010). The computed multivariate regression allowed the prediction of foam parameters: foam height, foam stability height and foam stability time.

Traditionally, Spanish cider is fermented at rather higher temperatures than its traditional English and French cousins: 22 °C is not uncommon. At such temperatures, alcoholic and malolactic fermentation usually occur simultaneously, or at least in overlapping succession. Laboratory-scale cider making using inoculation of must with *S. bayanus* and *Lactbacillus hilgardii* has suggested that simultaneous fermentations at 22 °C led to cider with increased levels some aroma compounds in the resulting cider. Alcoholic fermentation at 15 °C, followed by MLF at 22 °C resulted in a cider with normal levels of aroma constituents, suggesting that

this may be a way to a more delicate product rather in the French style, but which has had full MLF (Herrero *et al.*, 2006).

2.8.6 Large-Scale (Factory) Production of Cider and Perry: Fermentation and Beyond

Large-scale cider production is very different in England and France, so separate accounts are given in the following paragraphs, followed by discussion of factory made perry. UK and other northern European factory-scale cider making is not generally closely allied to the traditional method, as discussed in Section 2.8.5. Apple juice concentrate (AJC) is the common basis of most of these ciders and bulk AJC, purchased on the world market, is used for making the base ciders. Much of this is of Chinese origin (China is the world's largest apple producer) and is made from apple varieties such as Fuji, Gala, Ralls and Starking. Besides the bulk concentrate, the largest cider producers convert all their native apple juice to concentrates in the factory at the time of the harvest. In this way, juices of bittersharp apples, such as Bulmers Norman or Kingston Black, and bittersweet varieties, such as Dabinett and Tremlett's Bitter, with gravities of about 14 °Brix (∼1060) are (after sulfiting) converted to concentrates with gravities of around 70 °Brix or higher. The concentrates are kept in stainless steel or epoxy resin lined mild steel tanks of 2 500 000 l (or greater) capacity. The big advantage of using concentrates is that cider can be made the whole year round, like the brewing of beer, instead of being confined to the few weeks following the harvest. The two largest UK cider manufacturers (see Section 2.8.8) use in excess of 60 000 tons of (mostly local) apples per year, thus generating large amounts of dry pomace from the pressings. This is sold to companies for the extraction of pectin for use in jam making. Prior to fermentation, apple juice concentrate is diluted with filtered water, and glucose syrup or invert sugar is added to bring the gravity up to 1090–1100 (∼23 °Brix); this gives a cider of 12–13% ABV when fermented to dryness. If any natural apple juice is used, it is flash pasteurized first. Acidity is adjusted with malic or citric acid and yeast nutrients (diammonium phosphate and thiamine) are added. Pectinases are sometimes added at this stage to prevent haze formation at later stages. Before addition of yeast, the must is aerated with compressed air to provide oxygen for the rapid growth of the yeast population in the early stages of fermentation.

For fermentation, various strains of cultured cider and wine yeasts are used, the former imparting cider aromas and flavors and the latter promoting rapid, efficient fermentation. Strains of *Saccharomyces bayanus* (a Champagne yeast) are popular (Johansen, 2000). Although the larger manufacturers have their own strains of cider yeast, the tendency (unlike brewing) is to use a newly cultured yeast starter for each batch, rather than use the collected lees of one batch to start the fermentation of the next batch.

Yeast strain is important in cider production, as for other alcoholic beverages; the wrong choice can give less than optimum quality cider. In general, strains of *S. cerevisiae* used for brewing beer do not give the best cider, wine yeasts being more suitable. Even so, careful choice should be made, based upon correlation of chemical analysis (e.g. of aroma components by gas chromatography) and sensory analysis of the finished cider with yeast strain. Recently, the results of a combination of these two methods were analyzed by fuzzy comprehensive evaluation (FCE) (Peng *et al.*, 2008) in order to rank the cider making qualities of eight *S. cerevisiae* strains commonly used in Chinese cider production. The FCE technique overcomes the problems of imprecise subjective judgments from sensory analysis and incomplete objective data (from chemical analysis).

Fermentation occurs at ∼22 °C in thermostatted tanks (often of the cylindroconical type – see Section 2.6.5), fitted with agitators. These vessels are typically of from 100 000 l to over 600 000 l capacities, and are usually made of stainless steel. At 22 °C, fermentation is rapid, taking only 7–14 days, after which time the new cider is cooled to around 10 °C. After pumping the new cider off the yeast deposit (lees) into fresh tanks, metabisulfite and finings are added and the cider is allowed to stand for two days while clarification takes

place. The cider is then pumped into buffer tanks for a few weeks' maturation period. Some of these tanks, all of stainless steel or epoxy resin lined mild steel, are of 2 500 000 l capacities and higher (see Section 2.8.8) and are situated outdoors, like the largest fermentation/maturation tanks of the really big breweries (Section 2.6.6). These tanks are well insulated and thermostatted. The larger cider companies in the UK make six or so basic ciders in this way; eventually these will be blended in proportions that are decided by the blenders for each brand. High gravity ciders (12–13% ABV) will need dilution with pure water to the desired alcoholic strength (usually 5–8% by volume) before blending. Adjustment of acidity, sweetness or color may also be necessary. Blending can either precede or follow filtration, but adjustments are made at the same time as blending. Acidity is adjusted with either pasteurized sharp apple juice, citric acid or malic acid; sweetness is adjusted with sugar syrup or artificial sweeteners (Lea, 2004) and color is usually adjusted with caramel. Cross flow filter systems (using filters down to 0.2 μm pore size) or continuous membrane microfiltration systems are used for the final filtration and flash pasteurization may be applied before the cider is cooled to about –2 °C and carbonated in bright cider tanks, often of around 1 800 000 l capacity. It is from these tanks that the cider is packaged into bottles (glass and PET), cans and kegs. Some cider may be pumped into tankers and then taken off site to be packaged. Big companies that produce scrumpy cider brands (naturally hazy – see Section 2.8.8) apply only pasteurization to the ciders that are destined for these brands. The two largest producers of cider in the UK are H.P. Bulmer (Hereford) and the Matthew Clark group (Shepton Mallet, Somerset) (Section 2.8.8).

The rigorous sulfiting (addition of metabisulfite), filtration and pasteurization regimes of the UK large-scale cider producers ensure that the malolactic fermentation does not occur. This is in sharp contrast to the methods of many small-scale UK cider makers, as well as to those of some large-scale manufacturers in France. The roles of metabisulfite are to reduce the risk of infection by harmful microorganisms and to prevent extraneous oxidation. At the same time, of course, malolactic bacteria are inhibited. The active agent of metabisulfite is sulfur dioxide, SO_2 (Section 2.5.2). As in winemaking, SO_2 reacts with many components of must and cider or perry, both reversibly and irreversibly (Section 2.5.3). Bisulfites are formed with carbonyl compounds (acetaldehyde, α-ketoglutaric acid, pyruvic acid and others) or potential carbonyl compounds (sugars and their derivatives). Additionally, sulfonic acids are formed by reaction with polyphenols. In order to allow for this binding and to ensure that enough SO_2 remains in solution ('free SO_2') for optimum antimicrobial activity, metabisulfite is frequently added to apple juice (prior to fermentation) to give total SO_2 levels of ~100 ppm (Herrero *et al.*, 2003). The legally permitted limit in many countries (e.g. those of the European Union and the USA) is 200 ppm. Excessive binding of SO_2 is evident when apple juice concentrate is used or when significant amounts of damaged fruit are used to prepare the must. In the former case, this is probably because the heat treatment used to produce the concentrate results in higher levels of carbonyl compounds and other oxidation products (Jarvis and Lea, 2000). In the latter case, the action of acetic acid bacteria on the damaged fruit produces sulfur dioxide binding substances, notably oxo acids, such as 2-ketogluconic acid and 2,5-diketogluconic acid, as well as oxidized sugars, like 5-ketofructose.

Apple and pear juice concentrates are produced in many countries, including Argentina, China, Italy, New Zealand, the UK and the USA. Clean, sound fruit are crushed and pressed, and the resulting juice is depectinized and microfiltered before undergoing ramped low temperature vacuum concentration. Concentrates are usually of 70 °Brix gravity: *ca.* 1.35 kg/l density, with about 950 g/l sugar content. They are usually bright with turbidity ratios of less than 2 nephelometric turbidity units (NTU) (Section 4.6.3), although cloudy concentrates are also available. They can be purchased by the tanker load or in 200 l metal or plastic drums. Their lifetime is about six months if stored at temperatures below 7 °C. Beyond this storage time, the suppliers cannot guarantee insignificant deepening of color. Many juice concentrate firms also produce essence and flaked or powdered products. The larger UK cider manufacturers make their own apple juice concentrates from local apple varieties, such as Bulmers Norman, Dabinett, Tremlett's Bitter and others; they often add metabisulfite prior to subjecting the juice to the concentration process.

Factory production of cider in Brittany and Normandy is generally much more allied to traditional and small-scale practices than in most other countries. Under EU specific food law for France, only 10% of the must may be made from apple juice concentrate and additions of sugar (or sweeteners) and coloring agents are not allowed in the production of cidre. Interestingly, these additions are allowed if the product is given the English name 'cider.' In one particular kind of prefermentation treatment, some factory procedures differ considerably from the traditional method. This is the use of a counterflow diffuser. After securing the juice from the first pressing of the crushed apples, the pomace is conveyed along a long tube where apple juice is pumped in counterflow to extract extra sugars from the pomace. The pomace is then dried and sold to companies for pectin extraction, as in UK factory cider production.

The process of défécation or keeving is practiced by large and small French cider makers alike. Keeving occurs naturally in fresh apple must, as hydrolytic enzymes in the juice (principally pectin methylesterase) act upon pectins by hydrolyzing their side chain methyl ester groups to carboxylic acid groups. The demethylated pectins (essentially polygalacturonic acids) form gel-like salts with juice cations (mainly Ca^{2+}) and the gel rises to the surface to form a brown frothy crust (the 'chapeau brun'). Haze particles are trapped in the gel, so that when the crust is skimmed off, a partially clarified must is left behind. Weak interactions (probably dominated by hydrogen bonding) with pectin and cell wall debris also traps substantial amounts of proteins and higher polymers of flavan-3-ols, themselves potential haze producers (Hubert *et al.*, 2007). Thus, although keeving is a clarification process, it also leads to a less nutritious medium for yeasts, and additionally there is loss of bitterness and astringency caused by removal of some polymeric phenols.

Natural keeving can be a rather a slow process, often needing several days, so manufacturers often use pectin methylesterase (PME) from *Aspergillus niger*, along with calcium chloride as a supply of Ca^{2+}. The must is then often pressurized with nitrogen gas, so the gel of calcium pectinate rises quickly to the surface. This method, known as flotation, causes more rapid formation of chapeau brun. Although haze particles are still trapped in the gel, there is no time for effective interaction with polymeric phenols, which remain in the juice. Hence the flotation method gives a lower depletion of the higher polymers of flavan-3-ols, leading to more bitter juice (Hubert *et al.*, 2007).

After removal of the chapeau brun, the must is often further clarified by centrifugation or filtration before undergoing a long, slow spontaneous fermentation, sometimes at temperatures below 10 °C for several months. Malolactic fermentation may or may not occur, depending on the style of the manufacturer. After fermentation, clarification is effected with albumin, gelatin or bentonite, followed by either centrifugation and/or filtration, using a kieselguhr drum filter system. Some brands that are called 'cidre fermier' (farm cider) are not filtered at this stage.

The ciders are then blended and are stored in stainless steel or painted mild steel tanks at low temperatures (*ca.* 7 °C) before packaging. The largest producers (see Section 2.8.8) make both still ('table') and sparkling ('bouché') cider of varying degrees of sweetness and alcoholic strength (2–6% ABV). The sparkling cider is artificially carbonated before packaging. French cider is mainly packaged in bottles (glass and PET) of sizes up to 1.5 l. The wine bottle size (750 ml) is popular. The largest cider producers in France are Volcler and Loïc Raison (Section 2.8.8)

In Ireland, the method of production of factory made cider resembles those of some medium sized companies in the west of England. Rack and cloth presses are used, and the must undergoes spontaneous fermentation in oak or stainless steel vats at ∼20 °C for up to eight weeks. This is followed by several months maturation (some in oak vats) before filtration, blending, chilling, further filtration, carbonation and packaging. The main bulk cider producer in Ireland is Bulmer of Clonmel (not related to the Hereford Bulmer), known as Magners outside Ireland (Section 2.8.8).

There are fewer factory-scale perry producers and many of these actually manufacture both perry and cider (see Section 2.8.8). As in the case of cider, the must can be made of freshly crushed and pressed fruit or by

dilution of fruit concentrate. The must is often sterilized by addition of metabisulfite, but up to 150 ppm of total SO_2 may be required (compared with 50–100 ppm in apple juice) because of higher concentrations of SO_2 binding components in typical pear juice. These include acetaldehyde, a number of ketones, sugars and phenolic compounds. Although the legal limit for total sulfur dioxide in alcoholic beverages is 200 ppm in many countries, legislation may well reduce this limit in the near future, in response to constant lobbying by health organizations and consumer groups (Section 5.9.2). An alternative way to sterilize the must is to give it flash pasteurization treatment (Section 2.6.9). This kills both wild yeasts and lactic acid bacteria (as well as spoilage and pathogenic organisms), but it is certain to alter the flavor of the finished perry, compared with an unpastuerized product. The sterilized must is then fermented by addition of activated cultured yeast, often a champagne yeast, such as *Saccharomyces bayanus*. Sugar, acid and yeast nutrients may also be added at this stage. As with factory made cider, fining, centrifugation and filtration of perry can be performed at a number of stages in the manufacturing process. Blending of perry is as important as cider and tasting the blends may result in the decision to adjust sweetness by addition of sugar syrup or sweeteners. Centrifugation or filtration is always carried out after maturation and blending, but before committing the perry to bulk storage tanks at low temperatures for assessment of haze stability. The perry is next sulfited or flash pasteurized before packaging. It may well be given a final membrane filtration at this stage. The cooled perry is finally given an artificial carbonation in the bottle by use of counter pressure bottle filling machines. The best known brands of factory made perry are probably Babycham, Brothers, Kopparbergs and Pomagne (see Section 2.8.8).

2.8.7 Newer Techniques, Recent Developments and Innovations

Apple and pear harvests usually occur over a limited number of weeks in the autumn, the exact spread of harvest depending mainly on the varieties grown and the seasonal weather. The cider or perry maker is thus often presented with a large mass of fruit within the space of a few weeks at most, which (after appropriate sweating or storage) has to be converted into cider or perry. The net result is that the fermention and storage equipment is all used together until the spring or early summer of the following year; there are 4 or 5 months at least where the equipment is not in use, except in cases where maturation is extended over several months. This situation is particularly acute for large-scale producers during a protracted harvest; small-scale manufacturers can at least make separate vats of varietal ciders or perrys and blend them later, as required. For most large-scale and some medium-sized operations, the main answer to this situation is fruit (apple or pear) concentrates (Section 2.8.6), purchased and/or produced from their own fruit. These concentrates have useful lifetimes of around six months if stored at low temperatures (Section 2.8.6) and hence their use can extend cider and perry making activities over virtually the whole year, thus maximizing use of equipment, but without overloading at any one time. Careful blending can ensure consistency of flavor for particular brands. Thus, factory cider or perry manufacturers who use concentrates are in a similar situation to brewers: their basic ingredients can be stored for long periods and are used as demand dictates. In contrast, small-scale producers are in a situation similar to winemakers. Fruit juice concentrates have been used for many years, but it is only during the last decade or so that they have actually become the focus of factory made cider and perry.

Cider apples and perry pears, on the other hand, generally cannot be stored for much more than two weeks or so before being processed. Traditionally, the fruits are stored just long enough for them to ripen, but they are checked regularly for softness and processed immediately they are judged to be ready. Many pears and some apples do not store well (e.g. Jonathan, used for cider in North America); they are either processed immediately or are given just a day or two of storage time. Overextended storage of any fruit leads to age related deterioration (senescence) and other faults, even in sound (undamaged) fruit. It has been shown that

spraying fruit on the tree with ethephon – (2-chloroethyl)phosphonic acid – accelerates the ripening process, so that harvest can begin a full two weeks before the traditional harvest (Gómez-Cordovés *et al.*, 1996). This compound works by releasing ethylene, a fruit ripening hormone, as a result of hydrolysis inside the fruit. It also minimizes premature fruit drop in certain varieties of apples. On the other hand, application of seniphos delays ripening and extends the storage lifetime (without senescence) of the harvested fruit. In this way, use of these two agents may extend the harvest and storage time over several more weeks, thereby relieving pressure on equipment and other facilities.

Flavored ciders, mostly from the larger companies, have been available for the past few years (Section 2.8.8). The range of flavors in present use, however, is much narrower than with beer (Section 2.6.13), and at the time of writing (2008) is more or less limited to fruit flavors, such as bilberry, pear and raspberry. Cider flavored with lemongrass and ginger is made by Westons (Hereford, UK) (Section 2.8.8). Except when traditional cider or perry is fermented and/or aged in new oak vats or casks, oak aromas and flavors are not prevalent in these fermented beverages. Oak vessels are expensive and are not often renewed by cider and perry makers. Traditional producers use oak vats or casks largely for the accelerated aging that occurs via the gradual ingress of oxygen through the pores of the wood into the beverage, eventually resulting in a smoother, more mature product. Also important are the colonies of microorganisms that inhabit the pores of used vats and casks (Section 2.8.5); these organisms are responsible for both alcoholic and malolactic fermentation in traditional cider and perry. The oak aroma compounds (Section 2.9.5) are largely leached from the wood after a few seasons of cider or perry fermentation or maturation. So, all in all, oak aroma and flavor are not commonly associated with cider and perry. In any case, chestnut casks, with less intense flavoring capabilities, are used in place of oak casks by some producers, particularly in northern Spain. Socially, cider and perry are closer to beer than to wine, but in flavor and in most of the production methods they are closer to wine. Distinct oak aromas and flavors are common (and popular) in some wines (e.g. red Bordeaux and red Rioja, to name just two), but only very rarely in beer, cider or perry (see Section 2.6.13 for an oak flavored beer).

Oak chips have long been used by some wineries as an inexpensive means of producing red and white wines with oak character. So far, this has not been applied to the large-scale manufacture of cider or perry, or to the production of a speciality oaked cider or perry by a large company. It has been demonstated that the aroma profile of cider matured with added oak chips is attractively modified (Fan *et al.*, 2006). Moreover, as with wine, oak aroma can be fine tuned according to whether American, French or Chinese oak is used, and according to the level of toasting. Also, the modest oak chip maceration times gave typical oak aroma profiles without significant increase in tannic mouthfeel. This is noteworthy because both cider and perry (especially the traditional types) are already rich in polyphenols, including tannins. There is no doubt that the finest cider and perry rival many white wines in overall quality, so it would be interesting to witness an increase in the production of oaked cider and perry.

As with wine, the malolactic fermentation in cider and perry production presents something of a controversy. Traditional and small-scale cider and perry makers in England and France tend to encourage its occurrence, by the use of wooden vats or casks, long maturation times and minimal or zero levels of sulfur dioxide. Even here, however, occurrence of MLF is by no means universal (Johansen, 2000). In northern Spain, the general process and conditions favor MLF, which tends to occur simultaneously with alcoholic fermentation (Section 2.8.5). The situation with the largest cider manufacturers in Brittany and Normandy (including Loïc Raison and Volcler) is mixed: some encourage MLF, some do not, although whilst not taking steps to actively discourage it. On the other hand, factory-scale producers in England and northern Europe operate processes that actively discourage MLF (Section 2.8.6), the philosophy being to keep everything under tight control, largely for the sake of consistent product quality. The control option chosen is prevention of MLF, rather than the opposite option of adding activated lactic acid bacterial (LAB) starter cultures to ensure the occurrence of MLF. Indeed, it appears that the use of LAB cultures such as *Oenococcus oeni* or *Lactobacillus brevis* is

rare in commercial cider and perry production, with only a few smaller companies in the USA and Canada practicing this regime.

Recently, it has been found in the USA that a cider substrate (produced from noncider Braeburn, Fuji, Golden Delicious and Red Delicious apples) of between 6 and 11% ABV can be induced to undergo rapid MLF at 20 °C, by inoculation with *Oenococcus oeni*, provided the total SO_2 level is lower than 80 ppm (Reuss *et al.*, 2010). This resulted in a considerable drop in titratable acidity and suggested that the inoculation technique could be used by winemakers to produce high quality cider outside the winemaking season, thus providing a useful extra income and allowing more efficient use of winery equipment. Consumption of (hard) cider in the USA is in a definite upward trend.

The English and northern European factory cider manufacturers take the preventative option probably because, at the time of writing (2009), it is both easier and more reliable to prevent MLF than to promote it with certainty on an industrial scale. Nevertheless, there has been considerable interest and activity amongst research workers in the formulation of processes that both accelerate and maximize MLF in the production of wine, and to a lesser extent, cider.

Apart from spontaneous MLF and MLF brought about by addition of starter cultures, processes involving high cell concentration techniques have been described in the literature (Zhang and Lovitt, 2006). The latter techniques, which unlike the older former two methods, do not require the actual growth of the LAB in the medium during the malolactic process, include both batch and continuous versions. The latter can be subdivided into immobilized cell and membrane bioreactor (MBR) methods. Immobilized cell methods involve passing the juice or cider through packed bed bioreactors containing entrapped cells in matrices such as oak chips, poly(vinyl alcohol) hydrogel and calcium alginate. Malolactic fermentation of apple juice and cider has been described using hydrogel (Durieux *et al.*, 2000) and alginate (Herrero *et al.*, 2001) matrices, and simultaneous alcoholic fermentation and MLF of apple juice using entrapped *Saccharomyces bayanus* and *Oenococcus oeni* has been described using an alginate matrix (Nedovic *et al.*, 2000).

These processes are all described for small-scale (laboratory or pilot plant) conversions. They may suffer from the mass transfer and scaling up problems that have been encountered in the continuous production of beer by immobilized yeast cells (Section 2.6.8). Use of membrane bioreactors (MBRs) could improve large-scale continuous fermentation methods, since they perform both the fermentation and filtration (biomass medium separation) stages during a continuous process and furthermore, they do not have the mass transfer problems associated with matrix entrapped systems. At present, there is relatively little literature in the MBR field applied to cider manufacture (Zhang and Lovitt, 2006) and it remains to be seen whether the process will be adopted in large-scale cider or perry manufacture.

Some research in the present decade has focused on the effect of prefermentation fruit or juice treatments on both the juice and the resulting alcoholic beverage. Aiming at an assessment of different laboratory-scale prefermentation processes for possible use by a fledgling Ontario commercial cider business, Wilson *et al.* (2003) studied the effects of storage time, (Sections 2.8.5 and 2.8.6) (with and without addition of metabisulfite) and freezing/thawing on the chemical and organoleptic character of cider. Of particular interest was the possibility of extension of apple storage time by freezing whole apples sealed in ziPLOC® bags at –27 °C for 2–4 months. After thawing at 5 °C and then at ambient temperature, the juices extracted from these apples showed increased amounts of suspended matter and did not undergo keeving on standing. Moreover, this juice contained greater yeast and other fungal populations than juice from nonfrozen apples and the resulting cider had higher methanol levels.

Prefermentation clarification of beer wort and its influence on the character of the finished beer have been well studied (Section 2.6.2), but there are relatively few reports on the effects of analogous processes in cider and perry making. In a recent study (Hubert *et al.*, 2007), at both laboratory and commercial scale, the polyphenol contents and colors of cider apple juices were compared after five different treatments of the juice. The traditional method of keeving (défécation) had the highest impact on the reduction of both flavan-3-ol

content (Section 2.8.1) and the number average degree of polymerization. This corresponds to juices with relatively low bitterness and astringency. Fining with gelatin gave a lesser depletion of juice polyphenol content, but much more so than a depectinization method using a mixture of pectolytic enzymes. Flotation and gel fitration had little effect on polyphenol content, although the latter process led to a greater depletion of protein (as measured by nitrogen quantification).

2.8.8 Cider and Perry from Around the World

Cider and perry were staple beverages of northern Europe (along with beer), southern Europe (along with wine) and certain countries that were colonized by certain of these peoples, such as America and Canada. In many of these countries, the fortunes of both cider and perry waned in favor of either beer or wine (or other beverages) during the nineteenth century. Since the 1950s, cider and perry have had revived fortunes, firstly in their native heartlands of northern Spain, Brittany, Normandy and western England, eventually resulting in their almost global availability. Large companies grew up, especially in the UK, increased their output using modern equipment and methods, streamlined their products, implemented promotion schemes (marketing and advertising) and distributed their products widely. They bought cider orchards or commissioned farmers to produce cider apples, providing them with inexpensive saplings to enlarge their orchards or plant new ones, thus consolidating the supply of cider apples.

Nowadays (2009), there is the greatest area of apple orchards in northwestern Europe since the nineteenth century and cider apples are a more profitable crop than culinary or dessert apples. These companies pioneered the use of apple juice concentrate (AJC), so that cider could be made the whole year round, not just at harvest time. In the UK, drinks such as Babycham and Pomagne (sparkling perry) became popular in the 1950s and 1960s and this period saw the establishment of national brands, such as Bulmer's Strongbow, Taunton Blackthorn and Merrydown Vintage Apple Wine, which are now known internationally.

Western and northern Europe are the principal sources of cider and perry, but substantial quantities (especially of cider) are produced in Australia, Canada, New Zealand, some South and Central American countries and the USA. These beverages range from hazy, still drinks served from casks, flagons or bottles, to bright, sparkling drinks in sophisticated bottles, and alcohol contents vary from ~2% to over 12% (by volume), the norm being 5–6% ABV. This account emphasizes beverages made by indigenous companies from local produce; imported beverages or famous foreign brands made under license are mentioned here only in passing.

British Isles

Most cider and perry is produced in the western regions, notably Gloucestershire, Hereford and Worcester, Somerset, Devon, Cornwall, Wales and Ireland, for cider, and Gloucestershire, Hereford and Worcester for perry. The emphasis here is generally on traditional tannic cider apples (Brown's Apple, Bulmer's Norman, Dabinett, Foxwhelp, Kingston Black, Michelin, Somerset Redstreak, Tremlett's Bitter, Yarlington Mill and others) and perry pears (Barnet, Brandy, Gin, Moorcroft and others). However many cider makers in these regions also use a certain proportion of culinary apples (e.g. Bramley's Seedling and Grenadier) and dessert apples (e.g. Cox's Orange Pippin and Katy) in their blended ciders and even in a few single variety ciders.

The two biggest companies are H.P. Bulmer (Hereford), owned by Scottish and Newcastle Breweries (bought jointly by Carlsberg and Heineken) and Matthew Clark (Shepton Mallet, Somerset), part of the Constellation Group. They both concentrate their production on cider and use apple juice concentrate, but a large fraction of this is normally made on the premises from local apples of the varieties mentioned above. Producing nearly 300 million liters annually, gives Bulmer world top position, but Matthew Clark is not far behind,

with a yearly production of over 200 million liters. At both company sites, fermentation, maturation and all processing operations (as outlined in Section 2.8.6) are carried out using modern, highly automated equipment.

The Bulmer site possesses the biggest beverage container in the world (Guinness Book of Records) – a maturation tank of 7.3 million liters capacity. Blending is of prime importance in maintaining consistency of quality in the seven or eight brands produced by each company. The major brands of Bulmer are Strongbow, Woodpecker and Scumpy Jack, the last named being an unfiltered (but pasteurized) hazy cider that is marketed under the Symonds name. Matthew Clark's main brands are Olde English and Blackthorn. Bulmer also produces Pomagne, a sparkling perry and Matthew Clark make a similar product known as Babycham. Both these companies export their products to many countries and they both have stakes in or own foreign companies (e.g. H.P. Bulmer and Woodchuck Cider in USA).

In Ireland, the biggest cider manufacturer is C&C of Clonmel, County Tipperary, whose market name is Bulmers (not related to H.P. Bulmer) – known as Magners outside Ireland. The company maintains that this cider is made from Irish grown traditional cider apples, using oak vats for fermentation and storage for some of beverage. The production methods are rather more traditionally oriented than those of the big UK producers, resembling more the methods of some of the medium-sized English West Country producers. Like most of its English counterparts, Bulmers is chill filtered and carbonated prior to bottling. Clever advertizing has ensured a growing market for this cider in both Ireland and the UK, where it is supped from glasses that are half filled with ice (Section 1.3.8).

After H.P. Bulmer and Matthew Clark, Brothers (Somerset), Knight's (Malvern, Worcesterhire), Sheppy's (Taunton, Somerset), Thatchers (Sandford, Somerset) and Westons (Much Marcle, Herefordshire) represent the medium-sized cider and perry makers of the west of England, some of them making up to around 10 million liters annually. These companies use a mixture of traditional and modern methods, as described in Section 2.8.5. However, most of their products are pasteurized and/or filtered – only Knight's, Sheppy's and Thatchers produce traditional cask-conditioned ciders, but Westons make (pasteurized) bottled scrumpy.

Some of these companies make cider or perry for other companies as 'own' or 'special' label brands, a prime example being Duchy Original cider produced by Knight's. Brothers is a mainly perry producing concern that is run by four brothers who are descendants of Francis Showering, the originator of Babycham. Their major product is called 'pear cider,' an American term also used for perry produced by various Scandinavian breweries, although (apple) cider is also made.

Apart from the above-mentioned companies, there are numerous smaller producers (particularly of ciders) in the western British Isles, the majority of them being located in Devon, Gloucestershire, Somerset, Hereford and Worcester. In many ways, they maintain the tradition of farm cider or perry and many of their products are the equivalent of cidre fermier of northwestern France. Most of them concentrate on traditional beverages, although many of them use filtration, pasteurization and carbonation for some of their products. It is not possible to list them all here, as many are small businesses with only a local distribution area, but the reader is referred to www.ciderandperry.co.uk for an inventory of UK cider and perry firms and for general information.

Some of the better known west of England small producers are Bollhayes (Cullompton, Devon), Burrow Hill (Kingsbury Episcopi Martock, Somerset), Cornish Orchards (Liskeard), Countryman (Tavistock, Devon), Dunkerton's (Pembury, Hereford), Hayle's (Cheltenham, Gloucestershire), Orchard's (Gloucestershire), Perry's (Ilminster, Somerset), Minchew (Tewkesbury, Gloucestershire) and Westlake (Chilla, Devon). The Ross on Wye Cider and Perry Company produces both beverages matured in whisky or rum casks, as well as a standard range of traditional ciders and perrys. Burrow Hill is famous for the production of Royal Somerset Cider Brandy (Section 3.8.2) and various other beverages based on this, such as Pomona, a blend of apples and cider brandy, matured in oak casks for two years. Along with the Hereford Cider Museum's product, Royal Somerset is the only cider brandy produced in the UK at the time of writing (2009).

In Wales, there has been a recent revival of farm cider making, with present emphasis being on traditional methods (see www.welshcider.co.uk). Producers of Welsh cider (seidr) and perry (paeri) include Blaengawney Farm (Newport, Gwent), Ralph's (Radnor, Powys) and Tojola Orchards (Lampeter, Dyfed). Although cider and perry are made throughout Wales, there are concentrations of producers in Gwent and Powys, which are adjacent to Gloucestershire, and Hereford and Worcester, respectively. There is a lively interest in seeking out and maintaining local varieties of apples and pears and it is probable that some of these are unique to Welsh cider and perry. Apple varieties include Breakwell's Seedling, Broom Apple and Pen Caled; pears varieties include Gwenelog, Little Cross Huffcap and Welsh Gin.

There are two factory-scale manufacturers of cider and perry outside the western parts of the British Isles. These are Aston Manor Brewery (Birmingham) and Merrydown (Sussex). The former has a number of cider and perry brands on the market and sells much to supermarkets for selling as 'own' brands. This is one of just three breweries in the UK that brew cider and perry alongside beer on the same site (the others are Pitfield Brewery of Great Horkesley, Essex and Samuel Smith of Tadcaster), like some Scandinavian breweries. Merrydown, well known for its vintage cider (ex-vintage apple wine), is owned by the SHS group, and has recently switched its production site to Belgium.

Cider is made on the small and medium scale in most counties of England. Traditionally, the cider of East Anglia (mainly Essex, Norfolk and Suffolk) and those of the southeastern counties (especially Kent, East Sussex and West Sussex) were made from a blend of culinary and dessert apples and as such was lighter and more vinous than traditional West Country ciders. This is still much the case, but there are some companies using traditional cider apples and perry pears. Many of the ciders of the light, vinous type are produced by English vineyards, or by companies having an interest in English wine production. These include Biddenden (Kent), La Mare (Jersey), Rosemary (Ryde, Isle of Wight), Sedlescombe (Robertsbridge, East Sussex) and Shawsgate (Framlingham, Suffolk). In additional to these, Avalon Vineyard (Somerset) and Porthallow Vineyard (Cornwall) in the west of England also make cider using traditional cider apples; the former also produces apple wine from dessert apples.

East Anglia, like the Channel Islands, once had a strong cider tradition that waned considerably in the nineteenth and twentieth centuries to be revived toward the end of the latter century. In East Anglia, perhaps the best known cider makers are Aspall (Suffolk), Crone (Norfolk) and Whin Hill (Norfolk). The last named uses traditional cider apples and perry pears to produce a bottle-conditioned cider, as well as pasteurized, carbonated ciders (including single variety types) and a superlative perry.

Brittany and Normandy

Here too, the cider industry is undergoing a revival after an extended period of depression. Even so, the entire annual cider output of northwestern France is less than that of H.P. Bulmer alone. The largest companies are Loïc Raison (Brittany) and Volcler (Normandy), the former being part of the Pernod Ricard group. Annual production is around 30 million and 10 million liters, respectively. As in the western British Isles, these larger companies generally obtain their apples from both their own orchards and from local farmers, usually under contract. Only traditional cider apples are used, of which there are many ancient varieties, including Avrolles (sharp), Bedan (sweet), Binet Rouge (bittersweet), Douce Coetligne (sweet), Fréquin Rouge (bitter) and Kermerrien (bitter). To these can be added newer varieties that have been bred with in built disease resistance: Judeline, Judor, Juliane and others. There are around 800 cider apple varieties in the germplasm collection at INRA, Angers.

In France, unlike the UK, large and small cider makers generally use traditional methods in which the process of défécation (Sections 2.8.6 and 2.8.7) is important, although some companies, such as Volcler, use the flotation variant of this process (Section 2.8.7). The only additives allowed are sodium metabisulfite (with total SO_2 $<$200 ppm) and apple juice concentrate ($<$10%). Ciders from the larger (and many of the

smaller) companies are available in various bottled forms: cidre table (still), cidre bouché (bottle-conditioned or carbonated) and cidre fermier (unfiltered). With regard to sweetness, these can be brut (extra dry), sec (dry), demi-sec (semi-dry) and doux (sweet), residual sugar being (in % w/v) approximately 0.5 for brut, 1.5 for sec, 3.0 for demi-sec and 5–8 for doux.

Cidre fermier is made by numerous smaller producers, who use traditional methods with only relatively coarse filtration (or none at all), so that enough yeast is present for bottle fermentation; the favorite style of cidre fermier is bouché. Examples of cidre fermier producers include Bordelet (Charchigné, Normandy), Drouin (Pays d'Auge, Normandy), Dupont (Pays d'Auge, Normandy), Guillevic (Morbihan, Brittany), Kerisac (Guenruet, Brittany), Verger de Giverny (Giverny, Normandy) and Vergers du Val de Sée (Brécey, Normandy).

Unlike the British Isles, a considerable quantity of cider, especially in Normandy, is converted to apple brandy (Calvados) and pommeau, a drink made from apple juice and Calvados (Section 3.8.2). Calvados is matured in wooden casks for three, five and 10 years and pommeau for up to two years. Brittany has its own version of kir ('kir Breton'), made with cider (in place of white wine) and blackcurrant liqueur.

Perry is produced by a number of cider makers in Brittany and Normandy (although usually as a minor product) from the fruit of such varieties as Autricotin, Champagne, De Cloche, Plant du Blanc and others. Produced in a similar way to cidre fermier, French perry often has around 8% (w:v) of residual sugar, so falls into the doux category, even though the acid and tannins largely mask the sweetness. Elsewhere in France (e.g. Alsace), much perry or pear wine is converted to Poiré by distillation (Section 3.8.2).

Asturias and the Basque Provinces (Euzkadi)

Cider is the traditional drink of the provinces of northern Spain that border the Atlantic Ocean, principally Asturias and the Basque provinces of Vizcaya (Bizkaia) and Guipuzcoa (Gipuzkoa), but some cider is also made in Galicia, Cantabria and Navarre. Known as sidra in Asturias and sagardoa in the Basque country, it is made from the fruit of traditional cider apple varieties, nearly all of which are unique to northern Spain and many of which can be traced back through many centuries. The Centro de Experimentación Agraria at Villaviciosa (Asturias) houses a germoplasm collection of over 500 cider apple varieties. It is also a focus of research on all things relating to cider, although research of this type is also carried out at many of the local universities (e.g. Oviedo and Gijón).

The smaller producers of Asturias concentrate their efforts on making bottled cider in a traditional manner, often using chestnut vats or casks and always involving malolactic fermentation. Larger companies also make this type of cider, though not necessarily in a wholly traditional way, and some make Champagne style ciders in the French fashion, for export. In the Basque country, some cider is enjoyed on draught – it is served from jugs straight from the wooden maturation cask in the spring following the harvest.

Spanish cider is intermediate in style between the full bodied craft ciders of western England and the cidre de table of northwestern France; it is usually fruity, with a definite tang of acidity. It is very much an everyday social drink; most is consumed from unlabeled bottles (but with branded corks) by family and friends in local sidreries (Asturias) or sagardoategiak (Basque provinces), with an evening meal. Most of the cider makers use a high proportion (up to 40%) of sharp apples (Section 2.8.2) in their blends and single variety ciders are not generally known (compare with the UK and North America). English and French ciders generally possess greater proportions of bittersharp and bittersweet apples in their blends and sweet apples often form the greater part of North American ciders. Spanish cider apple varieties include Blanquina, Collaos, Coloradona, Durón Arroes, Limon Montes, Meana, Picona Rayada, Raxao and Xuarina.

In Asturias especially the traditional way of serving cider in a sidreries is the method of 'echar un culín.' The bar tender pours a small quantity (50–100 ml) into a wide glass by holding the bottle high with one hand

and the glass low with the other hand. The mechanical agitation not only puts air into the cider but also releases residual CO_2, thus giving the cider a short lived lightly sparkling head (mousse). It is then drunk immediately – before the head subsides. The textural characteristics of this mousse are important and traditional Spanish cider making practices produce cider with the balance of components that give the desired texture (Section 2.8.5). Notable producers of Asturian cider include Asturiana de Vinos (Xixón), Industrial Zarracina (Xixón), Llagar Castoñón (Villaviciosa), Llagar Herminio (Oviedo) and Sidra Acebal (Gijón). Basque cider producers include Petritegi, Sidreria Barkaiztegi, Zapiain (all Guipuzcoa) and Sidra Axpa, Sidra Edikoetxe (Vizcaya).

Europe

Some cider (and a little perry) is made in almost every country in Europe. In Germany and Luxemburg, cider is generally known as Apfelwein, Apfelmost or Viez (from the Latin vice, meaning second or substitute wine). German cider production is centered on Frankfurt (Hesse), Merzig (Saarland), Trier (Mosel) and Saarburg, near the Luxemburg border. German apple varieties are generally low to medium tannin, high acid (sharp) types and include Boskoop, Brettacher, Kaiser Friederich, Kaiser Wilmhelm and Winterambur. Consequently, most German ciders are very dry and racy, with 5–7% ABV. They are usually served still, from bottles. Some Luxemburg ciders, on the other hand, have rather more body and are reminiscent of English West Country scrumpy. Prominent German cider makers include Boller Fruchsäfte Stolz AG, Kelterei Hoppe GmbH, Kelterei Jörg Stier, Obsthof am Steinberg and Treuschs-Schwanen.

In Sweden and Finland, factory produced cider and perry have been popular since the late 1990s and some brands are exported to the UK and North America. Like Magners, they are seen as chic drinks for young people. The best known are probably Kopparberg, Herrljunga (both Sweden) and Upcider (Finland); they are produced by breweries and usually have 4.5–5.0% ABV. Some of these companies make cider flavored with fruit syrup or essence; cranberry, blueberry, and raspberry seem to be the most common. Elderflower is another flavor and both Kopparberg and Herrljunga make perry (called pear cider or pear flavor).

Unlike Finland and Sweden, Denmark makes only a little cider at the present time (2008), but production is growing, as is the import of cider. Fejø, Ørback Bryggeri and Pomona are the main producers. Scandinavian people like to make alcoholic beverages at home, including beer, cider, liqueurs, perry, sahti (Section 2.6.13) and schnapps (Section 3.4.3). Home brewed cider is made in southern Norway; there is an annual cider festival at Øystese in the Hardanger area, the country's main fruit growing region.

Central and South America

In most European countries, cider and perry are everyday social drinks, along with beer in the north and wine in the south. The two beverages hold a similar status in North America. In Central and South American countries, such as Mexico, Argentina and Chile, cider is usually of the filtered and carbonated type, often packaged in Champagne style bottles. It is often served on special occasions, taking the place of (more expensive) Champagne and other sparkling wines, rather like Babycham (a sparkling perry) did in the UK in the 1950s and 1960s.

Canada and USA

There are four main cider production areas of Canada: British Columbia, New Brunswick, Ontario and Québec. The ciders are mostly made from local indigenous apples, such as Delicious, MacIntosh, Northern Spy (sweet), Cortland, Golden Russet, Ida Red (bittersweet), and Paula Red and Sir Prize (sharp). Sometimes crab apples (e.g. Virginia Crab) are added to the blend for greater astringency, since none of the aforementioned apple varieties are really rich in polyphenols.

Until the last two decades, the cider industry in Canada was minor, although home brewed cider has been made and consumed in Ontario homes for many years. Furthermore, cider manufacture in Québec was suppressed in the early days of British rule in favor of beer production. Nowadays in Ontario, New Brunswick and British Columbia there are several companies making various styles of cider: still, carbonated and Champagne style bottle fermented. These include Applewood (Ontario), County Cider (Ontario), Gagetown (New Brunswick), Merridale (Ontario), Waupoos (Ontario) and Wyder's (British Columbia). Some perry (pear cider), fruit wines and pommeau (apple and apple brandy) are also made by some of these companies.

In Québec, cidre de table (still cider), cidre bouché (Champagne style bottle-fermented cider) and ice cider (pomme glace or cidre de glace du Québec) are made from indigenous apples by companies such as Domaine Pinnacle and Le Minot. The latter also makes a rosé sparkling cider from a red fleshed apple called Geneva.

Cider and beer were the most important alcoholic beverages of the early colonials of the USA, almost all farms making their own brews, including those of George Washington at Mount Vernon. As in many other countries, changing tastes and economic circumstances, coupled with two world wars, conspired against cider and perry production. Also, in the USA's case we can add the influence of temperance movements and the prohibition period of the 1920s. However, also like many other countries, there has been a revival in making and consumption of commercial cider and perry over the last two decades, although not on quite the same scale as the microbrewery revolution.

The main modern centers of cider and perry production are in the New England states of Massachusetts, New Hampshire and Vermont, but cider and perry are made in many states west of these, from New York to the Pacific states. The emphasis is on cider made from local apple varieties similar to those of Canada, with the addition of some others, such as Baldwin, Roxbury Russet and Red Field. The factory ciders are made from apple juice concentrate and sugar; other additives, such as apple essence or apple flavors may also be present. The best known brands are probably Cider Jack, Martinelli and Woodchuck, the latter coming from Vermont.

A considerable number of craft cider makers has come into being during the last two decades, some of them using classic European cider apple varieties, mostly from west of England, Norman or Breton stock that are becoming increasingly available at plant nurseries. Craft producers include Aeppel Treow (Wisconsin), Doc's (New York), Farnum Hill (New Hampshire), Oliver (Indiana), Original Sin (Vermont), Sow's Ear (Maine), West County (Massachusetts), Westcott Bay (Washington) and White Oak (Oregon). West County makes single varietal ciders from Baldwin and Red Field, a red fleshed variety, and many of these small companies make perry and flavored ciders. Although good ciders are already being made from local varieties, there are trials well underway to evaluate the potential of classic European cider apple varieties. Much of this work is centered on Cornell University and the USDA-ARS Plant Germoplasm Repository Unit at Geneva, New York state.

Asia, Australia and New Zealand

China grows more apples than any other country, though the emphasis is on a few varieties of dessert apples (e.g. Fuji, Delicious, Gala, Ralls, Red Star and Starking) and much of the produce, mostly from Shandong province, is converted to concentrate for export to many countries, including the UK and USA. Very little cider is actually made in China (e.g. Gansu Zhenghangde in Gansu province), although there have been some experiments and various foreign companies export cider or perry. Korea and Japan are also big apple producers, but cider in these two countries means a sparkling nonalcoholic drink, which may or may not be based upon apples. Some of the indigenous fruit liqueurs (called wines) (Section 3.9.2), as well as nonalcoholic juices, have apple juice as part of their constitution.

Much of the rest of southern Asia and much of Australia have unsuitable climates for the successful growing of apples, although cider is made from the apples of high altitude orchards in the northern Indian states of Himachal and Uttaranchal Pradesh. Probably the best known brand is Tempest, produced by Green Valley Cider of Himachal Pradesh. In Australia, cider making is limited to the cooler (or high altitude) regions of South Australia, Tasmania and Victoria. Probably the best known cider is the Mercury brand of the Cascade Brewery in Hobart, Tasmania, but Thorogoods and Henry make traditional ciders at Burra, South Australia and Harcourt, Victoria, respectively. The climate of New Zealand, especially the South Island, is more suitable for apple growing, although most effort goes into producing dessert apples such as Braeburn and Granny Smith. Nevertheless, scrumpy style (hazy, still) cider is popular (e.g. Ballydooly Cider, Hawkes Bay) and a number of wineries and country winemakers produce some cider, examples being Alpine Gold (Central Otago), Celtic Winery (Taranaki) and Cottage Wines (New Plymouth).

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2.9

Wine

My glass of wine, dear Valentine,
With what can it compare?
When ruby, with your lovely lips,
When golden, with your hair,
The rosé with your damask cheek
On which long lashes lie,
And in my glass of fizz I see
The sparkle of your eye. . .
—Cyril Ray

Wine is one of the world's oldest and most popular beverages. Wine (according to one definition in the Oxford English Dictionary, 1995) is: '. . . fermented grape juice as an alcoholic drink.' Hence, by definition, the grape (*Vitis vinifera* in Europe, and including other *Vitis* species such as *V. labrusca* in North America, etc.) constitutes the raw material for producing wines. This definition does not, of course, include any reference to the quality of wine made from the grapes, as quality is dependent on a wide range of factors other than the grape species. However, the potential quality of the wine lies (for the most part) in the grape, so it is important that components within the grape chemistry that determine the quality of the wine are preserved during the winemaking process. The winemaker should have good knowledge of these components and the factors that enhance or detract from them.

This chapter begins by considering important factors in the production of white wines (Section 2.9.1), red and rosé wines (Section 2.9.2), and sparkling wines (Section 2.9.3). Next, winemaking aids, such as fining and filtration are discussed (Section 2.9.4), followed by an account of the important factors involved in and major consequences of maturation and ageing (Section 2.9.5). The chapter is concluded by a discussion of some modern techniques and recent innovations (Section 2.9.6).

Recent technical books on wine or that include chapters on wine, include Margalit (2004), Zoecklein *et al.* (1995), Boulton *et al.* (1996), Jackson (2000), Lea and Piggott (2003), Goode (2005) and Ribéreau-Gayon *et al.* (2000; 2006). A useful general book is that of Robinson (2006). Books with much information on grapevine varieties include Galet (1958; 1962), Jackson and Schuster (1981) and Robinson (1986). There are also many informative websites, including those of wineries (e.g. Dunkery Vineyard, UK; http://www.winegrowers.info/) and those of groups, societies and research institutions, such as Australian and New Zealand Winemakers,

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

Australian Wine research Institute (AWRI), California Wine Institute, English Wine Producers, Office Internationale de la Vigne et du Vin (OIV), Geisenheim Institute and The Wine Institute and Wines of South Africa (WOSA). Additionally, there are websites of the many universities that possess departments or institutes of enology, wine or related subjects, including (this list is not by any means exhaustive) Adelaide, Deakin, La Trobe (Australia), Brock, Niagara (Canada), Victor Segalen (Bordeaux), Dijon, Montpelier, Strasbourg (France), Auckland, Lincoln (New Zealand), Stellenbosch (South Africa), California (Davis), Cornell, California State (Fresno) (USA) and Plumpton College (UK).

2.9.1 White Winemaking

Ribereau-Gayon *et al*. (2006a) state that grape maturity level is the first factor, and certainly one of the most decisive ones, in determining wine quality. Grape maturity is intricately related to vine variety, growing conditions or *terroir* (which includes soils, aspect of vineyard and climate) and vintage. One vintage (year of production) will differ in subtle and sometimes significant ways from another due to variations in the principal factors affecting grape development that year: light, heat and water availability. The variation of these conditions during the grape ripening period greatly influences a given grape composition and quality at maturity, but viticultural practices such as trellising, leaf stripping and bunch thinning also have a profound effect. It is therefore perfectly acceptable to state that a ripe grape is the result of a great number of complex physiological and biochemical phenomena, and that perfect ripeness in any vineyard is neither an easy thing to predict nor to achieve.

It is certainly possible to turn good quality grapes into poor quality wine through bad winemaking practices. If a winemaker is lucky enough to have access to good fruit, the task is relatively easy: to prevent quality deterioration and enhance the quality potential of the fruit.

Jackson (2000a) states that the division of still table wines (the most common category) into red, white and rosé, is the oldest wine classification system and takes into account distinct differences in flavor, use and production methods. The style of wine is determined by the grape variety or cultivar, the growth conditions of the grapes and the winemaking techniques used. White wines were originally intended for consumption with meals and are therefore still produced with an acidic character to accentuate and harmonize with food proteins and flavors.

Grape Varieties for White Wine Production

The following are brief descriptions of some of the better known and more widely grown white *V. vinifera* varieties of the hundreds known, which includes non-*vinifera* varieties and hybrids (interspecific crosses). It should be noted that many cultivars have local pseudonyms, so there may be many names for essentially the same variety (see Robinson, 1986).

Chardonnay

This is a highly popular green grape variety used to make white wine. It was originally from Burgundy, but is now grown wherever wine is produced. Because it gained so much popularity in the 1980s (the 'Bridget Jones' phenomenon), a glut of the wine on the market in the 1990s led a number of wine drinkers see the cultivar in a negative light. Despite this, it is planted in more wine regions than any other variety – including Cabernet Sauvignon. One of the reasons Chardonnay remains so popular is because it is so versatile. It is relatively easy to grow, and can adapt to different conditions of soil type and climate. It is a highly vigorous vine, and vineyard managers counteract this with careful pruning and canopy management so that the fruit

is not adversely affected. The grape itself is thin skinned and 'neutral,' with many of the flavors commonly associated with the wine coming from the effects of *terroir*, vintage and winemaking practices. It is made in many different ways, from the elegant, dry wines of Chablis to full, rich Meursault wines and more modern New World styles that emphasize the tropical fruit aromas. The neutral character of chardonnay and its ability to age make it an important component of many sparkling wines around the world, including Champagne. The 'green-apple' acidity (a trademark of Chablis wines) helps make some of the best ageing examples of dry white wines in the world. French oak tends to be favored over American for Chardonnay fermentation and maturation, as American oak may impart a sweet vanilla aroma that can overpower the white wine bouquet. Barrels are usually of the 'lightly toasted' variety rather than heavily charred for the same reason. The wines also are able to undergo malolactic fermentation without detrimental effect to the style, and in fact the buttery, fatty flavor of the diacetyl produced during the process will enhance the fuller Chardonnay styles.

Sauvignon Blanc

Sauvignon Blanc is a green skinned grape variety from the Bordeaux region of France and is also used to make the famous sweet dessert wines from Sauternes and Barsac. The vine ripens early, which allows it to perform well in warmer climates if harvested at the right time, and the cultivar flourishes in cooler climate areas such as New Zealand, and the south and west coast of South Africa, where it gets cool onshore sea breezes (Figure 2.9.1). If subjected to high temperatures, the grapes over-ripen and produce wines with cooked vegetable aromas and little acidity. The variety is now planted all over the world, and is usually made as a crisp, dry, white wine that shows very distinctive cultivar characteristics due to the aroma compounds present in the grape, which include some of the mercaptans, as well as a number of methoxypyrazines. Depending on the climate or conditions under which it is grown, Sauvignon Blanc is recognized as having two 'styles': austere, grassy green from cooler areas (very easy to identify) and softer and more tropical from warmer areas. Fine examples of these wines in the 'cooler' style come from New Zealand's Marlborough region where sandy soils with good drainage and poor fertility lead to low yields, and concentrated flavor. A producer can tailor his style through changing vine canopy management, harvesting at different times, varying yeast strain

Figure 2.9.1 *Healthy Sauvignon Blanc grapes at KWV in Paarl, South Africa. Note ripeness, lack of damage, lack of mould and healthy bloom.* Photo courtesy of Paul Gerber

and manipulating oxygen levels and fermentation temperatures. Sauvignon Blanc was one of the first fine wines to be bottled with a screw cap in commercial quantities, especially by New Zealand producers, some of whom declare their wineries 'cork-free zones.' The green style of the wine is usually consumed young, as it does not particularly benefit from ageing. The more tropical wines can be fermented or matured in wood to produce the Pouilly (or *Blanc) fumé* style.

Chenin Blanc

This is a white wine grape from the Loire valley in France. It has a high natural acidity and is a neutral cultivar, so it is used to make everything from sparkling to dessert wines. It is the most widely planted variety in South Africa, where it is called 'Steen,' but a lot of the grape is being grubbed up to make way for other cultivars, as fashions in wine drinking swing away from the semi-sweet style that was popular there in the 1960s. Chenin Blanc is not fussy about soil type, and is fairly resistant to vine diseases, but the tight clusters are prone to rot in damp conditions, and the grapes are thin skinned and prone to sunburn. In cool wine growing regions, the juice is sweet, but high in acid with a full bodied palate. In warm climates, the main problem is controlling the natural vigor of the vine. If this is not carefully managed, the grapes produce bland wines of mediocre quality that are really only suitable for blending. Chenin, as a dry wine (Anjou style), has flavors of quince and apples. An off dry ('demi-sec') style (like some Anjou and Touraine wines) will emphasize honey and floral characteristics. If the conditions are right (cool, damp conditions followed by warm dry spells), the grapes will be left on the vines so that they become infected with *Botrytis cinerea*, which concentrates the juice and produces rich dessert (noble rot) wine, which can be aged for years.

Colombard

Colombard is an early ripening white variety traditionally grown for distilling into Cognac and Armagnac brandies. It has a neutral character, and is grown in Bordeaux to make fruity white wines (both dry and sweet). This grape has good natural acid and is used to provide 'backbone' and 'structure' for white blends. In South Africa, it is known as Colombar, is the most widely planted variety after Chenin Blanc, and is used in blends, to make varietal wine, and brandy base.

Gewürztraminer

Sometimes known simply as Traminer, this is an aromatic wine grape with a strongly terpenic aroma profile and pink or red skin. Gewürztraminer has particular requirements for soil and climate – it performs best in cooler climates, does not do well on chalky soils and is very susceptible to disease. The vine is vigorous when grown in the right conditions, so needs careful vineyard management. It buds early, and ripens erratically. In hot climates it produces large amounts of sugar and loses acidity, which means it can make an unbalanced wine if the winemaker is not attentive to his grape composition. If it is picked too early, varietal aromas, which are very important in the aromatic cultivars like this one, fail to develop. The variety is usually used to produce wines that are off dry, with a bouquet of lychees, roses, passion fruit and flowers. Its aromatic flavors and richness make Gewürztraminer one of the few white wines that are suitable for drinking with strongly flavored food like curries, game and smoked salmon. In South Africa, the Neethlingshof Estate makes a well known example.

Muscat

Strictly this name refers to a big and ancient family of white grape cultivars, with four principle (and many minor) varieties, which reputedly originated in North Africa, and have been grown for millennia around the Mediterranean. Muscat d'Hamburg and Muscat d'Alexandrie have been cultivated as table or raisin grapes as

well as for wine production, particularly in Australia and South Africa. In South Africa, Muscat d'Alexandrie is known as Hanepoot, and used for making sweet fortified wines of the same name. The vines thrive in a hot climate and are sensitive to cold during the flowering season. Like Gewürztraminer, Muscat wines have high concentrations of terpenes and are therefore floral in aroma, which tends to favor a sweet or semi-sweet style. In South Africa, Muscat Blanc à Petit Grains or Muskadel is the variety frequently used to make late harvest style wines and Jerepiko (fortified grape juice). In Italy it is used to make the famous Asti Spumante, a sweet, semi-sparkling wine, whereas in France, Muscat d'Alsace is dry (although nowadays Muscat Ottonel tends to be predominant).

Riesling

The true Riesling (Weisser, White or Rhine) originated in Germany, where it is a very important cultivar and generally produces the best wine; some of the best in the world. It is an aromatic grape variety, which can display flowery, perfumed aromas as well as high acidity. It is used to make a range of styles of wine from very dry, right through to very sweet, and even sparkling white wines. Riesling wines are usually made without blending with other varieties and are seldom wooded. It is considered to be one of the best white wine varieties for producing top quality wine, as it has great character, good acidity and is expressive of its *terroir*, (i.e. the character of wine as influenced by its origin). Other than Germany, Riesling is grown in the French region of Alsace, as well as in most wine producing regions. In warm climates, Riesling does best in cooler locations, and prefers a long, slow ripening and proper pruning to keep the yield low and the flavor concentrated. Riesling wines are crisp, fruity and aromatic, have relatively low alcohol concentrations and are often consumed when young. They may develop complex aromas of green apples, grapefruit, peach, honey, roses or cut grass, and can undergo extended ageing. The cultivar does not, however, lend itself to barrel treatments, as the typical floral, fruity aromas of Riesling are not complemented by wood. The famous German sweet wines like *Beerenauslese* and *Trockenbeerenauslese* are especially suited for long maturation since the very high sugar content helps to preserve them. However, sometimes Riesling wines tend to acquire a rubber or kerosene aroma with age ('*goût petrol*') as a result of the development of trimethyl dihydronaphthalene (TDN) and vitispirane. Experienced wine drinkers accept the rubberiness that comes with age as part of Riesling's character, but is off putting to most consumers. Most of the best ageing white wines in Germany including dessert wines, noble rot wines and Eiswein are made with Riesling. In South Africa and Australia, the French cultivar Crouchen Blanc (now almost extinct in France) is known as Cape (or Paarl) and Clare Riesling respectively. There are many other (lesser) cultivars that have acquired the prestigious Riesling tag on their names; Goldriesling, Welsch Riesling and others (see Robinson, 1986).

Viognier

This variety was once fairly common, but by 1965 the grape was almost extinct and there were only eight acres remaining in Northern Rhône. Recently, popularity and price of the wine have risen and thus the number of plantings has increased dramatically. Viognier is difficult grape to grow because it has low and unpredictable yields and is prone to powdery mildew. Vines produce better wines when they are older (15–20 years), and prefer warmer slopes and a long growing season. Grapes need to be picked when fully ripe, or they fail to develop aroma and taste. When picked too late, (and some Californian and South African Viognier can be) the wine is flat, alcoholic and unbalanced. Winemakers may choose to carry out skin contact to enhance color and perfume, but this has to be managed carefully due to the high pectin and phenol content of the grapes. When optimally ripe the grapes have a deep yellow color and produce wine with a strong aroma (apricots, orange blossom, honey) and good levels of sugar. The color and floral aromas of the wine suggest that they should contain sugar, but most Viognier is dry. Although it is suitable for ageing if the acidity and alcohol

is appropriate, the wine is usually consumed young. It is sometimes blended with Shiraz in cofermentations to soften the tannins, stabilise the color and enhance the nose of the red wine. Surprisingly, for a floral, fruity variety, Viognier often benefits from malolactic fermentation, wood ageing (in oak and acacia) and *batonnage*.

For an account of many other widely grown white wine vine varieties, as well as many others that are rather obscure or their cultivation is perhaps limited to one or two small regions, the reader is directed to Robinson (1986) and Galet (1956; 1962).

Physical Composition of Wine Grapes

Wine grapes are smaller in size, have thicker skins and a more intense flavor than table grapes. Wine grapes are not particularly pleasant to eat as they have far higher acidity as well as a high concentration of tannins if they are red/black, but these physical and flavor differences determine the characteristics of the wine.

For winemaking purposes, the grapes are divided into stems, skins, pulp ('juice cells') and seeds. The juice, which contains most of the sugar and acid from the grape, is mainly contained in the flesh (pulp) in large, fragile cells within the grape interior. The major volume of a pulp cell is taken up by an oversized vacuole in which juice is stored, surrounded by a membrane, which is easily broken by pressure.

The main function of crushing the grapes is to break the berry so that the juice can drain from the pulp cells. Grape juice in most cases is pale in color (except in Tenturier varieties) and is extracted from the berries before fermentation in white wine production. In red wine production the skins, pulp and juice are fermented together. The size of the berry has an influence on the percentage of juice, e.g. Palomino has large berries with a relatively large amount of juice in comparison with a small berry such as Chenin Blanc. During fermentation on the skins in red winemaking, the cell walls become more porous and release juice more easily, so skin fermentation produces a higher yield of wine once the skins, or *marc* (fermentation cap), is pressed. If grapes are over-ripe or botrytized, grapes become very hard to press due to raisining and dehydration effects, and juice yield will be far lower.

The stems or stalks make up between 3 and 7% of the bunch depending on the variety and fruit set, with some cultivars, (e.g. Cinsaut) having a low percentage of stems, while others (like Cabernet Sauvignon and Muscat d'Alexandrie) have a high percentage. In the preparation of red and white wines in New World winegrowing areas, destemming is frequently carried out at the same time as crushing. In the case of mechanically harvested grapes the stems are not brought to the cellar, but left behind on the vine, and this leads to a difference in the tonnage delivered. The equipment used to crush the grapes should not damage the stems as this can release unpleasant, astringent substances into the juice, as the stems are rich in tannins and other phenolic compounds – around 20% of the phenolic composition of the grapes, according to Ribereau-Gayon *et al.* (2006a). The inclusion of stems in a red wine fermentation can lead to higher levels of tannin and potassium, and the winemaker will need to adjust the style of wine to take the higher concentration of phenolics into account.

The grape skin consists of the cuticle, epidermis and hypodermis, and is around 1.5 to 4 microns thick, depending on cultivar. It is covered by a layer of interlocking waxy plates called the 'bloom' which provides a waterproof coating and protection against injury, and contains fatty acids and sterols which stimulate yeast growth. Most winemaking varieties have small berries, which give a high skin to pulp ratio (for example, Cabernet Sauvignon). The thickness of the skin influences the resistance to diseases and damage, and consequently the health of the grape. The skin contains most of the polyphenols found in grapes, including the anthocyanins and tannins, so fermentation must take place on the skins in the preparation of red wines in order to extract these substances. However, as the tannins are astringent, a long fermentation on the skin can make the wine very harsh, and it will need a long maturation period before it will be pleasant to drink. The aromatic substances are also found in the skin, so white wines (particularly those that are dependent on varietal aroma for typicity like Sauvignon Blanc and Riesling) can benefit from cold skin contact prior to

fermentation. Winemakers may choose to ferment white wines on the skins to maximize aroma extraction, but as other substances can also be extracted which may be detrimental to wine quality, this process has to be carried out with care.

Seeds (pips) are rich in tannins, phenolic compounds and oils (mainly oleic and linoleic acid). If the seeds are damaged by the presses or the crusher rollers, tannins will be released, which will make the wine more astringent. Apparatus is usually adapted so that it does not have detrimental effects, and these days a lot of crusher rollers are inert rubber or pliable plastic and therefore less likely to cause damage to the seeds. Seeds contain between 20 and 55% of the polyphenols in the berry, depending on cultivar. During the fermentation of red wine the seeds are usually present, and these polyphenols are extracted by the increasing concentrations of alcohol, but seeds and skins are generally removed prior to fermentation in white winemaking so will make less of a contribution. Seed tannins can bind with anthocyanins after extraction, and help to stabilize color. As the wine matures, the seed tannins will also polymerize with other compounds and become less astringent.

Sugars

The sugars are important because they are fermented and converted to alcohol and carbon dioxide by yeast cells. Sugars also give the sweet taste to wines that contain residual or added sugar. The sugar level of the grapes determines the amount of alcohol present in the wine after fermentation, and therefore affects the style to be made. The most important two sugars are glucose and fructose, which occur in approximately equal quantities in ripe grapes. Fructose is twice as sweet as glucose. Sugar concentrations are measured as must density, and expressed as different units in different wine producing regions. In Europe, sugar concentrations are often measured in °Oeschle (related to the specific gravity of must; actually °Oe = [specific gravity – 1.00] × 1000), and in the New World, in °Brix or °Balling (both a measure of % w:v sugar). In physiologically ripe grapes, sugar content can vary from 18 to 26 °B (g/100 g), but in over-ripe and botrytized grapes the sugar quality may be well over 30 °B. See Table 2.9.1 for typical sugar content of ripe grapes, according to desired wine type.

Table 2.9.1 *Approximate grape ripeness criteria for different wine types. Given for major categories only. Typical values fall within regulation requirements, which can differ not only from between countries, but between regions within a country*

Wine Type	Criterion	Unit	Typical values
White wine	Sugar	°Brix	19–22
	Titratable acidity*	g/l	7.5–9.0
	pH	–	3.0–3.3
Red wine	Sugar	°Brix	22–24
	Titratable acidity	g/l	7.5–9.0
	pH	–	3.3–3.6
Fortified wine	Sugar	°Brix	24
	Titratable acidity	g/l	6.0
	pH	pH	< 3.8
Special late harvest	Sugar	°Brix	22 and higher
Noble late harvest	Sugar	°Brix	28 and higher

*Expressed as tartaric acid

Winemakers can use a rough conversion factor of 0.55 times the °Brix or °Balling measurement to predict the alcohol yield from fermentation, but this will depend on factors like yeast strain and fermentation temperature.

In most grape producing regions with warm climates, the juice for wines may not be sweetened (chaptalized) and sweetening wine for stylistic purposes may only be done with grape sugar or concentrated grape must. In cooler climates, where chaptalization is allowed, sucrose may be added before fermentation, but may not be used for sweetening. In grapes, some hexose and pentose sugars cannot be fermented by yeast, and may remain in the wine as residual sugar, which should be taken into consideration if wines are sweetened, in order to comply with legislation for the style. Usually a dry wine will contain less than 5 g/l of sugar. However, a wine containing less than 10 g/l of sugar (glucose + fructose) may be classed as dry, as long as the acidity is high enough. Legislation regarding sugar levels differs considerably between winemaking regions.

Other carbohydrates

Pectin is made up of polygalactoronic acid molecules, which are responsible for the 'sliminess' of the skins and the opacity of the must. Pectins are present in grapes in small quantities, especially around the vascular bundles and the seeds. High concentrations of pectins make it difficult to extract and/or clarify grape juice and filter wine. These substances are modified by heat and alcohol, so that during a red fermentation the skins become less gelatinous and easier to press, and the must becomes clearer. Pectins can be broken down by pectolytic enzymes, added during processing (see below).

Acids

The most important acids in grapes are (+)-tartaric acid and (–)-malic acid, which constitute more than 90% of the total acids present at harvest, and at maturity they are usually in a 1:1 ratio. These acids are responsible for the relatively high acidity and low pH of grapes compared to other fruit, and the crisp taste of wine. The increase in sugar concentration which occurs during maturation of the grape is accompanied by a decline in acidity. The total amount of titratable acidity (TA) in the juice of grapes varies between 3.0 and 12 g/l calculated as tartaric acid (in the New World), depending on ripeness level and cultivar, with the pH of the must usually between 2.8 and 4.3. In France, TA is calculated as sulfuric acid, which can be obtained from total acidity expressed as tartaric acid by use of a conversion factor of 0.6535. In other words, 5.0 g of TA expressed as tartaric acid is the equivalent of 3.3 g expressed as sulfuric acid. When Australian or South African winemakers discuss acidity with French winemakers, they may be surprized at the seemingly low levels of TA in the French wines, not realizing they need to multiply the French TAs by a factor of 1.530 to achieve equivalence. See Table 2.9.1 for typical total acidities of ripe grapes.

The sugar:acid ratio is used as an important measure of the maturity of grapes, with values from 2.5–3.0 regarded as desirable, but this differs from region to region, from cultivar to cultivar and even within vineyard blocks in a region. The buffer capacity of must is high due to the presence of large quantities of tartrate, malate, potassium and other ions, which has implications for sulfur dioxide use and even oxidation potential. Lactic, succinic and acetic acids may be present in very low quantities, but are usually an indication of microbial spoilage. The tartaric acid concentration decreases during fermentation due to the precipitation of potassium bitartrate which is less soluble in alcohol than in water. Malic acid can be reduced by some strains of yeast or malolactic fermentation.

Phenolics

Phenolics are a group of chemical compounds that affect the wine color, texture, astringency and bitterness. They are important in the style and quality of wine, even though they are present in relatively small quantities.

Anthocyanins and colored tannins are responsible for red wine color and are found in the skins of red grapes (Section 2.9.2). Tannins in the skins, stalks and seeds can all contribute positively to the structure of wine, if not present at excessive levels. Phenolic extraction is very important in red winemaking and will be discussed in detail in Section 2.9.2. Phenols that are present in the skins of white grapes are colorless, but may have implications for the oxidation potential (redox status) of the wine. These are usually nonflavonoids and include benzoic and cinnamic acids, but flavonols and smaller tannins may also be present. At high enough concentrations the latter compounds can be astringent and bitter, and lead to browning during maturation. See Section 5.4.3 for nutritional aspects of the phenolic content of grape juice and wine. Also, see Chapter 5.8 for a discussion of phenolic substances with regard to their general chemical characteristics, including their *in vitro* antioxidant properties, and possible health benefits.

Inorganic ions

These are not important in juice and wine, unless they are present in relatively high concentrations. If grapes are incinerated, the ash quality is from 0.2 to 0.6% of the fresh weight. The most important components in the ash are K^+, Mg^{2+}, Ca^{2+} and Fe^{3+}cations, as well as certain carbonates, oxides, phosphates and sulfates. Copper and iron are important oxidation catalysts (Danilewicz, 2003). At certain levels, metal cations give rise to complex hazes in the wine called iron, copper or aluminium 'casse.' Haze formation may occur with only a few mg/l of dissolved metals in total, but is far less common than it was previously when metal levels were not as carefully controlled. Iron, copper and aluminium may be leached into juice and wine from brass, copper and steel surfaces containing these metals, which is one of the reasons why stainless steel has become ubiquitous in wineries. Even stainless steel can contain welding sites that leach metal ions into solution, or act as 'electron guns' and promote oxidation reactions. Copper has also been implicated in the formation of protein hazes. The potassium content is important as the potassium salts of the organic acids may precipitate as potassium bitartrate, potassium tartrate or potassium tartromalate in the bottle if the wine is not properly stabilized. See Sections 4.3.3, 4.4.3 and 4.4.4 and Chapter 4.5 for chromatographic, colorimetric, atomic spectroscopic and electrochemical methods (respectively) for the determination of inorganic ions in beverages, including wine. Also, see Section 5.4.2 for nutritional aspects of minerals in wine and Section 5.10.3 for health aspects regarding inorganic residues, including pollutants.

Nitrogen containing compounds

Proteins, amino acids and ammonium salts all contain nitrogen. Yeast and bacteria need nitrogen sources to build the polypeptides and proteins in cell structures. The total organic nitrogen quality of must varies from as low as 60 mg/l to around 2400 mg/l, depending on cultivar, ripeness level and climatic conditions during growth (Ribéreau-Gayon *et al.*, 2006a). In cooler regions and vintages, concentrations of amino acids within the berry will be higher due to limited synthesis of proteins. The nitrogen compounds are present mainly as amino acid and proteins as well as ammonium compounds, which are the most easily assimilable by yeast. It is generally recognized that yeast prefer free amino nitrogen (FAN) concentrations of between 200 and 350 mg/l, but this will depend on the sugar concentration of the must. A higher sugar concentration will require larger concentrations of FAN (as high as 900 mg/l in the case of very sweet musts) in order that the yeast may complete fermentation. Nitrogen is also involved in the formation of higher alcohols during the synthesis of amino acids by the yeast cell, and it is recognized that nitrogenous compounds will affect the development of aroma, bouquet and foaming characteristics in sparkling wines (Section 2.9.3).

Proteins (which contain a significant proportion of nitrogen) denature and flocculate, and may give rise to hazes if they are present in wine at high concentrations (Section 2.9.4). These proteins can be removed by addition of bentonite or other fining agents before, during or after fermentation (Section 2.9.4). The

assimilable nitrogen concentration of the juice is often supplemented (not always necessarily) using diammonium phosphate (DAP) before fermentation by the winemaker in order to ensure a complete fermentation. See Sections 4.6.3 and 4.4.2 for digestion (or combustion) and infrared spectroscopic methods of assessing total nitrogen or total protein content (respectively) of raw materials and drinks. See also Sections 4.3.3, 4.4.5 and 4.6.1 for chromatographic, mass spectrometric and electrophoretic methods (respectively) of characterizing proteins, including their molecular weights.

A large variety of enzymes, extracted from the grape skins during pressing, occur in the juice or must. The concentrations of oxidative enzymes, including the polyphenoloxidases that cause browning of white must, vary between grape varieties. For example, the polyphenoloxidase content of the Cape Riesling (actually Cruchen Blanc) is relatively high, so its juice oxidizes very easily. Also, *Botrytis* contaminated grapes contain high concentrations of the fungal polyphenoloxidases laccase and tyrosinase, so botrytized juice tends to brown (oxidation) easily as a result. Although most enzymes will be inactivated by alcohol, some of the enzyme can remain in young wine and catalyze the more detrimental wine oxidation.

The most general method of deactivating undesirable oxidative enzymes is by moderate applications of SO_2, or through bentonite additions, which entrain and help precipitate proteins (and therefore enzymes) from juice or wine. Pasteurization by heating the crushed grapes for three minutes at 70 °C is also effective as it denatures the proteins within the enzymes and renders them inactive, but may have consequences for wine flavor. The use of winemaking enzymes is discussed in more detail later this section.

Pectic (or pectolytic) enzymes hydrolyze the pectins (water soluble colloidal carbohydrates) by breaking up the chains of polygalactoronic acid molecules, producing clearer juice and aiding filtration. The enzymes also help to effect an increase in free run juice (and therefore yield) from crushed grapes, especially the fleshier varieties. Commercial preparations of pectic enzymes are available to boost natural levels.

Aroma compounds

There are many aromatic compounds and precursors to wine aroma present in grape juice in trace amounts. The situation in wine is very complex, with synergy between groups of compounds as important as, if not more than, the individual compounds present. A distinction is made in wines between primary grape flavors, (e.g. terpenes in the aroma of Muscats), secondary fermentation flavors, (e.g. higher alcohols and esters) and tertiary flavors, (e.g. wood aroma compounds, bottle bouquet).

Ferreira *et al.* (2008) identified three 'odor patterns' in wine. Firstly, wines with a large quantity of distinct volatiles, deriving from grape types, which may be classified as 'aroma rich' – cultivars with clearly recognizable aromas in the grape that pass unchanged to the wine. Examples include Muscat varieties (various terpenoid components) and *Vitis labrusca* (methyl anthranilate). The author's second grouping are wines derived from grapes containing a large number of nonodorous precursors (glycoside or cysteine conjugates) specific to varietal flavor and released by microorganisms during winemaking. Cultivars with plenty of flavor in the grape, which produce characterful wines are 'aromatic' and include Sauvignon Blanc (grassy/tropical), Weisser Riesling and Gewürztraminer (floral and spicy), Cabernet Sauvignon (grassy and/or berry-like), Merlot and Shiraz (berry-like). 'Neutral' cultivars do not contain a specifically recognizable flavor and the fermentation bouquet and the flavors due to ageing and other processes are of more importance. These include Chardonnay, Chenin Blanc and Colombard. Chardonnay often produces a wine that has a fuller character. Ferreira's third aromatic division of wines rely on acid catalyzed hydrolysis and rearrangements during maturation and ageing, which yield highly odor active compounds eg: *cis*- and *trans*-rose oxide in Gewürztraminer or trimethyl dihydronaphthalene (TDN) in Riesling.

Despite the number and complexity of volatile compounds and their low concentrations, winemakers consider these components as crucial to wine quality, and try to optimize their extraction, and conservation during processing. The aroma and flavor characteristics of the grapes (the primary aroma compounds) are

paramount for a fruit driven wine style, but other contributors like esters from fermentation or certain volatile phenols from oak are very important in styles that are less dependent on primary aroma.

Alcohols

Alcohols should not be present in grapes unless there is rot, or wild yeast has fermented a small portion of the juice from broken grapes in holding tanks. The ethanol (a two carbon alcohol) that is present in wine is produced during fermentation by yeast from grape sugar. Trace amounts of other alcohols with three or more carbons (higher alcohols, fuselols or fusel oils) are also produced by rot, and during fermentation, and these contribute to the flavor of the wine and the complexity of the aroma. At higher concentrations, fuselols have unpleasant cloying aromas, and give a burning sensation on the palate. Glycerol, which can be produced in significant amounts when grapes are over-ripe or infected with *Botrytis cinerea* (noble rot), has a beneficial effect on the viscosity and sweetness of wine.

Berry Ripening

There are four stages in the development of grape berries. The first is the vegetative period (Figure 2.9.2), which extends from the formation of the berry (after fertilization of the vine flowers) until ripening starts. The berries are small, hard and green, and photosynthesize like the other green parts of the plant. Acid levels are high, and berries are bitter and unpleasant to eat due to the presence of tannins and stilbene phytoalexins, which help in the defence of the immature seeds. The second stage is veraison, which marks the beginning of the ripening period (see Figure 2.9.3). The berries change color from green to yellow (for white varieties) and to light then dark red (for red varieties). The color change is abrupt, and is accompanied by the grape becoming more translucent and less opaque. The sugar content of the berry increases. The berry ceases to photosynthesize, and becomes a storage organ. The third stage of development is the maturation of the berry, or accumulation stage. This stage lasts for 40–60 days. The grapes swell, the concentration of sugar increases and the acid concentration decreases. At physiological maturity the berries have attained their maximum diameter and sugar concentration. The accumulation stage is characterized by a steady increase in glucose and fructose. The vacuoles in the pulp cells become filled with water (juice), and this uptake is reflected in

Figure 2.9.2 *Early vegetative stage in Chardonnay berry development showing young inflorescence and growth points.* Photograph courtesy of Professor Piet Goussard

Figure 2.9.3 *Veraison in white grapes, showing translucency.* Photograph courtesy of Professor Piet Goussard

increases in berry weight, volume and diameter. During this stage the concentration of acids decreases. Malic acid is respired, as is tartaric acid if ambient temperatures are high during the ripening period. Anthocyanins and tannins accumulate in the skins of red grapes giving them their characteristic color, and this accumulation is influenced by the grapes exposure to light. The proportion of soluble polyphenols rises during ripening to a maximum then decreases. The flavor of the grapes increases with ripening, with the majority of the flavor volatiles accumulating later when the sugar increase slows down. For any one vineyard there is a general association between the progression of aroma and flavor development in the berry and sugar accumulation. Descriptive characters at the early ripening stage are usually associated with lower sugar levels (for example, acidic, tart, green, citrus). At maturity, descriptors for the fruit will usually be associated with the typical characters present in the wine (for example, peach-like, tropical, full, floral).

The last stage is when the berries are left on the vine and they become overripe (Figure 2.9.4). The fruit becomes shrivelled as it dehydrates and the sugar and acid in the juice becomes more concentrated. Flavor changes also take place, and the juice will take on raisin or madeirized characters.

Figure 2.9.4 *Over-ripe white Muscadel grapes, shrivelled, but with no 'noble rot.'* Photo courtesy of Professor Piet Goussard

In winemaking the ripeness criteria are different for different wine styles, and the decision to pick the grapes will be based on the sugar, flavor and acid levels required, and the personal experience of the winemaker. In some cases, grapes are picked a long time after physiological maturity has been reached, for example, red grapes are frequently picked over-ripe in order to achieve maximum color and tannins, when the stems are shrivelled and woody, and the skins are puckered. Chardonnay grapes destined for sparkling wine base, on the other hand, are picked before physiological ripeness when sugar is lower and acid higher than for a full bodied white wine. For aromatic and terpene rich wines the main criteria are the flavor and aroma of the grapes.

It is especially important to follow the flavor development of grapes for fruit driven white wine styles. Flavors are monitored by tasting the grapes as samples are taken for ripeness assays.

Berry Sampling During Harvest

Berry monitoring is a crucial part of the process of winemaking, and samples are usually taken from soon after veraison, throughout the maturation period. Taking a representative sample of berries from the vineyard is essential if the real state of maturity of the vintage is to be assessed. It is generally recognized that a minimum sample size of 200 berries per hectare of vines, taken randomly from vines, and ensuring that not only ripe bunches are sampled. Back at the laboratory, the berries are crushed, the juice strained, and the must is then tested for indicators of ripeness, such as total soluble solids, titratable acidity and pH. Lastly there is one ripeness indicator that is too often overlooked: the taste of the berry. Other sensory assessments of the berry might include firmness, elasticity, appearance of the seeds and greenness or woodiness of the stems. The juice should always be assessed for sweetness, acidity, fruit flavor and aroma.

Winemaking Principles

Winemaking is the process of turning grapes into wine, and it starts as soon as the grapes are picked. At a very minimum, winemaking techniques should include procedures to prevent or minimize three problems:

- microbiological spoilage
- problems related to too much or too little oxygen during winemaking and after bottling
- formation of other off odors and taints.

At the 2006 International Wine Challenge in London, where approximately 13 000 wines from all over the world were assessed, it was found that around 7% of wines were faulty, and of these wines, around 2% were found to have oxidative or reductive issues that were bad enough to disqualify them from competition (Thomson, 2007). Other problems included volatile phenols (usually associated with *Brettanomyces* infection), and cork taint. Although the author acknowledges that around half the problems are due to closures, it was also stated that winemakers needed to be aware of problems so as to deliver quality and consistency in their products.

The most important characteristics of fruit destined for the production of premium quality table wines are lack of rot or premature fermentation, and the correct degree of ripeness for optimum fruit aroma and flavor. Intact grape skins act as a barrier, which protects juice from the effects of oxygen, microbial spoilage and metal contamination, therefore it is beneficial to minimize damage to berries during picking and transport to the winery. Once the skin is broken the juice requires protection, and the extent of the use of antioxidants such as SO_2 and ascorbic acid should be proportional to the risk of spoilage. Good quality (rot free, ripe) fruit makes the task of the winemaker less arduous, but it is certainly possible to turn good quality fruit into poor quality wine through negligence. With good winemaking it is possible to make sound, ordinary wines

from mediocre fruit, and large volumes of such wines are made and sold at the entry level of the market. A winemaker will try to incorporate the following winemaking objectives into the production of quality white wines during the various stages of the process:

During harvesting of grapes, there will be selection of juice or wine fractions with best possible composition most appropriate to the style required and preservation of flavor.

During crushing and destemming, there will be avoidance of unnecessary damage to berries, seeds and stems, use of additional techniques such as skin contact and enzymes to increase fruit flavor extraction.

During juice collection, the winemaker will undertake correction of composition if necessary, e.g. by acidification or chaptalization. There will also be additions made to prevent major microbiological spoilage and oxidation.

During alcoholic fermentation (and malolactic fermentation, if appropriate), the most appropriate yeast (and bacterial strain) will be used and optimum fermentation conditions employed. The winemaker will also try to prevent or remove off flavors (e.g. undesirable fermentation characters).

The winemaker will employ appropriate clarification techniques for the style and correct potential instabilities which may reduce market acceptability (e.g. protein and bitartrate instabilities).

The winemaker will 'fine tune' the wine during stabilization and ageing to add complexity/balance/finesse (e.g. wood maturation, specific fermentation characteristics, blending).

Bottling the wine should be of sufficient quality to ensure protection from oxidative and microbial spoilage – so as not to undo all the good work carried out during the previous operations.

Naturally each wine style will have a slightly different phases and treatments. *Blanc de noir* and rosé table wines from red grapes are made in a similiar way to white table wines and may be prepared as dry, sweet, still or perlé wines. Red wines are fermented on the skins, and pressed after different periods of skin maceration, depending on the style. Considerations for red winemaking will be addressed in Section 2.9.2.

Wine Analysis and Monitoring the Ripening Process

Wine analyses provide the winemaker with essential facts concerning the quality and composition of the juice. This information may be used to modify the natural winemaking process, if necessary. Essential information is usually obtained by conducting the necessary analyses and includes the microbiological status, the chemical composition of grapes and wine, and the sensorial quality of the wine.

Sugar

The sugar content of grapes is one of the most important ripeness indicators, and is determined approximately by measuring the density of solution (total soluble solids) using calibrated hydrometers (in °Balling, °Brix or °Oeschle, depending on the country where the wine is being made). At 20 °C, pure water will have a Brix reading of 0 °B, 18% sucrose will read 18 °B, and 95% alcohol will read –7 °B (Morgan *et al.,* 2006).

Other than determining how ripe the grapes are, sugar concentration is also used for remuneration of growers, certification and monitoring of fermentation. The sugar concentration can also be used to determine the potential volume percentage alcohol (as previously described, °Balling × 0.55), but this is an approximation as the efficiency of conversion into alcohol is difficult to predict.

Refractometry (Section 4.6.3) also measures the density of juice, but through the refractive index of the liquid. Hand held refractometers are used in the vineyard to give a rapid indication of berry ripeness but as the °Brix level varies within the bunch and from vine to vine, it is not an accurate assessment of the ripeness of the whole vineyard. Laboratory refractometers are used for a more accurate assessment of sugar in juice expressed from larger, more representative sample of grapes. The measurement is very temperature dependant, the temperature of the refractometer being more important than the temperature of the sample.

Titratable acidity

Determining titratable acidity by titrating (Section 4.6.3) with an alkali such as NaOH, provides information on the ripeness of the grapes, the 'sourness' of the wine and also of wine stability. Generally speaking, riper grapes have lower acidity and unripe grapes are very acidic, with high levels of malic acid. This acid can be determined by enzymatic/colorimetric methods (Section 4.4.3), chromatography (see Chapter 4.3; especially Section 4.3.3), or (if the vineyard has access to technology) through the use of an infrared spectrometer (Section 4.4.2) like a Foss scanner. Malic acid levels will give an indication of whether malolactic fermentation needs to occur, and if decreases in this acid are accompanied by increases in lactic acid, the fermentation will be underway or may have completed.

pH

Measurement of pH (defined as $-\log [H^+]$) is determined by means of a pH meter (Section 4.5.1), and is important for microbiological and oxidative stability. Sulfur dioxide is far more effective at lower pH levels and oxidation reputedly happens faster at higher pH.

Volatile acidity

Volatile acidity (mainly acetic acid and ethyl acetate) can be determined by distillation (Section 4.6.3) or by titration and gives some indication of the microbiological health of the wine. If the technology or means of analysis are available, it is useful to determine tannin and color levels, esters and aldehydes, and to know whether metals (particularly Cu and Fe) fall within an acceptable range. Sensory evaluation of the grapes and juice are also essential, and inspection of the grapes should be carried out visually to determine the health of the harvest and seed and stem ripeness.

Sulfur dioxide

Knowing concentrations of sulfur dioxide throughout the winemaking process is important for the health and longevity of the wine, as it is crucial to microbiological stability and reducing capacity (oxidation condition) of the wine. As a generalization, half the added SO_2 will be bound to other components in solution (such as acetaldehyde) almost immediately on addition, and half will be available as free bisulfite to carry out the antioxidasic and antiseptic functions. The addition should be checked after a few hours when the equilibrium between free and bound will be reached. The ratio of free to bound SO_2 is affected by the amount of the addition (lower with smaller additions) and the temperature (lower with lower temperature). More detail on the activity of sulfur dioxide can be found in Sections 2.5.2 and 2.5.3, and health aspects relating to SO_2 content of wine and other drinks are discussed in Section 5.9.2.

SO_2 is determined by titration with iodine (the so called Ripper method; see Section 4.6.3), or by aspiration (Section 4.6.3) and titration with sodium hydroxide.

Alcohol

Alcohol, or more strictly ethanol, is determined most accurately by distillation, measuring the density of the distillate with a calibrated hydrometer, and then reading off the actual alcohol value corresponding to the density from a set of tables (Section 4.6.3). It is also possible to use an ebulliometer which operates on the difference between the boiling temperature of pure water and the boiling temperature of the alcohol mixture (wine) (Section 4.6.3). As the boiling temperature of water differs according to the barometric

pressure of the atmosphere, both boiling temperatures must be read each time the alcohol concentration is needed, and a boiling point of 100 °C cannot be assumed for water.

The alcohol level provides more information on the microbiological condition of the wine, but it is also very important in determining remuneration and the amount of excise duty to be paid.

Harvesting Procedures

During harvesting the grapes are physically removed from the vines and transported to the winery. The aim of this operation is to pick the grapes and transport them to the winery in the best possible condition, with the minimum delay. In so far as possible, the grape crop should also be harvested under favorable climatic conditions as rain dew, fog and mist may dilute must. Ideally, harvest should begin once the sun has dried the vines, but not so late in the day that the grapes are warm. There are two main options available for the actual picking operation: hand or machine harvesting. Hand harvesting is used when the grape quality is of prime importance, in small vineyards that cannot justify the cost of a harvester and in older vineyards, which were not been set up for a harvesting machine. Hand harvested grapes arrive at the winery in whole bunches. Whole bunch pressing is often used in premium sparkling wine production and always when grapes are to be used for carbonic maceration. Hand picking is also used when it is necessary to select bunches or parts of the bunch, as in the preparation of noble late harvest wines. Pickers can also be instructed to avoid unripe second crop bunches, or grey rot, and to avoid including leaves in the harvest. Machine harvesting has become very widely used since its introduction in the early 1970s (see Figure 2.9.5). It is fast, efficient and much cheaper than hand picking, particularly in areas where there is a lack of local labor. Large quantities of grapes can be picked quickly at the optimum ripeness, and it is possible to harvest at night when the temperature is lower. The drawbacks of machine harvesting include the high capital cost of harvesters, damage to sensitive fruit, and the requirement for suitable vineyard layout and vine trellising to accommodate the harvesting machines.

Figure 2.9.5 *Machine harvesting, Paarl, South Africa.* Photograph courtesy of Paul Gerber

Figure 2.9.6 *Spraying dry ice on grapes awaiting processing at KWV, Paarl, South Africa.* Photograph courtesy of Paul Gerber

Prolonged maceration of warm, harvested grapes in their juice should be avoided, as microbial spoilage and oxidation will result. Enzymes come into direct contact with substrates resulting in what Ribereau-Gayon *et al.* (2006a) describe as 'explosive' enzymatic reactions. For example, the laccase secreted by *Botrytis cinerea* will, in the presence of air (oxygen) cause oxidation of phenols, turning juice brown. Certain grape varieties (for example, Semillon) are more prone to damage than others and machine harvesting is a less desirable option then hand picking for these varieties. Low fruit temperatures can help reduce oxidation reactions and wild yeast growth. In warmer climates, picking is avoided during the hottest part of the day, and often carried out at night or in the early morning. Ideally grapes should be transported as quickly as possible to the winery, and in instances where grapes are transported long distances, provision should be made to keep them cool. Once the grapes have arrived at the winery it is easier to control the conditions they are kept under, but it is still important that processing is carried out promptly. In particular, care must be taken to protect the juice from oxidation and excess phenolic extraction. This can be done by sprinkling sodium or potassium metabisulfite over the grapes or preferably covering the grapes with an inert gas (N_2 or CO_2) or spraying them with 'dry ice,' as shown in Figure 2.9.6.

The treatment of the grapes at the winery depends upon the wine being made. In white winemaking the juice is separated from the grapes, then fermented, whereas red grapes are crushed and fermented with the juice

Destemming and crushing

Most grapes are crushed and destemmed during a white winemaking process. Crushing breaks the berries and allows the juice to escape, and the mixture of skins, pulp, juice and seeds produced is called must. The

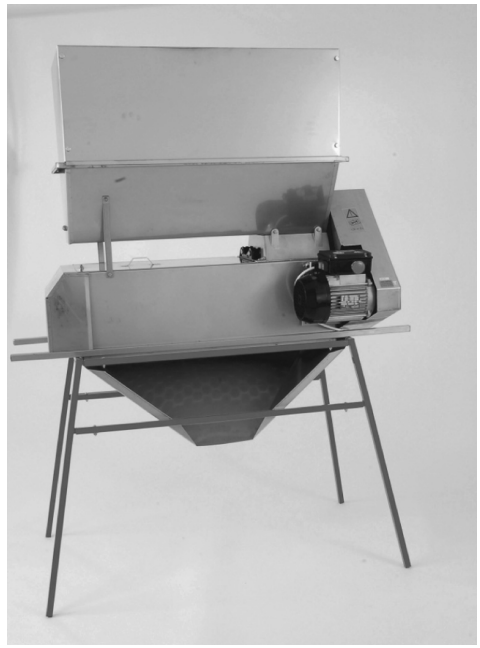


Figure 2.9.7 *Motorised crusher with stand.* Photograph Courtesy of Vigo Ltd., Dunkeswell, UK

winemaker can choose to leave in the stems or take them out, depending on the condition of the stems and the style of wine being made.

The grapes are normally destemmed either before or after crushing in the same machine (*egrappeoir*). The most common type of crusher is a roller crusher with fluted rubber or metal rollers to crush the grapes by a wringer action. This is normally coupled with spirally arranged, radial fingers which rotate over a perforated cylinder. The berries are knocked off the stalks and drop through the holes in the cylinder to be fed out through the crusher sump. The stalks are moved through the cylinder by the spiral fingers and thrown out of the end to be collected and removed. Additions made at the crusher may include sulfur dioxide as a preservative, and pectolytic enzymes to improve yield of juice from the grapes and juice clarification. From the crusher the must is pumped to a drainer or a press. Many crusher sumps are fitted with failsafe mechanisms, which ensure that the pump only operates when there is must to pump. The crushed pulp may be cooled by passing it through a cooling unit, if the grapes are warm.

During the crushing and destemming operation (see Figure 2.9.7), juice is aerated, and although a certain degree of aeration is essential for a healthy fermentation, too much oxygen can cause oxidation of the juice, especially if rotten grapes are crushed, due to the action of oxidizing enzymes from fungus and bacteria present on the rotten grapes. Herbaceous and astringent flavors may be increased if crushing is too harsh, breaks seeds and tears stems. If stems are left in the press (as when whole bunches are pressed), they can help to facilitate juice extraction by forming 'flow channels,' and preventing the press cake from becoming too compact. If a difficult pressing is suspected (as in the case of dehydrated berries, or difficult cultivars like Viognier), packing the press with alternate layers of grapes and stalks may facilitate release of juice. Fermentation in the presence of stems during white winemaking is rare.

If whole bunches are required (for example, in the preparation of sparkling wine base), then these must be handled and tipped manually direct from harvest trays or bins into the press.

Protection of the must and juice

Nowadays white must is usually protected against oxidation in order to avoid browning and loss of primary aroma. The most abundant phenolic compounds in white grapes are hydroxycinnamic acids, and when white grape juice is processed without sulfur dioxide, enzymatically induced oxidation occurs, leading to a precipitation of these phenolic compounds as insoluble brown pigments. Must resulting from extended skin contact will be high in flavones and endogenic enzymes, and therefore particularly susceptible to oxidation. The other changes caused by oxidation of the juice include color changes, and increases in bitterness and volatile acidity, with associated decreases in quality, particularly in the case of delicate, aromatic white wines where the wine depends on fruit aroma and flavor. The spoilage occurring as a result of contamination of grapes and juice by wild yeast and bacteria can also be slowed down considerably by the effective addition of sulfur dioxide, which is usually added as potassium metabisulfite powder or solution. Sulfur dioxide at levels around 50 mg/l will inhibit the action of oxidizing enzymes, and will also react with oxidation products like hydrogen peroxide and acetaldehyde to prevent further damage to the composition of the must and wine.

The extent of the use of antioxidants such as SO_2 and ascorbic acid is usually proportional to the risk of oxidation, which depends on the quality of the juice and processing conditions. Oxygen consumption is higher if the temperature of the juice is high and the grapes are moldy, as enzymes are more active. Two main oxidases exist (tyrosinase in healthy grapes and laccase in botrytized fruit). These transform the principal white grape phenolic compounds into quinones, which tend to polymerize, forming brown compounds.

The methods used for limiting oxidation, other than adding antioxidants, include using inert gas to cover juice and fill vessels, processing the juice as quickly as possible and cooling the juice to slow the rate of reactions, particularly those initiated by polyphenoloxidase. Ascorbic acid, also at around 50 mg/l, may be added at this stage as an antioxidant, but sulfur dioxide should be present if it is to be used, as it has been found to have pro-oxidant capabilities in the presence of phenols (Bradshaw *et al.,* 2003).

In some cases juices are treated oxidatively, as the resulting wine is less susceptible to faults associated with reducing conditions in the bottle (off aromas from sulfides and mercaptans). Working more oxidatively usually leads to a different style of wine which has less obvious fruit aroma and flavor, and which seem to display more resistance to oxidative quality degradation during ageing. Adding pure oxygen in limited quantities to unprotected juice before clarification can also improve the stability of white wine color without producing oxidation type flaws. This process is called hyperoxygenation (sometimes, and less correctly, hyperoxidation), and consists of oxidizing and polymerizing sensitive juice polyphenols to precipitate them so that they be easily removed during the normal racking process. When phenols are eliminated from must by enzymatic oxidation, they do not affect wine quality by undergoing chemical oxidation in the bottle later.

Thus, although oxidized musts are very dark, the resulting wines are lighter and more stable in sensory qualities than those produced by conventional reductive methods (Schneider, 1998). In order to precipitate must flavonoid contents lower than 100 mg/l (as catechin), one saturation concentration (9 mg/l) of oxygen may be sufficient. Higher contents of phenolics obtained by skin contact would require an oxygen concentration of about 30 mg/l, corresponding to approximately three saturations (Schneider, 1998). Excessive oxygen supply is usually highly detrimental to white wine quality, and this should be avoided. Hyperoxygenation is very cultivar dependent, and is more likely to be used for a neutral, full bodied style of wine (for example, Chardonnay) that may be given oak treatment. It can also used to remove color from musts from red grapes destined for white wines (Morgan *et al.,* 2006).

Microoxygenation is another process involving deliberate oxidation of wine, using a bubbler to deliver very small quantities of oxygen (about 3 ml/l month) to imitate the effect achieved during barrel ageing. This is reputed to 'open' wines up that would otherwise be 'tight' or 'closed,' remove reductive smells or problems developing on the lees, and smooth and soften red wines for earlier drinking.

As well as being more sensitive to oxidation than red juice, white must is also more susceptible to microbial attack because of the lack of polyphenols, which help to control sensitive organisms. If the conditions are suitable, microorganisms present on the grapes or in the winery will multiply rapidly and may spoil the juice. Other than the use of SO_2, good hygiene, low temperatures and rapid processing will also help to retard growth. Wine yeasts are adapted to low temperatures and sulfur dioxide and are therefore less affected than wild yeast and bacteria.

Skin contact and enzymes

If the grapes are healthy, destemmed and optimally ripe, it is often the case that the must is left in contact with the skins before pressing, in order to extract more flavor and aroma. This process can produce wines that often age better, with more varietal character, more body and a deeper, more attractive color. It is not usually used for light, delicate styles of wine, as the process also extracts phenols and tannins from the skins. Skin contact is used frequently when wine is made from certain varieties, notably Sauvignon Blanc, where volatile aromatic characters are crucial to wine typicity and quality. It can also be used in the case of red varieties like Pinot Noir and Merlot where some prefermentation skin contact at low temperatures allows color extraction without tannins, with a softer wine resulting. The temperature of skin contact is a key control feature and should be around 10 °C. Often the crush will be protected from excess oxidation by neutral gas (nitrogen) blanketing and around 50 mg/l sulfur dioxide, which also helps to weaken membranes and make cells more permeable. Maintaining the vintage at low temperatures is essential as high temperatures favor the extraction of harsh tasting phenolics, and the activities of microorganisms and enzymes. The time of contact is dependant on the level of extraction required, usually only 2–8 h for white crushes, but sometimes as much as 24 h if the grapes are in very good condition (Morgan *et al.,* 2006).

Use of enzymes

A variety of enzyme preparations exist for different winemaking purposes. Depending on the reason for use, they may be added at this stage, or later in the winemaking process. Commercial enzyme preparations are added to must at rates which vary from 20–100 g per ton, depending on the advice of the manufacturer. Enzymes are proteinaceous in nature, and are therefore inhibited by the factors that will denature proteins, i.e. low or high pH, low or high temperatures, high sulfur dioxide content and high alcohol concentration.

Commercial pectolytic enzymes are a mixture of enzymes, which catalyze the hydrolysis of methylated polygalacturonic acid (pectin) breaking down the colloidal polymers into lower molecular weight polymers or soluble galacturonic acid. The enzymes present include pectin methylesterase (PME), polymethylgalacturonate lyase (PMGL), polymethylgalacturonase (PMG), polygalacturonate lyase (PGL) and polygalacturonase (PG). PME hydrolyzes the methyl esters of galacturonic acid sequentially within the pectin macromolecule, liberating the acid functions and releasing methanol into the juice (Ribereau-Gayon *et al*., 2006c). PGL and PG sever the 1,4-glycosidic linkages between galacturonic acid molecules only when the carboxylic acid functions are free of their methyl groups. PMGL and PMG are able to act on 1,4-glycosidic linkages when adjacent galacturonic acid groups are methylated. The effect of breaking down the pectin is that the juice is easier to extract, draining is facilitated and the treatment enhances the yield of free run juice. Pectolytic enzymes can also be added after pressing at between 5 and 50 ppm to assist in juice clarification. The length of time that the enzyme takes to act depends on the strength of the enzyme solution, factors like pH and temperature, and the amount of pectin present, which varies with variety and season. Effectiveness of the treatment depends on temperature, holding time prior to pressing and the presence of natural proteases in must. Generally speaking, pectolytic enzymes are not inhibited by SO_2 unless the concentration is very high (around 400 mg/l), but are inhibited below 15 °C and above 60 °C.

Terpenes, essential components of varietal aroma in Muscat cultivars, Riesling, Gewürztraminer and others, exist in solution mainly as the glycoside form. When bound to glucose, terpenes are not aromatic (Ribereau-Gayon *et al*., 2006c). Normally the glycosides are separated from the terpenes through acid hydrolysis during ageing, helping to enhance varietal character later in the life of the wine. Glucosidase enzymes will hydrolyze the monoterpene from the glucose during skin contact, and thus release it as its aromatic form immediately, so improving the aromatic quality of early release wines. Malolactic bacteria have also been shown to exhibit limited glycosidasic activity (Liu, 2002).

Grapes that have some degree of rot (specifically an infection of *Botrytis cinerea*) will contain glucans (high molecular weight glucose polymers), which lead to clarification and filtration problems. Commercial preparations of the antifungal enzyme are produced by extraction from *Trichoderma* spp. Glucanase will hydrolyze the bonds and break the molecules up, thus aiding filtration and clarification. The enzymes are sensitive to temperature and ethanol (activity is halved at 10% v:v), so they should preferentially be used before or early on in fermentation. Like pectolytic enzymes, glucanases are also fairly insensitive to SO_2 (Ribereau-Gayon *et al.*, 2006c).

Pressing

The draining and pressing operations happen before fermentation in white winemaking (Figure 2.9.8), and after fermentation when making red wines. Once the grapes have been crushed and destemmed, in larger concerns, the crushed grape mass (or *marc*) may then be transferred (using a specialized crush pump) to static batch drainers. These act in principle like large sieves, which can be operated with a slight pressure of carbon dioxide or nitrogen to hasten juice removal. Drainers are generally completely enclosed to prevent juice oxidation (Figure 2.9.9). Continuous drainers or dejuicers have an inclined Archimedean screw inside a cylindrical screen, and the crush is carried up the drainer and continuously agitated so that juice runs through the screen, promoting high yields of free run. Inclined dejuicers are generally coupled with continuous presses and used in large wineries for bulk wine production. The juice quality from these drainers is generally good, as the bed of skins acts as a filter and retains a lot of finely divided solids. Draining may be speeded up by the addition of pectolytic enzymes. The disadvantage of drainers, and the associated movement of the marc,

Figure 2.9.8 *Loading the press.* Photograph courtesy of Paul Gerber

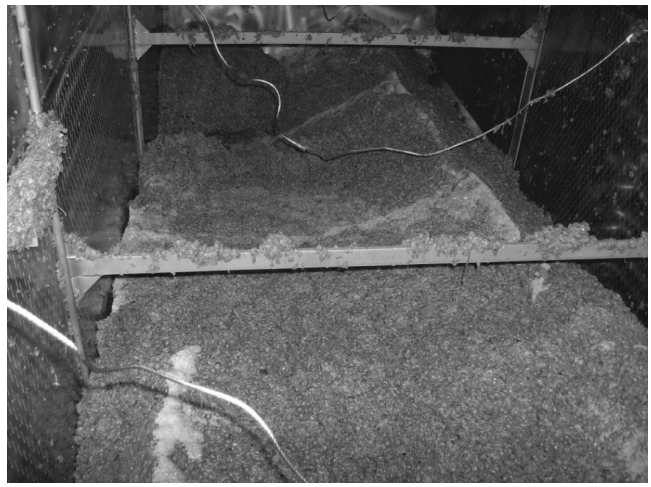


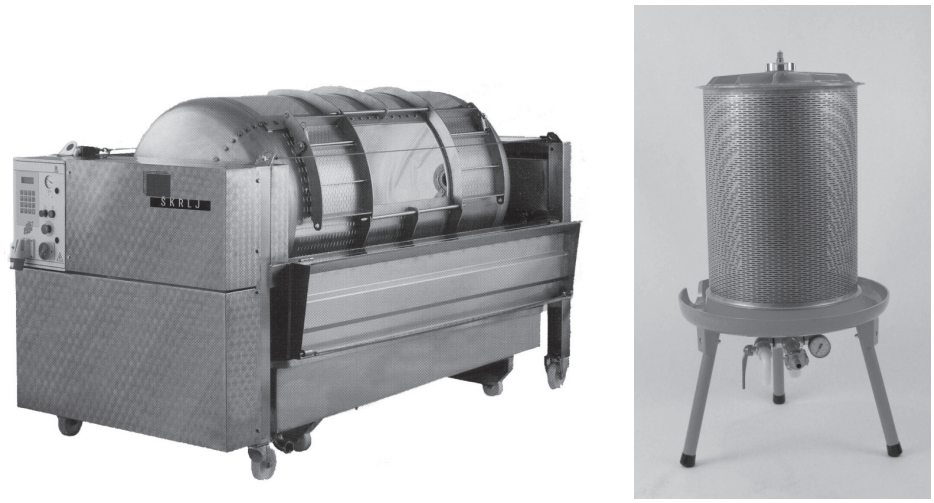
Figure 2.9.9 *A draining tank (Sauvignon Blanc) at KWV, Paarl, South Africa*. Photograph courtesy of Paul Gerber

is that damage to skins can occur, which results in lower quality pressings. The actual yield from the drainer and quality of the juice vary depending on the type of equipment used, the fruit variety, ripeness and quality, and any previous treatments that may have been made to the must (skin contact or partial fermentation on skins, pectolytic enzyme treatment and retention of stalks in white must all improve the yield, separation speed and quality of free run and light press fractions). When the free run (about 60–80% of the yield of juice, depending on cultivar and condition) has drained off, the remaining solids are pressed to extract more juice.

The simplest method of getting the juice from the grapes is by whole bunch pressing. This method is obviously only suitable for hand harvested grapes, as machine harvested grapes have been torn or shaken from their stems and are no longer in bunches. The pressing breaks the skins of the grapes and the quality of the juice released is good, but the yield is lower than for other methods of juice extraction. The first juice released (the free run) is low in phenolics and has the highest sugar, acid and flavor, and is frequently used for high quality wine production, like the *vin de cuvee* (Section 2.9.3). As the grapes are pressed, the juice composition changes. Sugar and acid decrease, and phenolics increase as the pressure increases.

In smaller concerns, unnecessary capital outlay on expensive equipment is avoided through presses such as membrane (bladder) presses being used for combined draining and pressing. This also avoids transfer between a separate drainer and the press and any associated damage to the skins. Depending on the winemaking goal, press program will have been set up to achieve the desired levels of extraction. The first of the pressings (approximately 10–20% of the yield) may be added back to the free run juice, but generally the subsequent, high pressure pressings are kept separate as they contain increased concentrations of phenols (including tannins), which may lead to astringency and bitterness. These pressings may be used for fortified wines or distillation, if local legislation allows.

The oldest and simplest form of press still used is the basket press. It consists of a basket of wooden slats seated in a steel juice collecting tray. The *marc* (crushed grape mass) is loaded into the basket and then compressed by a large plate which is forced hydraulically into the basket, and the juice is expressed through the gaps between the slats making up the sides of the basket. These presses are used mainly in small, traditional cellars and larger versions are used in Champagne making. Disadvantages of basket presses include the slow and inefficient nature of the batch process, the gap between the slats (which allows grape solids



(a) (b)

Figure 2.9.10 *A horizontal pneumatic press (a) and a hydropress (b).* Photographs by courtesy of Vigo Ltd, Dunkeswell, UK

to extrude), and the restricted ability to loosen the cake between pressure applications. Fairly high pressures are required, and juice may also be subject to oxidation as it runs out between the slats. Horizontal screw presses work on the same principle as the screw operated basket press, but the basket is horizontally mounted and revolves to loosen the cake between pressure applications. The pressed marc is emptied through doors in the basket.

Bladder (or pneumatic) presses initially consisted of a horizontal, rotating slotted stainless steel basket with an inflatable rubber air bag in the center. The marc was loaded through doors in the side of the basket, distributed evenly by rotating the basket and then pressed by inflating the bag. Because of the relatively short distance the juice had to travel to the basket only low pressures were required and the pressing action was gentler. More modern versions of bladder presses include tank presses, which consist of a fully enclosed vessel which contains a membrane and juice collection channels. These are gentle in action, and are used in applications such as sparkling wine production where recovery of phenolics is necessarily very low. Pressing occurs at relatively low pressures with minimal damage to the skins and seeds giving good quality juice. The horizontal pneumatic press (Willmes) is an inflatable cylindrical bladder or diaphragm with compressed air or cold water (Figure 2.9.10). Pressures are easily controllable, and very gentle, and yield juice of high quality. The 'tank' press (Bücher) is a totally enclosed variant of the pneumatic press, which has the advantage that it can be prefilled with nitrogen to minimize oxidation.

The continuous screw press gives the greatest yield of juice, is very quick and efficient, but does not yield the best quality juice. Grapes are moved through a perforated steel tube by an archimedean screw, pressure is easily adjustable, but pressures can get extremely high, and the quality of the juice expressed can be poor and fit only for distillation.

Prefermentation treatments and additions

According to Ribereau-Gayon *et al.*, (2006c) the total nitrogen contents of musts range between 0.1 g and 1 g of soluble nitrogen per liter with free amino nitrogen (FAN) or 'easily assimilable' nitrogen is usually

less than 100 mg/l, but can be as high as 400 mg/l. Slow or incomplete fermentations are often linked to a lack of FAN, as multiplying yeast is dependent on must nitrogen for the growth of their cell constituents. A satisfactory fermentation of 160–250 g/l of sugar requires between 70 and 140 mg/l of assimilable nitrogen. In marginal and cooler climates, a lack of nitrogen is generally not a common problem, however warmer conditions, later harvesting and a significant proportion of rot in the vintage will mean that natural grape FAN will be lower. There is also a winemaking requirement for higher FAN levels when yeast is likely to be stressed i.e. in cool, hot or very high sugar fermentations. Under these circumstances, yeast is likely to produce higher levels of hydrogen sulfide in the absence of FAN. It is therefore advisable to add diammonium phosphate (DAP). A 200 mg/l addition is usual if DAP is required.

Higher protein levels can cause filtering problems and can coagulate in the wine causing cloudiness (protein haze). Bentonite treatments have also been recommended in the past, to eliminate proteins in the juice which may be responsible for instability of juice clarity. Bentonite is a montmorillonite clay which in water swells to adsorb charged protein elements. Its use before (or during) fermentation removes heat unstable proteins and also solids that may darken and coarsen wine. Treating juice in this way is recommended mainly for wines which are to be further clarified (e.g. by filtering) and bottled soon after fermentation. However, despite this treatment, white wines may be unstable at bottling and require additional bentonite treatment. Also, if white wines undergo barrel or tank 'on lees' ageing, bentonite treatments are not necessarily recommended. The bentonite may damage the organoleptic quality of lees stirred wines and also even be redundant due to the protein stabilizing effect of yeast autolysis.

The usual dose employed is 0.5–1 g/l, but higher doses can be used for wines from vigorous vines as they have a high protein content. Some bentonite preparations can be used by just sprinkling on the surface of the wine (with mixing), but usually it must be mixed with 10 times its weight of water and allowed to swell for 24 h. Some fining may be done prefermentation, but most fining is carried out on wine. PVPP (polyvinylpolypyrolidone) is used to remove low molecular weight phenolics which may cause problems during ageing, and activated carbon may be used with caution to modify color if it is too deep. Further information on fining and clarification treatments can be found in Section 2.9.4.

Juice clarification

The removal of grape solids from juice is a physical problem so various physical methods are used. Juice clarification is widely practiced to reduce the risk of oxidation by removing the grape derived oxidase enzymes which tend to remain in the solid fraction. It also reduces the risk of wild fermentation as the wild yeasts are removed with the solids. Clarification produces cleaner fruitier wines, and is said to assist in removing herbaceous aromas. Although fermentation on solids, particularly when large amounts are present, may produce unclean wines with unpleasant cardboard and lees flavors, a number of winemakers are moving away from 'water clear' juice, and carrying out what is perceived to be a more natural 'high solids ferment.' With care, this can give a wine of interesting complexity and uniqueness, such as found in some white Burgundies. High solid ferments are not recommended for lighter fruit driven styles, but can also prevent problems which can occur with sluggish or incomplete fermentations.

When juices are highly clarified prior to fermentation, yeast may struggle due to the removal of nutrients, importantly long chain fatty acids and sterols, which are required by yeast growing in anaerobic conditions for cell wall production. A common approach is therefore to remove most of the solids, but to leave some fine material, giving an 'opalescent' rather than perfectly clear juice. The physical methods used for clarification are cold settling, centrifugation, filtration and flotation. These methods are discussed in detail in Section 2.9.4.

Following the clarification stage, the juice is theoretically ready for yeast inoculation and the start of fermentation, but there may be several steps a winemaker may wish to include before then, for example, sugar and acidity adjustments should be carried out after clarification.

Chaptalization

In cool climate vineyards, with reduced photosynthesis, continued vegetative growth and excessive crop yields, grape sugar accumulation is limited. Enrichment of must with sugar (chaptalization) in order to achieve 'normal' alcohol levels in wine is therefore usually permitted by local legislation. Although the given wisdom is that wine should be purely the product of the grape, and that the addition of sucrose from a sugar beet or cane is undesirable, it is generally recognized that wine made in cooler climates is greatly improved by the addition of sugar before fermentation. If the addition is carried out within the recommended guidelines, the extra alcohol improves the balance and body of the wine and helps in wine preservation. If the addition of sugar is too great, the flavor components will be diluted by the extra alcohol, and the wine may well be undrinkable. Therefore, the amount of sugar (potential alcohol) added is strictly controlled and permission from the relevant authority is normally required. In the EU, enrichment must not exceed 3.5% v**:**v in total (in a 'good' year) or 4.5% v**:**v in a 'poor 'year (Morgan *et al.,* 2006). Whether the vintage is a poor or good one is decided at an appropriate point by the local authority. The enrichment methods that are allowed include the addition of dry sucrose, concentrated grape must (CGM) or rectified concentrated grape must (RCGM).

Sucrose is converted to glucose and fructose by exocellular enzymic hydrolysis, and the monosaccharides are then imported and metabolized by the yeast. If sucrose is used, 17 g sugar/l of juice is required (white) and 19 g/l (red) would need to be added in order to raise levels of alcohol by 1% v:v, i.e. 180 g sugar becomes 92 g ethanol and 88 g carbon dioxide (Jackson, 2000b). RCGM (rectified concentrated grape must) is a purified form of grape juice, which, by law has a pH less than or equal to 5, and a refractive index of 61.7%. The nonpurified form is CGM, which is obtained by heating and can be dark in color. As it still contains acids, it can also have a very low pH. It is also rich in iron, which has implications for haze formation and oxidative stability in wine in which it is used (Morgan *et al.,* 2006).

The other implications of chaptalization are mostly associated with dilution. As stated, the natural grape contribution in terms of flavor and aroma is weaker. As potassium bitartrate, the most common salt of tartaric acid, decreases in solubility as ethanol increases in concentration, bitartate precipitation increases, and the total acidity decreases by 0.1–0.2 g/l for every percent volume alcohol added. In red winemaking, phenolic extraction increases by 5% for every 1% alcohol added.

Subtractive techniques can also be used in order to concentrate sugar in solution before alcoholic fermentation, but most of the methods are quite difficult to control in terms of how much the must is concentrated. An associated issue that other components are concentrated at the same time as the sugar: for example, iron and malic acid. Partial must concentration can be carried out by gently heating the juice (25–30 °C) and evaporating off water under vacuum. A thermocompressor can extract part of the must vapor and between 10 and 80 hl of must per hour can be treated, evaporation capacity: 150–1200 l/h (Morgan *et al.*, 2006).

Over-ripening of the grapes on the vine or in prepared areas is one way of concentrating the sugar in the grapes, but it will also lead to a completely different wine with possible madeirized or raisiny characters. This obviously has risks associated, such as loss of aromas and caramelization (browning) if the temperature is too high. A very nonquantitative method is to use cryoextraction, which involves the freezing of the juice and removal of the layer of ice from the surface. Reverse osmosis and ultrafiltration (see Section 2.9.4 for more detail on these methods) can also be used, with more controllable results. The maximum permitted volume reduction using subtractive techniques is 20% or an increase in potential alcohol of 2%.

Acidity adjustments

The sugar–acid balance of a wine is one of its most essential features. High levels of acidity, which are not accompanied by appropriate sugar levels will lead to wines that are tart and unpleasant, and acid levels that

are too low may mean the wine is bland and uninteresting. Good levels of acidity are necessary in white table wines for a number of reasons other than taste. A low pH favors the antioxidant and antimicrobial properties of sulfur dioxide, and microbial spoilage therefore happens less readily in a more acidic environment. A low pH also enhances freshness and fruitiness, and discourages oxidation.

Legislation concerning acidity adjustments differs according to climatic region, and usually tries to compensate for any deficits in the grape chemistry. Climatic regions that are cool will more than likely produce wines with high acidity and low sugar levels, so adding acid in these regions is prohibited, but chaptalization is allowed. There are few restrictions on must and deacidification in England and Wales, for example, but tartaric and malic acidification is prohibited in all circumstances – only citric acid may be added, and this is usually only used for ferric stabilization. In warm climates (e.g. Mediterranean winemaking regions like Spain, Portugal and South Africa), juice often has high pH and low acidity, and the wine runs the risk of being bland and easily spoiled, so the legislation in these regions will allow for additions of acid, but forbids sugar additions.

If must needs acidification, it is usually tartaric (preferred), malic or citric acids that are added. Acids that are not present in grape juice (for example, hydrochloric, sulfuric or nitric acids) are forbidden. Ideally, chemical addition would be avoided, and the winemaker would have had the foresight to harvest some of the grapes 'green' and freeze the juice in order to blend this acidic juice to that of grapes harvested later. In cases where tartaric acid is added, Ribereau-Gayon *et al.* (2006c) recommend addition before or during the fermentation, mainly to optimize volatile flavor compound production and also to ensure a balanced end product (addition after fermentation may make the wine coarser). Early addition of acid also confers the benefits of low pH during processing and allows the maximum time for integration of the acid. Must or juice with pH greater than 3.5/3.6 require acidity adjustment, ideally for white wines to 3.1–3.3 and for red to 3.2–3.4. Legal limits in Europe are for addition of 150 g/hl in musts and 250 g/hl in wine. The tartaric acid addition is best made at the crusher where the acid is weighed and dissolved in the minimum quantity of cold water or juice and added to the grapes before crushing or to the must immediately afterwards.

Deacidification (increasing the pH of the wine) happens naturally in wine during processing as most wines lose around 1 g/l of acidity by tartrate precipitation after the alcoholic fermentation. Under favorable conditions, lactic acid bacteria will transform malic acid into lactic acid during the process of malolactic fermentation, and this will increase the pH by around 0.2 units as well as 'rounding off' of the taste and aroma (discussed in more detail in Chapter 2.3). The malolactic fermentation is fairly normal in the elaboration of red wines, and is also used in dry white wine production when acidity levels warrant it. Certain yeasts belonging to the genus *Schizosaccharomyces* break down malic acid completely to carbon dioxide and ethanol (Swiegers *et al.*, 2005). Certain strains of *Saccharomyces* will degrade malic acid by up to 45%, and research is ongoing to try and increase this.

If deacidification is to be carried out 'artificially,' only three additives are authorized for the process: potassium tartrate, calcium carbonate and potassium bicarbonate. These are available in different combinations in commercial products specifically for acidity reduction in wine and beer. In principle, the tartaric acid will react first with the bases to form insoluble tartrate salts, which can be racked or filtered off. If calcium carbonate is used alone, it is accepted that 100 g of tartaric acid will consume 67 g of calcium carbonate in 100 l of must to form calcium tartrate, which will precipitate at wine pH.

Once the tartaric acid has been neutralized, malic acid then reacts to form malate salts with the excess base. These do not precipitate at wine pH and can have a bitter taste. If the deacidification is carried out in a large quantity of must, the deacidification agent will be 'used up' by tartaric acid, and malic acid will be left in solution, which will change the tartaric to malic ratio and has implications for the chemical and microbial stability of the must and wine. Therefore, if large acidity reductions are needed (above 2 g/l), it is recommended that they are carried out initially on a smaller quantity of the must. This is known as the

'double salt deacidification' method, which neutralizes a portion (around 10% of the volume of the must) to around pH levels of around 5–6, at which pH the malate salts also precipitate, and this portion is then added back after the precipitates have been removed.

The method reduces malic and tartaric acids simultaneously, and ensures that the remaining acid in solution will be a representation of the original acid structure, and not artificially high in malic and other acids. It should be stressed that the success of the double salt deacidification does depend on the initial tartaric:malic ratio, which needs to be as close to 1:1 as possible, and the procedure should only be carried out if musts require large deacidifications. It is prudent to quantify the acids before carrying out this procedure, but in must from unripe grapes it can be assumed that malic acid will be present at significant levels.

Again, legislation exists for this operation in the EU, which must be sanctioned by the control agency in Europe. Also, for example, table wines must have a minimum final acidity of 4.5 g/l as tartaric acid. This treatment must be declared and cannot be combined with an acidification.

White Wine Fermentation

The biochemistry of fermentation is discussed in general detail in Section 2.2.9, so a more technical, but less biochemical account is given here.

All winery surfaces normally support a residential or winery yeast flora. The viability of this flora will depend on a number of factors, including the type of surface; for example, wooden surfaces are almost impossible to sterilize, whereas stainless steel is relatively easy to clean, as well as the cleaning and sanitizing program within the winery. Equipment, particularly if it has areas that are difficult to reach and clean, forms the major reservoir for spores and live yeast, especially in older wineries. Mechanical harvesters, delivery containers, crushers and pumps can all act as a source of yeast and bacteria if they are not kept dry and clean (preferably sterilized).

Although a natural fermentation can give a complex, interesting product that might be viewed as more 'organic' and expressive of the *terroir*, it should also be noted that wild yeasts are known to be unpredictable in their efficiency to convert sugars to alcohol, can produce off tastes, including hydrogen sulfide, and may not finish the fermentation to dryness. The natural microflora may be of high quality and produce an excellent result, but there is no guarantee of this for the producer. As white wine is more sensitive to off flavors and usually dependent on expression of some delicate fruit character, wild fermentations are not as common. White wine is seldom fermented on the skins, and the natural yeast population in the juice may not be sufficient to kick off a healthy, active fermentation.

Aeration of must is beneficial for the growth stage of the yeast colony as it ensures the presence of sterols in the yeast cell wall, which later assist in the transfer of components, and resistance to the toxic effects of alcohol. Most yeast strains produced commercially are able to grow very well in totally anaerobic conditions for up to three generations before their viability will be affected by anaerobiosis. Insufficient oxygen during the initial growth phase of the population may result in a lack of 'survival factors' in the yeast population. These survival factors include sterols (particularly ergosterol) and certain long chain fatty acids, and if oxygen is lacking, they do not develop in the membranes sufficiently to ensure resistance to accumulated toxins such as ethanol and acetic acid later in the fermentation, which may result in an incomplete metabolism of the sugar in solution. Another disadvantage of working anaerobically has become obvious as greater quantities of wine (white and red) are bottled with screw caps rather than corks. While corks have contributed their fair share of problems to wine (including sporadic oxidation, and 'cork taint'), they are not generally accused of causing reductive problems. Although the extent of reductive problems is at times overstated, it is generally accepted that wine that is destined to be bottled under screw cap needs to be made under more oxidative conditions in order to prevent the development of off odors associated with reductive conditions in the bottle later.

The yeast population, for example, should be given sufficient oxygen and assimilable nitrogen so as to prevent the development of hydrogen sulfide. Even at levels that are undetectable at bottling, sulfides can undergo reactions in the bottle to form disulfides and mercaptans with much lower detection thresholds, and associated 'eggy,' vegetal and sulfurous smells. Many winemakers will carry out an automatic copper fining (copper sulfate at a few mg/l) in order to strip out any sulfides that may have developed during fermentation.

The wine should not go into the bottle in an oxidized state, but equally, should not contain compounds that can be converted under reductive conditions. Wine that is destined for cork may certainly be treated differently. Cork, arguably, allows the very slow permeation of oxygen into the bottle, thereby avoiding reductive conditions. Cork itself contains oxygen in small cells and pockets, which may also be exchanged at the wine surface.

Another cause of incomplete fermentations is a lack of available nitrogen, sometimes due to overclarification of the must before fermentation. It is common practice in modern wineries to add diammonium phosphate (DAP) to the juice in order to ensure adequate nutrition for the growing yeast population in the form of the ammonium ion, which is easily assimilable by the organisms. In the event that the temperature is too cold in white must, fermentation slows down or becomes stuck, risking the production of off flavors. If this happens, it is usual to raise the temperature of the fermentation to around 18 °C, and to pump the wine over to oxygenate it. Between 10 and 15 mg/l DAP may be added, as may amino acids L-phenylalanine at 2 mg/l and thiamine at 50 mg/l. If fermentation does not restart after these measures, a new yeast starter culture will be introduced, as the old population may have become unviable.

Ideally, a yeast strain for white wine production has the following characteristics: it will be resistant to high alcohol levels, and tolerant of high initial sugar levels and normal doses of sulfur dioxide. It will enhance the quality of the wine by adding fresh, fruity aromas, glycerol and esters, and it will not contribute negative characteristics like mercaptans, sulfides, acetic acid or ethyl acetate. It will be able to convert sugar to alcohol efficiently and completely, at a specified (and sometimes very low) temperature, using low levels of vitamins and oxygen.

The onset of fermentation will be rapid, even in highly clarified juice, and the fermentation will proceed predictably and will be controllable through temperature manipulation. Minimal amounts of foam will be produced during fermentation, and the yeast will flocculate rapidly once the wine is dry, and contribute pleasant autolysis character if required. This is a lot to ask of a simple eukaryote, but centuries of selective breeding by vintners have ensured that almost every wine yeast used these days fulfils most of the criteria listed.

The ADWY (commercial active dry wine yeast) strain selected has a significant effect on the progress of the fermentation (fermentation kinetics) and the development of aromas. Some yeasts produce high ester concentrations such as QA 23 and these are used for neutral grapes as the yeast characters can add to the wine. Other yeasts such as VL3 are used with Sauvignon Blanc as they intensify the varietal aroma. In some cases yeasts such as CY 3079 are chosen for their influence on the body of the wine. In red winemaking, yeasts such as F10 are chosen for color and flavor extraction for early drinking reds. If fermentation difficulties are encountered then EC1118 is a good yeast strain to use for restarting stuck ferments, but this strain is known to produce a 'medicinal' aroma profile associated with elevated levels of vinyl guaiacol, as is Vin7, a popular yeast strain for Sauvignon Blanc fermentations in South Africa.

Yeast lees (also called yeast ghosts or hulls) are sometimes added to musts to provide nutrients for the yeasts and absorb chemical spray residues and long chain fatty acids, as well as other substances that may retard the progress of fermentation. The only yeast nutrients permitted by legislation in most wine producing areas are diammonium phosphate (up to a limit of 0.96 g/l in the EU) and thiamine (1 mg/l). Ammonium sulfate may be used instead of DAP at similar levels (Morgan *et al.,* 2006).

If these nutrients are lacking in the must because the grapes are rotten or juice is very highly clarified, their addition will produce a more efficient, purer and more complete fermentation, so a number of yeast

manufacturers now sell products which contain these compounds in combination with yeast lees as a fermentation supplement. If yeast is producing detectable levels of H_2S, it is a sign of yeast stress and may be caused by a nutrient deficiency, so adding DAP is usually one of the first actions taken on detection of the gas.

Preparation/inoculation of yeast starter culture

Once white juice has been clarified, it is transferred to the fermentation vessel. Approximately 10% of the vessel volume should be left empty to avoid the overflowing of foam produced during the tumultuous phase of alcoholic fermentation.

The freeze dried wine yeast will be activated following the manufacturer's directions for the specific yeast, on a tank by -tank basis (in buckets, drums or small tanks), or through large propagation tanks of yeast starter maintained with the addition of sugar or grape juice. White grape juice should be inoculated with yeast at a concentration of 10–15 g/hl (or $5–10 \times 10^6$ cells/ml of juice) (Ribereau-Gayon *et al*., 2006c). The culture is normally pumped over from the lowest valve in the tank, via an open bin, to the top of tank. Fermentation usually begins within 24 h, is vigorous after four or five days (Figure 2.9.11) and typically lasts two or three weeks, depending on conditions.

Similarly, for crushed red grapes, these should only be filled to 80% of the tank capacity to allow for the large volume increase in the pomace cap during fermentation. Enzymes can be added at this point to improve the aroma, and other attributes of the wine. The vessels used for fermentation nowadays are mainly stainless steel tanks (Figure 2.9.11), but glass fiber and epoxy-lined concrete are in common use. Barrel fermentation is also used for white juices, and to finish off fermentation in drained/pressed red wines.

Additional information on red winemaking can be found in Section 2.9.2. The fermentation should be started up as soon as possible, in order to limit the amount of time in which the must is liable to oxidation and to microbial spoilage. If the fermentation is too slow, yeasts and bacteria can produce volatile acidity and by-products, which are coarse or even foul tasting. If the fermentation is too fast, there is a great loss of volatile aromas and those formed are coarse, leading to a considerable loss in finesse. For these reasons, it is very important to monitor the alcoholic fermentation and to control its development.

Figure 2.9.11 *An active fermentation of white grape juice.* Photograph by courtesy of Derek Pritchard

To monitor the fermentation, it is essential to measure the temperature and density of the must in each vat at least once a day. This monitoring will enable the producer to follow the development of the fermentation, and anticipate possible problems, such as overheating and stuck fermentations.

If the fermentation rate is high, the temperature will increase, carbon dioxide will be generated more quickly, and there will be a concomitant loss of volatile components. If a fast fermentation is allowed to continue, the heat produced may be sufficient to kill the yeast, and the fermentation will not complete before all the sugar is consumed. This type of problem is more likely to occur in production facilities with large tank volumes at high ambient temperatures. It is generally recognized that the control of temperature is vital in the production of fine white wines as high temperatures encourage oxidation, microbiological spoilage and instability. Desirable aroma and flavor compounds and alcohol are depleted by volatilization, and above 35–38 °C, yeast will be compromised. Different beverages will have different requirements in terms of ideal fermentation temperature, but for white wines, the temperature is adjusted to obtain a uniform sugar reduction of approximately 1.5 °Brix per day, which corresponds to a fermentation temperature of 12–16 °C. For red wines a reduction of around 3 °Brix per day is achieved at temperatures between 18 and 24 °C.

Cooling systems range from low temperature water in an insulated vessel to direct glycol cooling through heat exchangers. The entire facility may also be subject to temperature control through air conditioning, and this will probably be sufficient to keep smaller fermentations at lower temperatures, but high volume fermentations need to be conducted in jacketed tanks so that temperatures can be controlled in individual tanks. A sudden fall in temperature can also adversely effect the fermentation, and in the final stages a significant temperature drop can drastically slow or stop it completely.

It is important to act quickly if off odors are seen in fermentations, as they are usually warnings of yeast stress. As stated previously, if H_2S is detected, DAP should be added. If the rate of fermentation has slowed significantly, the must may be aerated, or the temperature increased to boost metabolism. If the acetic acid concentration rises, the fermentation rate and temperature should be checked and if a wild ferment is being conducted, reinoculating with a known culture may help to alleviate the situation. When altering the fermentation temperature it is important to do so incrementally, as sudden changes can affect the yeast adversely. The aroma compounds produced during fermentation and the factors affecting them are discussed more fully in Section 2.2.9. Even without the means to control (reduce or increase) tank temperature, following the course of the fermentation temperature and density will give a good indication of the progress and health of the process. A further check is to measure yeast colonies/ml of solution.

It needs to be noted at this point that the carbon dioxide generated by fermentation poses a hazard to workers as it has a higher density than air and collects in the production area, in tanks, and in low cellars. It is potentially lethal, and good ventilation is essential where fermentations are carried out, as are precautions such as never entering a closed fermenter (or empty tank) without measuring for adequate oxygen levels. Large concerns should be equipped with alarms for low oxygen levels.

The alcoholic fermentation of a white wine seldom exceeds 12 days, except in the case of exceptionally high sugar concentrations. Most red wines and dry white wines are fermented to dryness (contain less than 2 g/l sugar), which should be confirmed by chemical analysis. A complete fermentation should not leave residual sugar in which further growth of bacteria or yeast could occur. The density of the fermenting solution is measured daily to monitor alcoholic ferment kinetics, and when it drops to *ca*. 0.993–0.994, sugar concentrations are then quantified to verify completion. In red wines (because of solids extraction) the finished density is higher, usually *ca*. 0.996–0.998. It must be noted that hydrometers are calibrated for dissolved solids in water solution. When alcohol (less dense than water by a factor of 0.7893) is present the hydrometer no longer gives a true reading and therefore sugar concentrations should be checked chemically using a full reducing sugar test. In a winemaking context, a stuck fermentation is when more than 2 g/l of sugar are left at the end of fermentation. This can be very risky if not spotted straight away, as lactic or acetic spoilage can result, with the production of off odors and flavors. An incomplete fermentation implies a higher

residual sugar (usually fructose which tastes significantly sweeter than glucose) than originally planned for by the producer, leading to an unbalanced wine.

Towards the end of fermentation residual reducing sugar levels are measured. A number of producers use Clinitest® (tablets formulated for monitoring low levels of reducing sugars in urine by Bayer Healthcare) for an initial screening test and later they use a more accurate method such as the Lane and Eynon method (Section 4.6.3) or the Rebelein method for quantifying residual sugar (Iland *et al*., 2004). If a residual sweetness is desired in the end product, the fermentation may be stopped by racking, cooling, filtering or centrifugation and by the addition of large doses of sulfur dioxide (200–250 mg/l), or alcohol, in the case of fortified wines, by alcohol addition.

If the must does not contain sufficient sugar to generate the required alcohol level before the fermentation is stopped, rectified concentrated grape must or 'sweet' reserve may be added to boost sugar levels. This needs to be in compliance of local legislation for sugar levels for the style of wine. Any beverage containing natural sugars needs especially clean and careful handling, close monitoring and maintenance of free sulfur dioxide levels and a sterile filtration (preferably through a 0.45 μm membrane filter) before bottling. At this point, unless malolactic fermentation or a long lees contact is desired, wines are racked and the sulfur levels checked. In the event that previous additions of sulfur dioxide have been moderate, 60–100 mg/l of sulfur dioxide may be added to bring the free sulfur dioxide level up to 30–40 mg/l. It is important to add SO_2 as soon as the wine has cleared and is racked off lees. If a residual sweetness is desired in the wine, the fermentation can be stopped by racking, cooling, filtering or centrifugation and by the addition of large doses of sulfur dioxide (200–250 mg/l), or alcohol, in the case of fortified wines.

Any wine containing natural sugars needs close monitoring of free sulfur dioxide levels, which should be maintained at around the 50 mg/l level before bottling. The additions need to be in compliance with local regulations for sulfur dioxide maxima. In South Africa, for example, the legal limit on sulfur in a dry white wine is 210 ppm or mg/l over the entire winemaking process. Even in the event that total sulfur measure only 150 ppm before bottling in the wine, if 210 mg/l have been added, the wine is not permitted any more sulfur.

Once the wine has fermented to dryness, the yeast will settle out naturally if the wine is left in a cold place, and the clear wine can be racked off and filtered. It is important to monitor wines regularly after fermentation has finished. Attention should be paid to the level of wine in the tank (ullage), the surface of the wine, the level of SO_2 and the temperature. The greater the ullage, and the higher the temperature, the more chance there will be oxidative or microbial spoilage. The wine is no longer protected by actively fermenting yeast and the generation of carbon dioxide and poor storage conditions will quickly lead to spoilt wine.

White wines may be run into barrels for ageing after fermentation, or the whole fermentation may take place in the barrel in the first place. Although the modern tendency is to make fruit driven styles of white wine in stainless steel, fermentation in oak is still used for some styles.

If lees contact (or *sur lie*) is required, but malolactic fermentation is not desired, the wine may be cooled to around 10 °C and the lees stirred by pumping over, avoiding oxygen dissolution. Wines are given time on lees to improve the mouthfeel and structure, and they should be stirred or pumped over every 2–3 days to keep the lees in suspension and maximize contact with the wine. Pumping over or stirring lees through the wine prevents the formation of reduction odors (H_2S) in the lees, and helps with the breaking up or autolysis of the cells. The release of the cell contents, including mannoproteins from cell membranes, has been found to assist in the protein stabilization process. After one to two weeks the wine is sulfured at to the appropriate level and racked off the yeast lees.

Wines which have been barrel fermented are often left on lees for extended periods to improve the body and mouthfeel of the wine. They acquire flavors and aromas which can be described as creamy, yeasty, bread-like or toasty, which certainly suits more neutral cultivars like Chardonnay and Chenin Blanc. This property is used to improve the flavor of 'Muscadet sur lie,' which can even be bottled unfiltered. The flavor and creamy

texture of Champagnes, especially Bollinger (up to 10 years on its lees) is reputedly due to the time left on lees.

The aim of the producer is a smooth, reliable and controlled fermentation giving the results desired for the style of beverage being made. The fermentation should be carried out with no off flavors and where possible a positive contribution to the flavor and aroma. These can be influenced by the choice of yeast, the nutritional factors and the environmental conditions of the fermentation.

Postfermentation Operations

If wine is saturated with air the oxygen is consumed slowly in chemical reactions with wine components and is usually undetectable after a week. The oxidation reactions are chemical in nature and the exact reactions depend on the composition of the wine. Some of the more readily oxidizable substrates in wine are phenols, sulfite and ascorbic acid. The capacity of wine to consume oxygen is roughly proportional to its total phenol content, which in the case of most white wines is low. Any oxygen not taken up by phenols, sulfur dioxide or ascorbate will be free to cause oxidation reactions which may damage the wine's color and flavor profile. The slow insidious progress of oxidation can go unnoticed until irreversible damage has occurred, the wine contains detectable levels of acetaldehyde and ethyl acetate, and the color has taken a brown tint. It is judicious, therefore, to limit contact with oxygen in the latter stages of a white wine's processing, and atmospheric oxygen contact can be substantially reduced by displacing air above the wine with inert gas, either CO_2 or N_2, or a mixture of the two. This is a basic level of protection and prevents the initial dissolving of oxygen in the wine. The displacement of air from the headspace of storage tanks helps prevent the growth of film forming microorganisms, but complete control requires additional treatment such as SO_2 addition. It is now general practice in some wineries to flush all hoses and equipment with inert gas before any transfer or operation and to fill receiving vessels with inert gas before wine is pumped in. Low temperatures reduce the risk and rate of both oxidative and microbial spoilage. It is desirable to have low temperatures at virtually all stages of winemaking. When temperatures are high the winemaker must pay extra attention to inert gas use and SO_2 usage.

Malolactic fermentation

If MLF is to occur successfully SO_2 levels must be very low (none should be added after primary fermentation) and the temperatures should be 20 °C or above. This means the only protection available against oxidation and film yeast growth is air exclusion. This is achieved by either inert gas usage or ensuring all vessels are completely filled and air tight. While MLF is being encouraged extra care must be taken to ensure effective air exclusion and to detect the early stages of spoilage. This is especially important in white winemaking as the effects of spoilage would far outweigh the gains hoped for by having an MLF. MLF should be achieved in as short a time as possible, and the course of MLF should be followed so that action can be taken. If MLF is slow or incomplete the conditions should be checked. The wine can be reinoculated and nutrients added or MLF may be terminated. As soon as MLF is completed the wine should be checked and pH, SO_2 and/or temperature correction be carried out.

More on malolactic fermentation and its effect on the wine can be found in Chapter 2.3.

Fining and Filtration

Most white wines, particularly those that contain sugar, will undergo some form of filtration before bottling, although recently winemakers have moved away from excessive filtrations and overprocessing of wine towards gentler alternatives. A number of white wines are now marketed as 'unfiltered.' If fining is carried out for any

reason, (for example, a bentonite addition for protein stability), the wine will need to be filtered to remove fining residues. The wine should also be cold stabilized to precipitate any excess potassium bitartrate, and the winemaker may want to filter out any microscopic crystals that have not settled out during the process. It could be argued, however, that a well made dry white wine that has been in contact with clean, inert surfaces and is protected with appropriate levels of sulfur dioxide should not need any filtration. See Sectoins 2.9.4, 2.6.9 and 2.8.6 for more detail on fining and filtration of wine, beer and cider, respectively.

Removal of reductive odors

Despite the use of low sulfide producing yeast strains and the addition of yeast nutrients new wines still often have sulfide off colors at low levels. At low levels, these mask desirable fruit flavors while at higher levels they may be responsible for a range of off odors from 'struck flint' to 'canned vegetable' and even 'garlic.' It is preferable to remove any sulfide off characters as soon as possible after fermentation before they develop into more complex problems in the bottle. A simple addition treatment for removing and/or preventing sulfide is copper sulfate, at levels of up to about 1.5 mg/l of copper (6 mg/l copper sulfate). This treatment is generally effective against hydrogen sulfide odors, and has been shown to be relatively effective in the prevention of formation of mercaptans and disulfides later in the wine's life. The wine should be clarified, and small-scale trials conducted to determine the most appropriate treatment and addition rate of the copper sulfate.

Blending

There are several reasons for blending wines. These include: producing the best wines possible using some or all of the wines available, producing a consistent product, year after year, 'stretching' good quality wine by blending it with wine of average quality, correcting problems such as excess acidity or sweetness and blending away bad or faulty wines. Producing a consistent product is especially important with branded wines, in order to satisfy the consumers' expectations, and it is essential that the blender has appropriate experience of the product. Skilful blending can add to the value of the wine and maximize the contributions of different cultivars or wines to the whole. A great number of the world's best wines are blends.

There are many considerations to be taken into account when blending wines. Blending can be undertaken at any point during the winemaking process (from grape batches to finished wines), although it is most often carried out after individual batches have been taken to completion and, therefore, just prior to bottling. It could be said that in some cases (Portugal, for example) blending actually starts as far back as the vineyard, where different varietals are planted in the same vineyard and no efforts are made to keep them separate at harvest. If blending is carried out after the wines have been stabilized it is essential that the stability of the blend is checked as the new composition of the wine may have adversely affected some of the components. If the pH changes, for example, the sulfur level may need to be adjusted, or if the alcohol level changes, the blend may no longer be tartrate stable. Newly blended wines are generally allowed time to 'marry' before final stability testing and (potentially) final filtering prebottling.

Blends may include different cultivars, different vintages, different fermentation lots or even wine from different countries. The legal situation varies, but usually there are limits which must be kept to in order to label wines in certain ways, for example in South Africa, in order for a wine to be considered a 'cultivar wine,' 85% of the grapes in the blend must be of one particular varietal.

Bottling

Bottling lines are capital intensive pieces of equipment, often used only once per year and for many small wineries, third party (i.e. 'contract') bottling is the most prudent and economical choice. In turn, this

consideration has prompted many wineries with bottling lines to offer the service themselves. If the winemaking has been careful, a brilliantly clear, stable wine should be presented for bottling. The bottling process cannot correct any faults that are inherent in the wine, and can certainly add faults that were not there previously, if all is not in order. A poor bottling, in other words, can undo months (even years) of hard work and care on the part of the viticulturalist and winemaker, and therefore it is crucial that problems are foreseen and prevented. The common problems associated with bottling include faulty filtration, faulty pumps and nonsterile lines; excess dissolved CO_2 in the wine, exposure to oxygen; faulty corks, screwcaps and closure, nonsterile bottles and capsuling problems.

Prebottling checks are absolutely essential if the product is to have the best possible chance. The final filtration should not perform a 'dirt removing' task, but should be a sterile operation at below 45 microns. Bottles must be washed and sterilized with SO_2 solution and/or hot water or steam. If the manufacturer has guaranteed bottle cleanliness, and the bottles arrive in sealed packs, it is still worth checking random samples before the bottling starts. The filling operation itself requires slow opening inlet and nonreturn valves to help protect the wine feed, and preferably the bottles should be prefilled with nitrogen gas to avoid oxidation. The filling operation may also include vacuum corking and/or use of CO_2 in the bottle's neck to reduce oxygen pick up. The bottling operation itself, whether carried out 'in house' or by third party should be under conditions of scrupulous hygiene. This chiefly concerns sterilization of the bottling line equipment and all wine contact surfaces (e.g. pumps and pipes) with a caustic solution (e.g. 1% NaOH) followed by water rinsing, acid dilution (e.g. 1% citric acid) and final rinsing with sterile water. The most effective systems also incorporate pressurized steam cleaning of the lines. Cleaning must also include the corking equipment, and, especially, the filter system and wine filler heads. A typical six head filler in an English winery is shown in Figure 2.9.12.

The bottle closure, traditionally cork, is another key feature that may cause loss of wine quality. In a fault monitoring exercise that was carried out at the 2006 International Wine Challenge, around 'fifty percent of the faults detected were related to the type of closure' (Thomson, 2007). Bird (2001) notes that natural cork has the benefit of being a good oxygen barrier, by virtue of its composition and the way its elasticity compresses

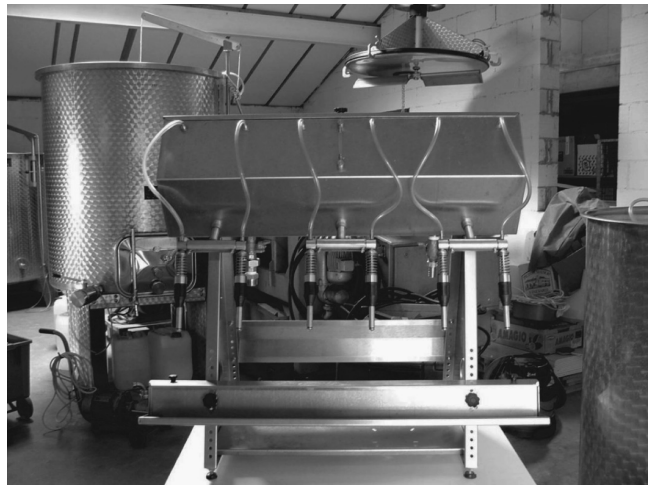


Figure 2.9.12 *A six head wine bottle filler, typical of those used in small wineries.* Photograph used by courtesy of Derek Pritchard, Dunkery Vineyard, UK

it tightly in the bottle's neck, but the problem of cork 'taint' has resulted in a significant decrease in the popularity of natural cork. One of the prime causes of taint is a mold in the cork's crevices, which releases 2,4,6-trichloroanisole (TCA) (amongst other compounds) as a metabolite. TCA has a powerful moldy aroma, which, at the very least, deadens the fruit character of the wine. Most cork producers avoid any kind of chlorine based sterilants now and use peroxide based products instead, but the problem of natural cork 'taints' is not yet resolved. Wines under cork are still showing around 2% taint, which equates to a large volume of wine annually.

Alternatives to natural cork have already made great headway. Initially plastic 'replica' corks were the leader, but the recent rise of the metal screw cap (ROTE, or 'roll-on-tamper-evident'), mainly for white wines, has taken off in recent years, particularly in New World winemaking areas. By 2009, screw caps accounted for 65% of South Africa's bottled wine production, for example. More and more red wines are being put under screw cap as consumers become more accepting and winemakers become adapted to new bottling requirements.

The choice of bottle (or other container) is important, based on (legal) sizes and protective and robustness functions. If glass is chosen, it is usual for red wine to be in a 'green' bottle, which will help avoid light causing oxidative bleaching of the wine color. Glass is by its nature heavy, and producers are looking to lighter options; in 2010, 750 ml wine bottles closed with screw caps and weighing only 350g will be available to wine producers. These compare with an average bottle weight of over 500 gs just four years ago. Lighter bottles are essential in reducing transporting costs and associated greenhouse emissions and are excellent in providing visible evidence of a producer trying for a smaller carbon footprint.

In addition to glass, plastic bottles, aluminium cans, bag in box and cardboard brick packs are now in common usage.

Final Wine Analysis and Records

For most quality wine schemes all over the world, a final analysis is required to be carried out. This can be by self-certification, or by an accredited laboratory. In addition to such final wine analysis, the legislating authority usually requires the keeping of records in such a format as meets their needs for the information shown on their preferred record format.

2.9.2 Red Wine Production

Although red and white wine have major similarities in their making, unlike white wine, red wine fermentation takes place in the presence of grape skins, seeds and sometimes stalks. During fermentation, color, tannins, fruit flavor and other chemical components are extracted from the skins and to a lesser extent from the seeds, giving the wine its color and character, which may be further modified by malolactic fermentation (if carried out) and the ageing process. The main consideration in red winemaking is the management of the extraction of phenolics, which takes place during fermentation.

Grapes for Red Winemaking

Cabernet Sauvignon

This variety is believed to have Cabernet Franc and Sauvignon Blanc as parents, and is probably the best known red (or black) wine grape in the world. Cabernet Sauvignon is easy to cultivate – the vines are hardy and resistant to rot and frost, and the grapes have thick skins, which resist disease and express the

typical character (typicity) of the variety to produce some of the world's finest wines. The vines are planted just about anywhere wines are made, but the grape is only successful in regions that are warm enough to encourage phenolic ripeness. If grown in cool areas, or if the grapes are picked too early, the wines may show herbaceousness (leafiness or green peppers). As the grapes are small, with thick skins, there is a high ratio of skin to juice, and seed to pulp, which means that there will be a large extraction of phenols and tannins during periods of maceration (skin contact before, during or after fermentation). In Bordeaux, Cabernet Sauvignon is an important species, and the maceration period was traditionally around six weeks, giving extremely harsh, rich wines, which needed to mellow or mature in barrel or bottle for about 10 years before drinking. Nowadays, winemakers try to make wines that may be consumed far earlier, so maceration times are reduced to as a little as a few days. In Bordeaux, the wines of Pauillac are dominated by Cabernet Sauvignon, but in many others, Cabernet Franc (as in the wines of Château Cheval Blanc, St. Émilion) or Merlot (as in the wines of Château Palmer, Margaux and in Pomerol wines in general) may be more in evidence. As a pure varietal, Cabernet may lack 'middle palate' and for this reason, in the New World it is often blended with Shiraz or Merlot, both of which help to give add more fullness and complexity. The 'Bordeaux Blend' of Cabernet Sauvignon, Merlot and Cabernet Franc is copied all over the world. Cabernet Sauvignon may also be blended with Tempranillo and Sangiovese before, during or after fermentation, to give full bodied red wines that last well in the bottle. Good Cabernet Sauvignon may also be found in Spain (Navarra), Bulgaria (Suhindol), California, South Africa and Australia. The wine suits the use of oak during production, which brings out the fruit and spice nuances of the grapes, and helps to boost the middle palate. Maturing the wine in small oak barrels before bottling Cabernet is common in order to achieve the best quality wine. Cabernet Sauvignon aroma and flavor characteristics include deep color, good tannin structure (astringency), blackcurrant and red berry flavors, also cherry, cedar wood and tobacco, with some 'green' characteristics like green pepper or olive.

Shiraz/Syrah

This grape is known as Syrah in France and Shiraz in the New World winemaking areas. The vines grow very vigorously, but when planted on the right vineyard site may produce grapes that give wines of intense color and flavor, well suited to wood maturation. In warmer climates like Australia, the grape produces wines that are sweeter and riper tasting. In cooler climates like the Rhone valley of France, the grapes often give a wine that has more pepper and spice aroma. Shiraz may be consumed while it is still fairly 'young' (i.e. within two years of harvest), or it may be aged for a long time before release (e.g. Hermitage in France and Penfold's Grange in Australia).

In France, Syrah is often blended with Grenache (a more neutral red grape) and is an essential grape in the production of the famous and very expensive wines of Chateauneuf du Pape. The grape is the most widely planted red grape variety in Australia where it is sometimes blended with Cabernet or occasionally with Mourvèdre (a strong, tannic cultivar).

Wines made from this cultivar are often quite powerfully flavored and full bodied, with a wide range of flavors, depending on the climate and soils where it is grown, and vineyard management. Normally, aromas range from violets to dark berries (as opposed to strawberry and raspberry), as well as chocolate and coffee, with black pepper, and blackberry and pepper in young wines. After time in the bottle, leather, smoke and truffle aromas may emerge in the bouquet. In the Rhône, as well as in most other areas, Syrah is famous for producing big spicy wines, with intense color, capable of considerable ageing. Hermitage and Côte Rôtie produce well known examples. The latter may be blended with up to 20% Viognier (a white variety) to give aromas of orange peel, cinnamon and plums. Amongst the New World winemaking countries, Australia has put its stamp of ownership on the Shiraz cultivar, with very good examples made in the Barossa, Eden and Clare Valleys, and McLaren Vale.

Merlot

This variety is a close relative of Cabernet Sauvignon, but ripens earlier and hence is able to mature its grapes more readily in cooler seasons or in cooler climate regions. However, it is very sensitive to growing conditions (particularly at flowering time), the vines are susceptible to fungus and mold, and therefore quality of the wine depends on location and vineyard practices. In hotter growing conditions, the grapes may tend to produce flabby wines. This variety was first successful in Bordeaux, in France, and dominates certain blends there, particularly in the wines of Pomerol and St-Émilion. A famous example of Bordeaux Merlot, Chateau Petrus, is one of the most expensive wines in the world. Generally Merlot is lower in tannins than Cabernet, and therefore makes wines that are soft, fruity and smooth, with ripe berry components in the bouquet. It can make good varietal wine on its own, or be blended with Cabernet to produce wines that are more approachable and less aggressively tannic when young. Merlot can have long ageing potential, but most are ready to consume within five years. The wine can be served at slightly lower temperatures than the normal 20–25 °C serving temperature of red wine.

Tempranillo

This is a thick skinned Spanish varietal, which is also known as Cencibel and Tinta de Toro (and Tinta Roriz in Portugal), the ability of this cultivar to thrive in the most unfavorable climates has led to it being grown in most red wine producing regions. It blends well with fruitier varieties and suits oak treatments, which give it the smooth vanilla notes commonly found in the wines of Rioja. It can produce light, early drinking wines right through to deep colored, tannic wines with spice, tobacco and strawberry flavors.

Pinot Noir

This variety is one of the most difficult grapes to grow and work with, particularly in warm climates, but if made well can produce very fine wines. Pinot produces a small crop of grapes with low tannin and color levels, and fairly high acidity, with strong cherry and strawberry aromas (Graves, 1990). The grapes need warm days and cool nights to mature optimally, and are fussy about ripening conditions. In Burgundy, if days are too cool, the cultivar gives thin wines with little tannin and color. Excessive heat during the ripening season can, however, lead to jammy, characterless wines which do not age well. It is the most abundant red grape in the Burgundy (Bourgogne) region of France, and has also become important in Oregon in the United States, as well as in New Zealand. It is known as Spatburgunder in Germany. The ageing potential can range from three to 12 years depending on the quality and style of the wine, and unusually for a red wine, is a good match for grilled seafood. Pinot Noir is also used in Champagne production, where it adds length and body to the blend of Chardonnay and Pinot Meunier (Section 2.9.3).

Pinotage

This cultivar can be considered *the* South African red wine variety; it was created in South Africa (in 1925) by Professor Perold, the first Professor of Viticulture at Stellenbosch University. Perold attempted to combine the best qualities of the robust Cinsault (known as Hermitage in South Africa) with Pinot Noir, a grape that can be difficult to grow. Pinotage is fairly easy to cultivate, and is a good cropper, producing high sugar levels (and therefore higher alcohol concentrations in the wine), but has had a few criticisms. The production of isoamyl acetate during fermentation can lead to the aroma of banana, and varnish (duco). Since its first appearance, the grape has been a victim of fashions within the South African wine industry, sometime experiencing a resurgance in popularity, but more often being maligned. Pinotage, if made well, makes smooth textured wines with lots of fruit flavors which can be drunk early. It stands up to wood ageing very well, and produces coffee and chocolate characteristics when matured in oak barrels of particular sources.

Tinta Barocca

Tinta Barocca is a fast growing, early maturing cultivar, frequently used in the production of port. It is grown primarily in the Duoro region in Portugal, but is also planted in South Africa. The vine produces large, thin skinned berries, and wines with delicate and subtle flavors. It grows best in cooler situations and can be blended with other varieties in order to soften the blend, add perfume to the aroma and lengthen the finish (aftertaste). In South Africa, a few producers make it as a varietal (containing 85% of the named cultivar) wine, but it is often used in blends.

Cabernet Franc

This is one of the major red grape varieties worldwide. It is usually blended with Cabernet Sauvignon and Merlot, but can also be made into a varietal wine. It is one of the five varieties legally allowed in Bordeaux blends (the others being Cabernet Sauvignon, Malbec, Merlot and Petit Verdot). Typically having lighter color and less tannin than Cabernet Sauvignon, Cabernet Franc produces fragrant, slightly green wines with blackcurrant leaf aromas. In Canada, it can be made into ice wine (wine made from the juice of frozen grapes). It shares many of the same phenolic and aroma compounds as Cabernet Sauvignon, but with some noticeable differences. Cabernet Franc may be served slightly chilled, and has pronounced aromas of raspberries, blackcurrants, violets and graphite. It has slightly less pronounced tannic structure than Cabernet Sauvignon and tends to produce a wine with a smoother mouthfeel. The grape is less common in New World regions, but in South Africa, acreage of Cabernet Franc has slowly been increasing, and producers often delay harvesting the grapes to try and minimize the green leafy notes. The grape contributes finesse and a peppery perfume to blends with more robust grapes.

Barbera

This Italian variety produces wine of good fruit flavor, with a high level of natural acidity that has no doubt helped its popularity in warmer climates. Great examples of Barbera are to be found in South America and Australia, and the grape is increasingly being planted in other warm regions like South Africa. Barbera blends well with Cabernet Sauvignon and Sangiovese. Overproduction on the vine encourages acidity, but low yields give beautiful, balanced wines.

Gamay

Gamay Noir à Jus Blanc is the dominant red grape of Beaujolais in South Burgundy. Whole bunches are placed in a tank pressurized with a carbon dioxide seal and the grapes undergo ‘carbonic maceration’ to produce wines with low tannin levels and fruit flavors of berries, ‘tutti-frutti’ and banana. The wines are traditionally drunk young. Although Gamay will never make a big, full bodied wine, California, South Africa and Australia are producing some excellent examples of the light, scented red style of the grape.

Grenache

Grenache grows across huge regions of southern France and Spain, and is also grown in the New World (notably Australia). It is high yielding variety, and is typically overcropped and under-rated, producing thin wines with little character. Due to its affinity for warm climates, if subjected to proper management, the vines produce fruit that makes dense, peppery wines with high alcohol content. In Rioja, Garnacha is blended with Tempranillo, and in France, it is either made as a varietal or blended to produce reds such as Châteauneuf du Pape.

Figure 2.9.13 *Pea sized berries in the early stages of Pinotage fruit development*

For an account of many other red wine vine varieties, some of which, such as Nebbiolo and Tannat are as important for quality (but are perhaps less widely cultivated) as the above varieties, the reader is directed to Robinson (1986) and Galet (1956, 1962). The reader will also find here information on 'workhorse' red wine varieties, such as Aramon, Alicante Bouschet, Carignan, and others – big yielders of basic, everyday wine.

Fruit Maturity and Phenolic Content

The main difference between white and red wines is the phenolic content from grape seeds and skins of the latter, which gives red wine its color, structure and longevity. Up to the point of veraison (see Figures 2.9.13 and 2.9.14, below), red and white grapes look very similar. During veraison, the red grapes will start to

Figure 2.9.14 *Veraison stage for Pinotage. Black, red and green grapes are present in the same bunch.* Photograph by Prof. P. Goussard, used with kind permission of Wineland, South Africa

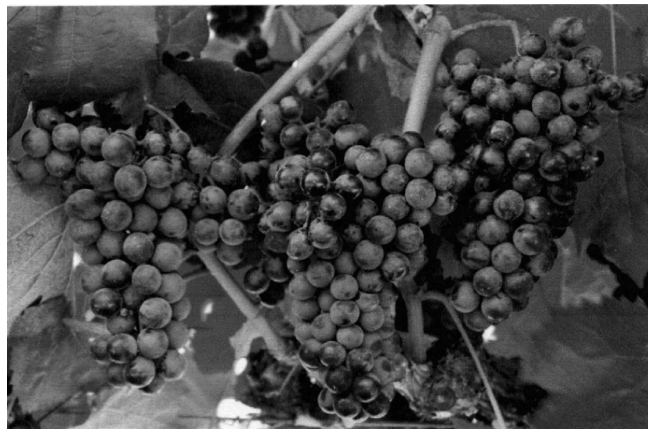


Figure 2.9.15 *Black grapes ready for harvest. To the eye, the berry skin color is quite uniform.* Photograph by courtesy of Prof. P. Goussard

gain pigment, and gradually become darker and darker in color until they attain full maturity (Figure 2.9.15). White grapes will become more translucent, and gradually may become more gold or for some varieties (e.g. Gewürztraminer, Pinot Gris) pinkish in color, but will never attain the same concentration of phenols as red grapes, even in advanced stages of ripeness. White wine is also made from juice that is separated from the skins before fermentation, and is therefore low in polyphenols.

Red grapes are frequently harvested after physiological maturity (maximum sugar accumulation and berry size) as winemakers seek intense color and flavor in their wines. It is not unusual for red grapes to be picked when they are starting to pucker and dehydrate (though it is unlikely that the grapes will be as over-ripe as those shown in Figure 2.9.16). The making of red wines involves extraction and management of high concentrations of polyphenols from skins and seeds during fermentation, with the extraction rate increasing

Figure 2.9.16 *Over-ripe black grapes. Some of the berries have shriveled and may have less skin pigmentation.* Photograph by courtesy of Prof. P. Goussard

with time of contact (maceration), higher temperature and higher alcohol content. The other sources of phenolics in wine are oak treatments and additives such as commercial tannins.

Research into the types of associations and interactions between polyphenols in wine has produced reams of information, some of which are relevant and interesting to winemakers (see Waterhouse and Laurie, 2006; Fulcrand and Cheynier, 2006 for two recent papers, and Chapter 5.8 and Sections 2.6.9, 2.8.2, 2.10.7 and 2.11.2 for examples and citations; see also Part 4 for methods of separation and analysis of phenolic substances).

In short, a phenolic compound has phenol as its basic structural unit – a six membered carbon (C_6) aromatic benzene ring backbone with at least one hydroxyl functional group attached. This structure gives compounds in which it occurs great stability due to the delocalization of electrons around the ring. Here is presented a brief, general account of phenolic substances in black grapes and red wine – for details of the major families (including structures) of phenolic compounds found in wine and other drinks, see Figures 5.8.6 and 5.8.7 in Section 5.8.6. For information on individual phenols (and their reactions) in other beverages or their raw materials, see Sections 2.8.2, 2.10.7 and 2.11.2, and Chapter 3.2.

There are two main groups into which phenolics are usually divided. The *nonflavonoids* are all based on either the hydroxybenzoic acid (C_6-C_1) backbone, or the hydroxycinnamic acid (C_6-C_3) backbone. With the addition of extra functional groups on the basic structure, C_6-C_1 compounds include gallic, vanillic, salicylic acids. The C_6-C_3 compounds in wine include coumaric and caffeic acids. These nonflavonoid acids are usually present as esters – combined with sugars or alcohols and form polymers called 'hydrolyzable' tannins. Grape derived nonflavonoids are not possessed of strong aromas or tastes, but may act synergistically to enhance the mouthfeel and aroma of the wine. When they polymerize, they form relatively weak bonds, which can break and reform under the acidic conditions in wine. Yeast metabolism may provide other nonflavonoids that include tyrosol and tryptophol.

Few volatile phenols or phenolics derivatives appear to come from grapes, but those that do fall into the nonflavonoid group. Methyl anthranilate is a phenol derived ester which is an important component of the characteristic foxy aroma (loved by some; hated by others) of some *Vitis labrusca* varieties. The rose-like fragrance of 2-phenylethanol often distinguishes varieties of *V. rotundifolia*. In addition, several volatile phenol derivatives, such as 2-phenylethanol, vanillin and zingerone, occur in nonvolatile conjugated forms in several *V. vinifera* cultivars. Release by enzymatic or acid hydrolysis could significantly influence the sensory impact of the compounds. Wines matured in oak will contain high levels of hydroxybenzoic acid type phenols (such as gallic acid) by hydrolysis of ellagic acid, pentagalloylglucose and similar hydrolyzable tannins, which themselves arise from the breakdown of phenol–sugar polymers within the wood. Breakdown of lignin will also provide cinnamaldehyde (cinnamon aroma), benzaldehyde (almond/maraschino cherry odor) and the important aroma contributors, eugenol (clove-like notes) and vanillin (vanilla or caramel-like notes).

Another source of volatile phenolic aldehydes involves the heating of must or wine. Fructose, for example is rapidly converted to 5-(hydroxymethyl)-2-furaldehyde during baking or very slowly during ageing. Furfural is commonly produced during distillation and the 'toasting' of oak staves during barrel construction.

The transformation of hydroxycinnamates into volatile phenols occurs under the action of enzymes derived from either *Brettanomyces* spp. or lactic acid bacteria. The derivatives, 4-ethyl guaiacol and 4-vinyl guaiacol, could add smoky, vanilla and clove-like notes to wine. Eugenol, a volatile phenol that smells strongly of cloves, may also occur. Guaiacol which has a sweet, smoky odor may be generated by *Brettanomyces* and by the barrel toasting process, but the concentration is usually insufficient to influence the bouquet directly. Guaiacol and 4-methyl guaiacol may be involved in an off odor derived from contaminated stoppers, and have definitely been implicated in the smoky smell associated with vines and grapes that have been exposed to bushfires, as has happened recently in Australia and South Africa.

The other main group of phenols in wine, the *flavonoids* have a name that is derived from the Latin word for yellow ('flavus'), and have a C_6-C_3-C_6 structure, i.e. two C_6 aromatic rings separated by a chain of three

carbon atoms. The C_3 chain usually, but not always, forms a six membered heterocycle, using an oxygen atom to complete the ring (along with two carbon atoms of the benzene ring). The flavonoid group can be further subdivided into subgroups such as flavones, flavanones, isoflavones, flavonols (yellow pigments), chalcones (yellow-brown) and anthocyanidins (other colors). These subgroups each have a distinctive structure, depending upon what other functional groups are attached to the basic C_6-C_3-C_6 backbone. Classification of flavonoids is based on the ease of hydrolyzing tannins, and tends to be more of historical interest than practical use. Copolymers can be formed from flavonoids and nonflavonoids, making this classification system somewhat complicated, as previously noted. Some examples of specific flavonoids are quercetin, malvidin, catechin (also called flavan-3-ols), and leucoanthocyanidins (also called flavan-3,4-diols), but see Figures 5.8.6 and 5.8.7 in Section 5.8.6 for further details.

Procyanidins (polymers of catechins and cyanidins) condense in turn to form tannins, each containing between two and five procyanidin units with molecular masses of between 500 and 3000 amu. They are very stable and have a high protein binding property. Sugars bond to flavonoids and give rise to molecules such as malvidin-3,5-diglucoside, which is used as a marker for testing juice from *Vitis labrusca* species, where it is present. Diglycosides are found only in trace quantities in *Vitis vinifera* grapes.

Polymerization continues as the wine ages, so the concentration of larger molecules containing flavonoids and nonflavonoids gradually increases in wine, and the concentration of monomers and smaller polyphenols decreases. Polymerized flavonoids are known as condensed tannins, and as these are held by strong covalent bonds, they are relatively stable and will not easily be hydrolyzed in the conditions found in wine.

Pigment molecules such as *anthocyanins* can also be bonded to tannins to form colored copolymers. Anthocyanins are glycosides of anthocyanidins, that is, the free anthocyanidin (or aglycone) is bonded to glucose which increases the chemical stability and water solubility of the free form. Examples of anthocyanidins (with their main color in parentheses) include malvidin (purple), cyanidin (magenta), delphinidin (blue), petunidin (purple) and peonidin (magenta). Although abundant in young red wines, anthocyanins contribute little to the taste of wine. The most common anthocyanin in *Vitis vinifera* species is malvidin-3-glucoside.

The concentration and form of anthocyanins markedly influences the hue and color stability of the wine, both properties of which are directly affected by the hydroxylation pattern of the anthocyanidin B ring, and the charge on the central heterocycle, which in turn is influenced by pH. Blueness increases with the number of free hydroxyl groups, while redness increases with the degree of methylation.

Color in red wine comes primarily from the small proportion of anthocyanins that exists in the red colored flavylium (positively charged) state, which is favored by low pH. At pH above 4, anthocyanins will lend blue and purple hues to the wine, while at lower pH (3.5 and below), the red form predominates. Sulfur dioxide bleaches free anthocyanidins, and renders them colorless, but reaction is reversible, so if the sulfur dioxide concentration decreases, the equilibrium will shift, and sulfur dioxide will be released from the bound form, restoring the color. The sensitivity of anthocyanins to oxidation is influenced by the presence of vicinal *o*-diphenols (adjacent hydroxyl groups) on the B ring, which are particularly reactive to both enzymatic and nonenzymatic oxidation. Except for laccase, most polyphenoloxidases oxidize only *o*-diphenol sites. Because of the sensitivity of free anthocyanins to oxidative degradation, it is preferable that polymerization occur early during wine maturation.

In the fruit of red grape varieties, anthocyanins tend to exist in loose complexes held together by hydrophobic interactions between the anthocyanin moieties and hydrophilic attractions of the glucose components. Other flavonoid phenols, hydroxycinnamoyl esters and polyphenols are often involved in these complexes. Heating grapes or must (thermovinification) initially helps to release anthocyanins into solution, but can lead to a serious loss by precipitation of these compounds during wine maturation if insufficient tannins are extracted from the grapes to help stabilise the color in other pigment complexes.

Alcohol also destabilizes the hydrogen bonding between anthocyanin aggregates, so must fermented in contact with skins for only a few days may show a loss in color as fermentation continues. In addition to

anthocyanins, flavonoid tannins are extracted from the grape skins, stems and seeds (pomace or cap) later on in the fermentation. Grape stems are known to contain significant amounts of polyphenolic compounds, especially phenolic acids, flavonols and flavanonols. Tannins in the stems mainly consisted of (–)-epicatechin units along with smaller amounts of (+)-catechin, (–)- epicatechin gallate and (–)-epigallocatechin (Souquet *et al.,* 2000). These compounds begin to polymerize with free anthocyanins and anthocyanidins. Polymerization is an important factor in stabilizing wine color by protecting the anthocyanidin molecule from oxidation or other chemical modifications. When they are attached to one or more other phenolics, anthocyanidins are more resistant to decolorization by sulfur dioxide or high pH.

As tannins are released during fermentation, more stable pigment complexes are formed. Initially, the procyanidin molecules found in wine are small and highly soluble, and form complexes with anthocyanins. By the end of a normal red wine fermentation, around a quarter of the anthocyanins have polymerized with tannins, and this level may rise to nearly 50% within a year, depending on storage conditions. Polymerization, especially when associated with oxidation, increases the proportion of both the flavylium and quinoidal states, but will change the color of the molecules to yellow-brown. In combination with red color loss, the yellow-brown of the anthocyanin/tannin polymers results in the wine progressively taking on a brick red shade. After this, polymerization continues at a slower pace until all the phenols in the wine will be in polymeric form after several years. Higher degrees of polymerization will cause the wine to darken (which is not the same as intensifying) and eventually to go brown. Even very stable pigment complexes will become colloidal and form hazes and deposits, given enough time and the presence of oxygen.

Reduction and oxidation affect the degree of polymerization of the pigment molecules, as do temperature, pH and light (Section 2.9.5). Procyanidin/anthocyanin polymerization does occur in the absence of oxygen, but it is a relatively slow process, and it is generally accepted that most polymerization in wine is due to acetaldehyde induced copigmentation after oxidation. Acetaldehyde is produced from ethanol through the action of hydrogen peroxide released during the oxidation of phenols and, by reacting with anthocyanins, acetaldehyde forms a complex that facilitates bonding with catechin and procyanidins. This helps stabilize the color in red wine by limiting anthocyanin oxidation and decolorization by sulfur dioxide. In addition, it consumes acetaldehyde and restricts the development of a stale, oxidized odor. This type of polymerization helps to explain the color enhancing and stabilizing effect of exposing young red wines to small amounts of oxygen (about 40 mg O_2 per year) before bottling.

It is now accepted that phenols are involved in both the progression and limitation of oxidation through their own oxidation and reduction. This apparent anomaly results from the involvement of various phenols in the generation of peroxide and/or the reduction of oxygen to water. As a consequence, oxygen is consumed by phenols, and is therefore unavailable to oxidize other wine constituents. One of the initial reactions in must appears to involve the enzymatic oxidation of phenols, such as caftaric acid, to quinones. The latter may be reduced back to phenols through the coupled oxidation of procyanidins or ascorbic acid, which has the advantage of inducing the polymerization and early precipitation of readily oxidizable phenols before or during fermentation. In the presence of oxygen, *o*-diphenols are oxidized to *o*-quinones, and the oxygen reduced to hydrogen peroxide. Subsequently, the hydrogen peroxide oxidizes the predominant substrate in wine, ethanol, to acetaldehyde. Because quinones react with other phenols, they enhance phenol polymerization. By slow structural rearrangement, quinone/phenol dimers can generate new *o*-diphenol dimers, which may subsequently react with additional oxygen molecules, producing more peroxide, and ultimately even more complex polyphenols.

The antioxidant (oxygen assimilating) action of phenols is improved if oxygen penetration is slow or infrequent. Because of the efficient removal of oxygen, phenols can help maintain the low redox potential of a wine. This is considered beneficial in the development of an aged bouquet during long in bottle maturation. The low phenolic content of white wines makes them particularly susceptible to oxidative browning.

The two main effects of phenols, other than contributing color and contributing to the redox balance of the wine, are astringency and bitterness. Astringency (Section 4.7.2) is due mainly to tannins, and is caused by the hydroxyl (–OH) groups in tannins binding with the protein molecules in the saliva, causing them to lose the ability to lubricate the surfaces of the mouth. Bitterness is a taste (see Section 4.7.2) and is caused by the lower molecular weight phenolics, particularly from seeds. Most phenolics acids, such as caftaric acid, are slightly bitter, but occur at concentrations well below the detection thresholds in wine. Combinations of phenolic acids have lower thresholds than the individual components, and may contribute jointly to the bitterness and flavor of the wine phenolics, especially at higher alcohol concentrations. Higher sugar content tends to mask bitterness, but higher alcohol levels will enhance it. Bitterness is masked by astringency, so when tannins are removed bitterness may become more obvious. Hydrolyzable tannins, such as those extracted from wood cooperage, tend to be more astringent than condensed tannins.

Phenols are also known to exert antimicrobial action, which may be due to the binding of tannins with proteins, which limits enzyme activity by modifying enzyme solubility and structure. The antimicrobial activity of phenolics could also be the result of tannins binding membrane phospholipids and proteins, disrupting membrane function. Phenols also have strong chelating properties that may restrict access of sensitive microorganisms to essential minerals.

Under normal fermentation conditions, phenols do not inhibit the growth or metabolism of yeasts, but high levels may complicate the initiation of the second fermentation of sparkling wine. For this reason, red wines are seldom used in the production of sparkling wine, and the recent trends to make rosé and red sparkling wines are restricted to light styles.

The interaction between tannin and proteins, which leads to flocculation and precipitation, is often used in wine fining to remove excesses of either compound. Red wines are usually protein stable due to their tannin content, and if they contain excessive amounts of tannin (making them overly astringent), they can be treated with protein fining agents like gelatine or isinglass to strip out some of the tannin and make them more palatable. Unlike red wines, white wines (particularly certain cultivars like Sauvignon Blanc and Schönberger) may contain high levels of colloidal proteins, and enological tannins can be added to white wine to precipitate colloidal proteins and prevent haziness. Fining can also, to some degree, selectively remove phenolics by molecular weight and therefore reduce bitterness or astringency separately. Gelatine is commonly used to remove tannins and so reduce astringency in wine. Casein and polyvinylpolypyrrolidone (PVPP) react with the lower molecular phenolics and so may be used to reduce bitterness in wine. These principles are discussed in more detail in Section 2.9.4.

Harvest

The criteria for ripeness and harvest date will be determined by the style of wine which is being made. For a light red wine designed for early drinking, the sugar concentration in the berries will not need to be as high (19–21 °Brix or °Balling) as for a full bodied red designed to withstand ageing (23–26 °Brix or °Balling). Acidity for red grapes is generally lower than that of white grapes, as acidity enhances astringency, and is not as necessary for microbial stability in red wines due to the presence of phenols. For full bodied red wines, the winemaker may wait until the grapes are over-ripe and puckering in order to achieve the phenolic (color and tannin) levels required in the finished wine. Other factors that are taken into account when deciding on the time of picking would be the fruit condition, weather and the logistics of picking and processing the grapes.

Harvesting equipment and transport of grapes to the winery for red winemaking have the same issues as white wine (see Section 2.9.1). As there is no draining and pressing, and as red wine is 'protected' (to a degree) by its phenolic composition, there is less opportunity for oxidation or the growth of unwanted wild yeast and bacteria during the early stages of red winemaking. The grapes may be transferred straight after crushing to a

tank and the addition of an actively fermenting selected yeast culture added to scavenge any oxygen present and prevent growth of unwanted wild yeast and bacteria. Before fermentation, the effective incorporation of additives into the must is challenging, as the grape mass is not homogenous. Materials such as pectolytic enzymes and sulfur dioxide can be added to the receival bin so mixing takes place as the grapes are processed or liquid additions can be metered in to the crushed grape mass after destemming and crushing using an automated in line metering or dosing pump. If additions are made into the fermentation tank it is necessary to pump over the must to ensure adequate mixing.

Crushing and Destemming

Most red grapes, except those destined for carbonic maceration, are crushed to release juice before fermentation or pressing. The best crushers (usually those with rubber rollers) will break, but not excessively damage the skin, leaving the stems and seeds intact.

In most modern wineries, crushing and destemming are carried out in the same machine. Destemmers will usually incorporate crushing rollers, which ensure that the berries are broken, liberating the juice, before the crushed grape mass is transported (manually or with a crusher pump) to the fermentation tank. There are systems that rely solely on the pump from destemmer to tank to do the crushing. Modern destemmer/crushers remove stems before crushing, and thus the extraction of sap from stems, which may contribute colorless, bitter and astringent phenolic compounds to the wine, is avoided. If the stalks are green the extracted character may also be herbaceous and unpleasant. Despite all their negative characteristics, stem phenols are known to improve color stability, so a proportion of ripe stems is sometimes added back to the fermentation. It is also well recognized that stems contribute potassium ions to the maceration. The benefits of destemming also include increased tank capacity (around 30% better usage), removal of source of color and alcohol sapping (absorbing) stems and minimizing losses of acidity through precipitation as potassium bitartrate.

If bunches are crushed but not destemmed, or stems are added back to the crush, there will be better initial aeration within the must so that lag phase of fermentation is shortened and the fermentation will proceed more quickly. With stems present, heat is dissipated more evenly through the cap of skins and there is better juice extraction during pressing, although larger press capacity is needed. Stems can help protect wine color from oxidasic casse (because laccase is inhibited by stem phenols), and sometimes stems will be deliberately included in the fermentation in order to increase tannins. Despite overall increase in phenolics with stems present in a red wine fermentation, color diminishes as the stems absorb a certain amount from the fermenting juice. However, as less stable color compounds within the juice react to form more stable polymers with the stem tannins, color in these wines will decrease less on ageing. Up to a quarter of the tannin in a red wine fermentation may come from stems if they are present, and these tannins tend to be harsher and more astringent than skin tannins.

Aliphatic carbonyl compounds (like hexenals), identified as ‘green’ odorants in macerated stems, are higher in leaves and berries, but the methoxypyrazine (also herbaceous and vegetal) content of stems is generally higher than those of the berry or leaf samples (Hashizume and Samuta, 1997), so if they are included in the fermentation, these compounds may extract and contribute to the wine odor. This issue is gaining relevance, particularly in South Africa, where herbaceousness is seen as a problem in Merlot and Cabernet Sauvignon wines, is the presence of insects in the macerating grape mass. Harlequin ladybirds (*Harmonia axyridis*) are known to release a potent mixture of pyrazines as a defence mechanism during stress. Locusts (*Locustana pardalina*), common in vineyards in the Western Cape, may also release compounds that can detract from the quality of the wine if they are included accidentally in the harvest, but there has been little research conducted (probably unsurprisingly) to quantify the effects. Even the presence of ‘jacks’ (the broken off remains of the pedicel in the berry) can increase herbaceousness in the wine, which does not usually fit in with the red

berry/'warm' aromatic profile of a red wine. Ribereau-Gayon *et al*. (2006c) noted that, in general, when a fine quality wine is desired, destemming is indispensable.

As stated, the presence of air in the stems acts as a growth factor for yeast, and the fact that the stems do not ferment brings down the overall temperature of fermentation, which also helps in yeast survival. Stems modify wine composition as a result of their low sugar content, and additionally, they have the effect of lowering alcohol concentration by up to 0.5% due to absorption of alcohol from the fermented mass. Stems are rich in K^+, which may contribute to the precipitation of tartaric acid as less soluble potassium bitartrate as alcohol levels increase.

After crushing and destemming, in areas with warm temperatures during the harvesting season, the crushed grape mass is usually chilled from ambient to between 5 and 10 °C, especially if there is a waiting period before processing, or prefermentation cold maceration is to take place in order to extract color. Cooling helps to limit fermentation by wild yeast, and oxidation reactions.

Sulfur Dioxide Additions

Sulfur dioxide behaves differently in red juice and wine to how it behaves in white juice. In red juice, it becomes lightly bound to the anthocyanins causing them to lose color. When the wine is analyzed for free sulfur dioxide by the aspiration method, these weak bonds are usually broken by the addition of acid to the wine.

Sulfur dioxide is frequently added just after crushing and destemming at levels between 50 and 100 mg/l, depending on the state of the grapes. The main reason for using it is to counteract the increased oxidation associated with damaged fruit, which is also likely to have an increased population of wild yeast, mold and bacteria. For fruit in sound condition little sulfur dioxide is required, but many winemakers add a nominal amount, often 50 mg/l. It is known to inhibit wild yeast growth and favors dominance by the added yeast culture, which is usually more resistant to the effects of the compound. The amount used is kept to a minimum as it has an effect on color, and residual SO_2 after fermentation, although bound to other compounds, does inhibit malolactic fermentation. Normal residual levels of total sulfur dioxide are between 20 and 80 mg/l, depending on the initial level and the yeast strain used. The current trend for decreasing the use of sulfur dioxide may account for the increase in the amount of wines infected with *Brettanomyces* seen in various wine competitions, and the decreasing ability for wines, particularly white wines, to undergo ageing successfully.

The practice of cold maceration or cold soaking of the skins in the juice has been shown to give extraction of different color and flavor components. Sulfur dioxide will usually be added at levels up to 150 mg/l (depending on local legislative restrictions) to a prefermentation maceration. The grapes may be allowed to soak for up to four days either at ambient temperature or cooled (often through the addition of dry ice). There is no cap as there is no fermentation and the skins remain distributed throughout the juice. The high levels of SO_2 increase the permeability of the grape skins and allow the diffusion of the pigment and flavor into the liquid. The juice (which should be red from extracted pigment) becomes pink in color due to the bleaching effect of the sulfur dioxide. After maceration the juice may be separated and the skins pressed, and then the juice fermented to completion without the skins to give a light fruity red wine. The juice may also be fermented on the skins for a time to increase the color and flavor extraction. Sometimes desulfuring of the juice is carried out using hydrogen peroxide, but this is not legal in all winemaking areas (Morgan *et al*., 2006). If this is carried out, the color of the juice increases dramatically as the sulfur dioxide is removed and the bound pigments are released. Alternatively, prefermentation maceration may be carried out at less than 10 °C with a lower level of SO_2, such as 50 mg/l. In this case, less color will be extracted and fermentation will be carried out with the skins for a few days. During prefermentation maceration the extraction of phenolics with the juice gives a different result than when the phenolics are extracted with alcohol, as during fermentation. The color and

fruit flavors increase, but bitter or astringent tannins are extracted to a lesser degree and there is usually a marked increase in color in the juice within a day or two.

Acidity Adjustments

The pH in red wines varies considerably, but is usually between 3.3 and 3.8. Higher values are more common in hot climates, but winemakers recognise that useful advantages of lower pH include reduction of the incidence and effects of undesirable bacteria, and slower rates of oxidation. Improvement of the intensity, hue and stability of color are also benefits of lower pH, as well as improved flavor balance as high pH wines sometimes suffer from bitterness, possibly as a result of oxidation of certain polyphenols. During malolactic fermentation, pH is also an issue, as *Lactobacillus* or *Pediococcus* species will dominate at a higher pH, rather than the more beneficial *Oenococcus oeni*. The pH may rise by 0.2 units during alcoholic fermentation due to the extraction of cations, especially potassium, from the skins, and subsequent precipitation of the acid as potassium bitartrate, which is less soluble in the alcoholic solution. It will increase by an additional 0.2 units if malolactic fermentation takes place because of the metabolism of malic acid.

The acid addition rate to reduce the pH of the juice to the desired level should be determined by small-scale trials, but it is generally accepted that 1 g of tartaric acid per liter of must reduces the pH by 0.1 units (Morgan *et al*., 2006). If pH adjustment is required it should be done as soon as possible in order to gain the full benefit, and reduce risks associated with high pH. Additional discussion on must adjustments can be found in Section 2.9.1.

Carbonic Maceration

This is a process for making early drinking red table wines by preliminary storage of whole uncrushed grape bunches in an inert (oxygen free, anaerobic) atmosphere, usually in a tank pressurized with carbon dioxide gas (hence 'carbonic' maceration). The grape berry usually has an aerobic metabolism (sugar and oxygen metabolized to water and carbon dioxide), using respiration to produce its energy needs. Whole, uncrushed berries may develop anaerobic metabolism (fermentation) if deprived of oxygen, in order to meet their cellular energy needs. During this anaerobic metabolism, ethanol is produced as a by-product in the berry, along with volatile flavor compounds. As grape berry cells are not tolerant of alcohol, the level of production is only 1.5 to 2% (v:v) before cells being to atrophy and die. The internal cell structure of the berries becomes compromized, and alcohol, flavor compounds and anthocyanins ar able to diffuse through the berries. The weight of the berry mass in the tank will eventually crush the weakened grapes below them, and they will release their juice. A limited fermentation by wild yeasts may start in the juice at the bottom of the tank, generating more carbon dioxide. Carbonic maceration fermentation is allowed to continue for an extended period (usually between 7 and 12 days) after which the fermentor is emptied and the pomace pressed. Free run and press wine are blended together, and the wine finishes alcoholic fermentation without the skins present.

The main difference between wines made this way and those made through a 'normal' fermentation are that a much lighter style of wine is produced with lower levels of phenolic compounds, particularly tannins. The wines are therefore softer, and the intracellular fermentation also gives a unique 'carbonic maceration' aroma that has been described as 'tutti frutti,' with strong strawberry, maraschino cherry and plum notes. The technique is ideal for making early drinking 'primeur' wines, such as the Beaujolais Nouveau wines of the Gamay cultivar. Carbonic maceration is carried out on a large scale in the south of France, and these wines are amongst the first released each year in that country (usually November), with associated festivals and celebrations to mark the event.

The fermentation is then continued in a normal fashion. This method is also used as a way of increasing the complexity on Pinot Noir wines, sometimes by leaving a relatively high proportion of uncrushed grapes

in the ferment, which then undergo carbonic maceration, and release the juice when they are pressed, adding small amounts of sugar, and interesting fruit flavors to the wine.

Yeast Selection and Inoculation

The yeast used for fermentation has an influence on the resulting wine, as discussed in Section 2.9.1. Yeasts are selected based on similar criteria for those used in white wine (they must carry out the fermentation to completion and with a positive contribution to the flavor), but with the obvious proviso that the red wine strains are resistant to higher alcohol levels and concentrated phenols. Producers of dehydrated yeast will have recommendations for strains for different cultivars and red winemaking applications. As more research is being carried out on the specific attributes of wine yeast strains, interesting properties are emerging which go some way to explaining the products that are formed. Anchor yeast, for example recommends WE372 for most red wine fermentations, as it enhances the red berry characteristics of the grapes. It has also been recently implicated in forming fairly high levels of succinic acid during fermentation, so for low acid/ high pH musts, this yeast could be advantageous. Other Anchor yeasts include the 'NT' range (NT116, NT202 and NT 50), as well as Fermicru XL (for 'New World style Merlot') and Collection Cepage Cabernet, which is said to enhance blackcurrant, chocolate and tobacco in the aroma of this cultivar.

Lallemand also have a range of different strains for different red winemaking purposes, with yeast listed in the annual catalog under aroma attributes. BM4×4 and BM45 for example, are listed as 'plum' aroma producers, and are recommended for fermentation of Grenache, Shiraz and Cabernet Sauvignon. Product catalogs also contain a wealth of information on rehydration and nutrition of yeast and, usually, lactic acid bacteria and their requirements and characteristics. For commercial yeast strains used to make red fruit and country wines, see Table 2.11.4 in Section 2.11.3.

Red grape juice is a good growth medium for wild yeast. Unlike white must, red must is rarely cooled, has a relatively low SO_2, is partially aerated, rich in nutrients and has abundant solids which provide a physical substrate for yeast growth (Morgan *et al.*, 2006). Skins are present and wild yeast cells cannot be effectively reduced by clarification, so rates of inoculation are usually higher than those used in white winemaking even though it has not been proven that the resident population of ambient yeasts are dominated, or killed off, by an early inoculation of selected culture. The lyophilized cultured yeast is usually rehydrated and added to the filled tank according to the manufacturer's recommendations, and an appropriate dose containing 250×10^6 cell/ml ensures rapid onset of fermentation with the desired yeast strain.

In more traditional institutions (and more adventurous modern concerns), winemakers will conduct natural fermentations, using the population of yeast present on the skins, and from spores in the air and tanks in the winery. Investigations have shown that these spontaneous fermentations do contain some interesting variants like *Kloeckera, Candida, Metschnikowia* and *Pichia* in the early stages, but that these genera are usually killed off as the alcohol level approaches 5% v:v after which *Saccharomyces cerevisiae* will dominate. To conduct a wild fermentation, a winemaker will prepare a '*pied-de-cuve*' or starter yeast preparation (Morgan *et al.*, 2006). The method is as follows: A small proportion of healthy, ripe grapes (up to 5% of total vintage volume) is picked a few days early. They are then crushed and destemmed, and the crush is divided into two lots: A (around 10% by volume) and B (the other 90%). A is heated to around 30 °C to encourage fermentation, and the equivalent of about 30 mg/l sulfur dioxide is added to B in order to acclimatize the yeast to sulfur dioxide. When A is fermenting strongly B is added to A very slowly. The culture is then ready for use, and is placed at the bottom of the first few tanks to be filled (the 'pied' of the tank). Additional tanks are inoculated with fermenting musts from the first tank. Sometimes a proportion of juice is separated from the must immediately after crushing. This may be done to increase the skin:juice ratio for better color, or for the production of rosé or white wine from red grapes.

Nutrients are not usually added, as the inclusion of the skins and grape solids provides adequate nutrients to ensure complete fermentation. Diammonium phosphate (DAP) may be added to prevent or cure the production of H_2S during fermentation. This is more of a problem if wild yeasts are used for fermentation. In studies conducted with Shiraz must by Ugliano *et al.* (2009), it was found that addition of DAP to a final yeast assimilable nitrogen (YAN) level of 250 or 400 mg/l resulted in an increased formation of hydrogen sulfide by *S. cerevisiae* compared to nonsupplemented fermentations (100 mg/l YAN). Supplemented fermentations also showed prolonged formation of hydrogen sulfide into the later stage of fermentation, which increased this compound in the final wines. *S. bayanus* showed a different H_2S production profile, in which production was inversely correlated to initial YAN. For both yeasts, DAP supplementation yielded higher concentrations of organic volatile sulfur compounds in the finished wines, including sulfides, disulfides, mercaptans and mercaptoesters. The authors commented that these results raise questions concerning the widespread use of DAP in the management of low YAN fermentations with respect to the formation of reductive characters in wine.

Fermentation Conditions

During red winemaking, the aim is to extract the phenolic compounds (color and tannins), through maceration (mixing juice and skins) during and after the fermentation.

The conventional fermentation on skins is the most common method of red winemaking used in most premium wine producing countries of the world. It relies on the increasing concentration of alcohol making the skin cell membranes permeable to allow extraction. In order for the extraction of color and phenols to occur there must be intimate contact between the skins and the liquid.

During fermentation with skins, carbon dioxide production and the buoyancy of the skins combine to cause the skins to rise to the surface and form a 'cap' (sometimes called the *marc*). The cap may rise above the surface of the liquid and become dry if the skins are periodically immersed in the wine. If the skins are not in sufficient contact with the liquid, diffusion of the pigments cannot occur, and the wine will lack color and structure. 'Cap management' is therefore a crucial aspect of red winemaking, and a great deal of time and energy is devoted to its practice. Methods of cap management will be discussed in detail a little later in this chapter.

Open fermentors obviously expose the fermenting juice to the air, which stimulates yeast growth and provides a faster onset and higher rate of fermentation, leading to lower alcohol yields and more biomass. Extraction of anthocyanins and tannins is enhanced at higher temperatures, and it is not normally usual to cool red mash, unless a cold prefermentation maceration is required. Aeration can also be achieved in the must by pumping over (extraction of must from the lower racking valve and pumping it back over the floating cap of skins), or punching the cap down into the wine on a regular basis. These actions also help to extract color, which will be discussed in detail later in this section.

The availability of oxygen increases the possibility of bacteria spoilage in the cap, especially towards the end of fermentation when the generation of carbon dioxide by the yeast slows down. Pumping over and punching down will help to discourage bacterial spoilage due the presence of alcohol and anaerobic conditions in the juice.

A limited exposure to oxygen is important for promoting the condensation of tannins and anthocyanins to enhance stability of color in the final wine. The phenolic compounds and color intensity usually increase rapidly for the first five or six days, with maximum color being attained a little before the end of fermentation, when around three quarters of the sugar is utilized. After this, total phenolics continue to rise mainly due to tannins being extracted from the skins, but the color intensity decreases slowly due to polymerization, adsorption on solid particles and decolorization by alcohol. It is generally recognized that wines that retain the most color later in the bottle are fermented dry, or even left on the skins for up to 40 days, as in the case

of some of the great French examples. Wines pressed at the peak of color density during fermentation may precipitate color during the early months in the bottle, as the stability of color compounds is dependant on sufficient tannin being present to stabilize the anthocyanins by complexing with them. Addition of tannin, as enological tannin powder, or by stalk addition, to early pressed wines may also help to stabilize the extracted color, and the use of tannins is far more common than most winemakers will admit.

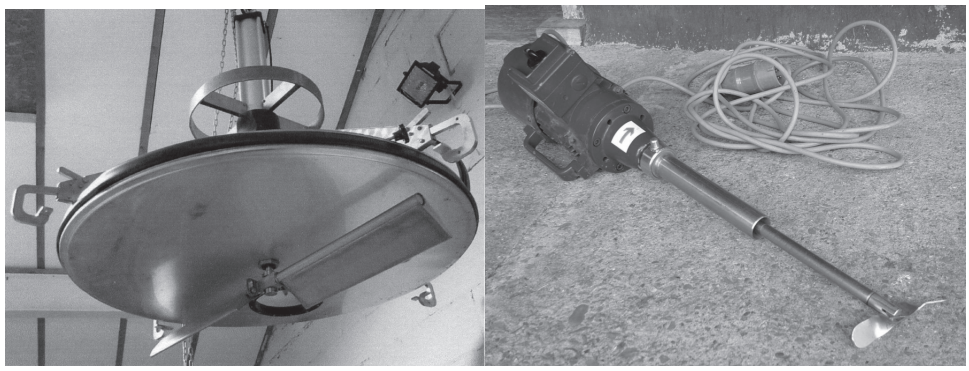
Temperature has a strong influence on the success of color extraction, and the start of fermentation (and its ultimate progress) depends to a large degree on initial temperature. Red ferments are carried out at significantly higher temperatures than for white, where the objective is to extract and preserve aromas and flavors. The dissolution of compounds occurs more quickly and completely at higher temperatures as heat degrades grape tissues, increases dissolution and accelerates maceration. However, at the temperatures necessitated by color and tannin extraction, the yeast may be less efficient at metabolizing, and a stuck fermentation may result. Even for the red fermentations a measure of temperature control is therefore desirable, if not essential. Tank temperature controls may thus be most useful in attaining the desired wine style, and jacketed tanks that can circulate water through their outer skins are popular. These tanks may also help if the grape crop is too cold for normal ferments to start, e.g. fermentation initiation needs to be at 20 °C to assist yeast in its growth phase, and the must can be warmed.

Early aerations at the beginning of fermentation will help to prevent stuck ferments. At this time, the yeasts are in their growth phase and oxygen is used to improve their growth and produce survival factors. The temperature chosen by the winemaker for a red wine fermentation will reflect the desired wine style. A lower temperature of fermentation (e.g. around 18–20 °C) usually leads to a wine in which the fruit aroma derived from the grapes is retained along with the estery fermentation bouquet. The wine may lack complexity and body, as lower levels of color and tannin are extracted at these temperatures, and wines will be suitable for early drinking. Wines that are fermented at more moderate temperatures, i.e. between 20 and 27 °C (the traditional fermentation temperature for red wines), will lose a proportion of the fruit aroma by volatilization, but yeast metabolism at these temperatures gives characteristic metabolic by-products, which contribute to traditional red wine bouquet. The wine may be complex, full bodied and able to age fairly well, but may lack fruit character when compared to a wine fermented at a low temperature. In standard winemaking, moderate temperatures favor primeur style wines; good color extraction is allied to fruity aromas and minimal tannin.

Higher temperatures (30 °C) extract the tannins required for premium wines capable of long ageing, but wines fermented at above 27 °C may also run the risk of having a cooked, caramel bouquet. They lack fruit aroma and are therefore not as complex as wines fermented at a lower temperature. More color and phenols are extracted at higher temperatures, but the amount of extra color is not significant and under normal conditions the detrimental effect of added astringency and/or bitterness may negate any benefits. High temperature fermentation may result in stuck ferments and a high volatile acidity and higher alcohol concentrations, as the yeast becomes stressed and produces by-products that are unpleasant. In lighter colored varieties, such as Pinot Noir grown in cool conditions, the argument for higher fermentation temperatures may have merit. It may be advantageous to let the temperature of the fermentation rise (to 28 °C, for example) for a short time before lowering it to 20–25 °C, in order to give some wine complexity, but at the same time allowing the retention of some fruit character.

Phenolic Extraction During Red Winemaking

As previously mentioned, the essential feature of red winemaking is the extraction of colored anthocyanin pigments and phenolics. The extraction of color, tannin and aroma from the grapes is a complex issue, and the decisions taken during fermentation of red wines affect the style and quality of the final wine. Other than the temperature of the fermentation, the degree of extraction of phenolics is dependent on many factors, including the cultivar, the ripeness of the berries (which in turn depends on canopy management in the vineyard and the



(a) (b)

Figure 2.9.17 *Red wine fermentation KTM tank lid, showing blades for breaking up compacted cap of skins during fermentation (a) and, for smaller scale red wine fermentation, an electric rouser (b).* Photographs by courtesy of Derek Pritchard, Dunkerley Vineyard, UK

climatic conditions of the vintage), the duration and type of maceration, the pH, sulfur dioxide concentrations and the alcohol content of the must. Other factors affecting extraction are the mechanical damage to the cell structure which occurs during crushing, the length of time the skins are in contact with the liquid, and, significantly, the treatment of the cap.

The skin cells are the only cells containing pigment in the big majority *Vitis vinifera* red varieties. There are certain *Vitis vinifera* species, the 'Teinturier' varieties, such as Alicante Bouschet, Grand Noir, Dunkelfelder and Roobernet (in South Africa), which have colored flesh as well as skins. These are often used to boost the color of juice from paler red varieties. Here, we are concerned with the problems of extraction of pigment from grape skins. The permeability of the cell membrane is bought about by the killing of the grape skin cells. The methods of bringing about the cell death include exposure to alcohol, heat, SO_2 or asphyxiation with carbon dioxide. The diffusion of the anthocyanins requires contact between the dead cells and the juice/wine. This is achieved in various ways, such as immersion of the skins in the liquid, by use of adjustable rotating blades attached to the lid of the vat (Figure 2.9.17), by 'pumping over' (Figure 2.9.18) or fermentation in a rotary fermenter. The proportion of the phenols extracted and the relative composition of the wine are very much affected by the processing techniques and equipment used. The conditions that encourage extraction of anthocyanins also encourage the extraction of other phenolics, but it is fairly well known that anthocyanins extract early on in the fermentation, and tannins later, being more alcohol soluble. The latter compounds may contribute high levels of astringency and/or bitterness to the wine. Extraction and diffusion are increased by the mechanical breakdown of cells during crushing, but excessive pulverizing of the berries may have a detrimental effect as the bitter and astringent tannins in the stalks and seeds may also be extracted. The presence of sulfur dioxide, as mentioned previously, assists in making cells more permeable, but bleaches color temporarily. Bisulfite also increases color extraction in wines made from partially rotten grapes due to the inactivation of oxidasic enzymes like laccase, which are then not able to cause browning of color compounds.

Phenolics extracted from red grape skins during primary fermentation may be extracted into fermenting must and stay there, but they may also be adsorbed on to yeast cell walls, precipitate with grape proteins or react with acetaldehyde produced during fermentation to give colorless or insoluble polyphenols. Colorless phenolics found in grape skins are mainly monomeric or dimeric flavonoids and these are released along with the anthocyanins. Grape seed polyphenols, mainly small to medium sized tannins, are not released until

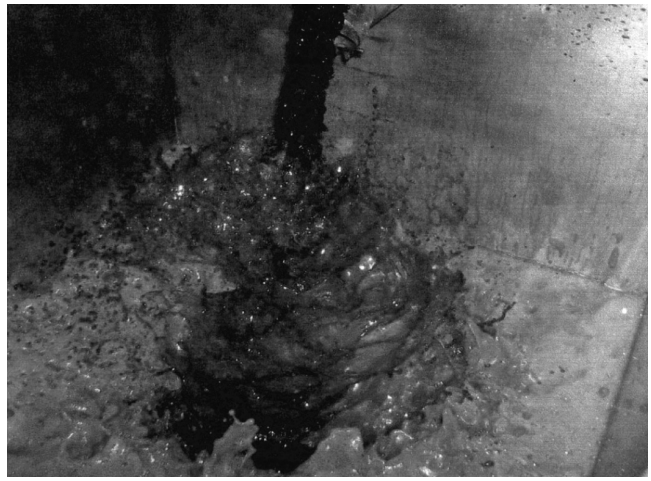


Figure 2.9.18 *'Pumping over' an active red wine fermentation.* Photograph by courtesy of P. Gerber

fermentation is well advanced and alcohol has had time to dissolve the protective layer of oils and pectic substances around the seeds. Seed tannins are more easily extracted if the seeds are damaged, and contribute to the total phenol of the wine. The ratio of nonpigment phenol to pigment is increased by fermenting on skins for a long period and allowing extended maceration after fermentation. The tannin astringency is softened during extended skin contact after fermentation due to polymerization of the phenolics.

Heat treatment (sometimes called thermovinification) of crushed red grapes, generally in the range 45–80 °C, damages cell membranes and thereby assists in extracting color from the skins without fermentation. The juice produced is densely colored, has low tannin and produces a soft early drinking wine. It is not generally practiced outside Europe where it is used to produce large amounts of beverage quality of wine. The crushed grapes are heated to a specific temperature in a variety of ways, for example by using a double mantle tank provided with stirrers, or through the use of a canal heat exchange apparatus. Depending on the winemaking objective, a number of treatments may be used. These include heating of must and skins to 45–65 °C for a few hours, heating of must and skins to 60–78 °C for an hour or so or heating of a portion of the must and skins to higher temperatures for a short time (flash heating methods). During the latter option, whole grapes may be heated in a pressure chamber for a very short time (10–20 s) with the aid of superheated steam, or crushed grapes are heated by means of a heat exchange unit to between 70 and 85 °C and then cooled. The effect of heat includes the denaturing of proteins and pectins, which makes the cell walls more permeable so that the juice and coloring matter can be released more easily. Heat treatment of grapes, which are infected with mold is effective in destroying the fungal oxidase enzymes which may otherwise cause problems later in winemaking.

The problems related to heat treatment of grapes are mainly connected to color losses as a result of oxidation of phenolics by enzymes and/or precipitation of oxidized pigment, difficulties experienced with clarification, high cation extraction giving high pH, undesirable changes in color and a loss of bouquet due to protracted exposure to heat. Energy/electricity expenditure obviously increases with the application of heat to large volumes of grapes, but appropriate heat recovery equipment may assist in keeping the cost of the energy required within reasonable limits. Thermovinification for a longer period to a more moderate temperature (e.g. to 45 °C) was developed as part of automated or large-scale winemaking and is used during continuous production to speed color extraction and enhance the efficiency of the winemaking process.

Phenolic Extraction During Fermentation

Red wines were traditionally fermented in open vats, and a great deal of red wines are still produced this way, with regular punching down or pumping over of the cap of grape skins. Nowadays, red wines are fermented in a wide variety of different tanks and vats, with varying degrees of oxygen exposure and automation in their making.

The manner in which the cap is managed will influence color extraction, the degree of maceration of skins, ease of temperature control, draining efficiency, marc disposal, duration of maceration and nett cost, and control of oxidation and spoilage. The physical separation of the skins from the juice and tendency for cap to become hot leads to stratification of temperature in the tank, and therefore temperature may be monitored at several points in the tank, particularly in closed systems. An important facet of conventional red wine fermentation is the surface area and depth of skins in the buoyant cap, which can influence the degree of juice/skin contact and the heat exchange properties of the cap. Shallow, wide tanks with a large surface area have a large area of contact between the juice and the cap, which encourages extraction and heat transfer, but also have a large surface exposed to oxidation. These tanks are costly and inefficient in their use of space. Tall, narrow tanks, which give rise to a much thicker/deeper cap of skins, are also able to dissipate heat more easily from the cap due to the relatively large tank wall surface area in contact with the skins.

Methods Used to Enhance and Control Phenolic Extraction During Red Winemaking

In order for most of the color and moderate quantities of tannin to be extracted efficiently from the cap of floating skins, it needs to be submerged and mixed effectively with the fermenting wine, which will leach the phenolics out of the skin cells. Other than extraction of phenols, keeping the skins wet with alcoholic solution also prevents overheating in the cap, the development of aerobic/acetic bacteria and oxidation of components by prolonged exposure to the air. The pressed juice from the cap will also have higher levels of color and phenolics, and higher level of cations, particularly potassium, which leads to lower acidity and higher pH. If contact is reduced, insufficient color and tannin will result, and the wine may be thin and lacking in flavor. The appearance of aerobic bacterial spoilage and high VA production in a neglected cap is common, especially at the end of fermentation.

The efficacy of cap management treatments in extracting phenolics and enhancing the color of the wine will depend on the way in which they are carried out. The best method of mixing cap and wine is to irrigate the cap evenly and gently until it is submerged, as physical damage to the skins leading to harsh flavors within the wine. If wine is fermented in open tanks, the most common methods of cap management for color extraction, prevention of oxidation and microbial control are pumping over and plunging (or punching down).

Plunging or punching down

This involves pushing the cap under the surface of the juice using a wooden (often handmade!) or stainless steel plunger to break the mass of skins up and mix it with the liquid. It is an effective method of achieving juice/skin contact, and can be carried out in a number of different ways depending on the size of the operation. Treading by foot is the traditional method used in shallow open tanks, during which the grapes are crushed as well as being submerged (see Section 2.10.7). Manual plunging is often used in smaller volume open fermentations, but is not practical for larger volumes. In small, open fermentations, punching down is carried out fairly frequently to ensure the cap does not dry out and become liable to oxidation and microbial spoilage. It is hard work, and in larger tanks, can even be dangerous due to the generation of carbon dioxide through the cap, which may be inhaled and cause dizziness.

Additionally, handheld electric rousers (Figure 2.9.17) or rotating tank lid blades, like those of the KTM red wine fermenting tank systems (Figure 2.9.17), can be used to break up the cap in a gentle manner. The former is particularly useful for small pilot or specialist red wine fermentations and can be used on nongrape wine pulp fermentations too (Section 2.11.3).

In larger fermentations, mixing is usually achieved using a variety of mechanical or semi-mechanical fermenters (see later in this section) which are able to handle much larger volumes and operate either automatically or as programed by the winemaker. Punching down may be combined with pumping over (see below) to may be carried out on its own.

Pumping over

Pumping over (see Figure 2.9.18) involves circulating the must from within the tank over the cap of skins by means of a pump, which provides a percolation effect where the juice passes through the skin bed. The weight of the must on top of the skins, and the physical action of the pressured liquid hitting the skins acts to break up the cake and wet it. Pumping over may be ineffective if the juice travels through fissures in the cap leaving much of the cap untouched, but if done well, it provides effective mixing and temperature equilibration between the cap and juice and increases the extraction of anthocyanins and other phenolics. It is common to add deflector plates or sprinklers to deflect the juice over a wider area so that channels do not develop. In smaller wineries a racking plate is sometimes added to the delivery tube to sprinkle juice back over the cap, while in large operations special rotating irrigators are used. Pumpover frequencies vary according to wine style, but satisfactory results may usually be obtained by pumping over twice a day for an hour each time. After pumping over the cap temperature rises quite rapidly. Pumping over is a popular method of color extraction in larger concerns as it can be carried out on large volume fermentations, and uses equipment that is already present in the winery, so no additional capital expenditure is required. It is also physically a lot easier to carry out than punching down. As the juice is aerated at the same time the tannins partially polymerize and become softer, making the wine less tannic and astringent. In France, in certain wineries, the aeration during pumping over is even greater as wine is run along open channels between tanks before being returned to the cap.

Heading down boards

As exposure of the cap to air increases the risks of oxidation, growth of acetic acid bacteria, and inefficient extraction of color, some producers choose the option of keeping their caps permanently submerged during fermentation. In order to keep the cap positioned below the surface of the liquid, ‘heading down boards’ will be fitted into the fermentation tank to hold the skins in place. Heading down boards may be employed in open or closed tanks, but their use is usually limited to smaller fermentation tanks which must be designed or adapted to cope with the large forces involved in static headed down ferments. As a result of the compression of the skins by the pressure from below, ‘hotspots’ or areas of increased temperature (differences of over 20 °C) may develop due to the heat produced by fermentation, lack of liquid flow and insulation. The juice inside the cap (also called the pomace) will ferment faster as a result of higher temperatures, and the possible presence of air trapped in the skins. At pressing, the free run juice may still contain significant amounts of sugar, while the pressed juice from the cap is dry. If there is sufficient circulation of juice through the submerged cap, color extraction is good, because of the constant contact between the juice and the skins. The juice in the tank may also be fermented cool so that the temperatures in the cap are controlled to a degree, and some tanks will be fitted with temperature monitors so that temperature can be checked regularly. Aerobic spoilage is minimized as the cap is kept below the fermenting liquid surface in an oxygen free environment. The cap is not continually punched and

disturbed as it is during punching down or automated mixing, and so extraction of harsh phenolics from pulverized skins will be decreased. Pumping over is mainly used for large volume fermentations without heading down boards, but it may also be used with heading down boards to provide circulation through the cap.

Rotary tanks are horizontal, cylindrical, closed fermentation tanks, which revolve around a central axis. They usually have a fixed device inside, which helps break up the cap and assists in emptying the fermentor after use.

Closed tanks have the same problems of color extraction and temperature control as open tanks, but are more versatile, and can be programed. With an enclosed top the wine is less susceptible to oxidation and bacterial spoilage as there is less exposure to air, but throughput is relatively slow and ease of operation depends on the design of the tank. The color extraction is moderate to good depending on the rotation regime used, but as oxygen is excluded, color stability may not be as good. Some enclosed tanks have good draining efficiency with a screen to separate the skins, excellent marc disposal with a sloping bottom, well placed opening and door, which makes them able to double as drainers (also for white winemaking), as well as storage tanks. Some rotary tanks are very easy to operate, the rotation may be programed and emptying is very simple. Although these tanks are expensive, they allow rapid fermentation and are versatile in their applications. As the cost and the availability of manual labor becoming more of an issue, this type of tank is becoming more common in larger operations.

Pressing the Fermenting Pulp

After fermentation, depending on the style of red wine being made, there may be a period of extended maceration on the skins. This will lead to a very robust style of wine, rich in phenolics and tannins, which lends itself to a long maturation period. In past times, maceration periods of up to 40 days were the norm in France, but nowadays, it is more usual to separate the skins and wine directly after fermentation is complete (i.e. as soon as the sugar has fermented out and the wine is dry). The fermented skins are prone to shredding and release of suspended solids, giving turbid, astringent and sometimes bitter wines. Marc (fermented skin) disposal is labor intensive, slow and messy, especially if it has to be heaved up over the wall of a concrete tank using spades. Sometimes the skins will be drained of free run wine, which will be kept separate, but more usually financial constraints will mean that free run and press fractions are added and matured together. The decision to blend press with free run wines is crucial and relates to wine style desired, as press fractions are often drier than the free run due to the increased fermentation rate within the cap, and the sweeter free run is perceived as fruitier, softer and more pleasant. For premium wines some press wine is usually desirable for body, and herbaceous notes can decrease over time, or get masked by other characters as the wine matures.

The main press types are as those for white wine grape juice extraction, namely vertical and horizontal hydraulic presses, pneumatic presses and continuous presses. Dependent on press type and levels of pressure, the resultant press wine may constitute up to 15% of the total finished wine made, as the chosen method of pressing will affect the composition of the 'press' fraction, with quality being enhanced if pressing is carried out in slow and regular pressure increments. Usually the first portion of the press wine ('first press') is more easily obtained and is better quality, and later press fractions ('second' and subsequent) are only obtained after breaking up the press cake one or more times, and reapplying pressure. Later press fractions are likely to be herbaceous and astringent, adding to the already higher tannins in the first press, and the overall make up of press wine is 'concentrated,' i.e. higher in phenolics, residual sugars and cations. However, press wine may also contribute fruit flavor and complexity, as the volumes involved are usually small in relation to the free run. Delaying the blending of free run and press fractions until after natural or fining induced clarification may improve wine quality overall.

Malolactic Fermentation

Malolactic fermentation (MLF) has been discussed in more detail in Chapter 2.3. During red wine production, MLF is often spontaneous and difficult to stop due to the presence of naturally occurring bacteria on the skins, warmer fermentation temperatures and lower sulfur levels used, which encourage the growth of indigenous lactic bacteria. Winemakers allow it to proceed or actively encourage it early in the life of a wine, rather than have it happen in an uncontrolled fashion in the bottle. After undergoing MLF, the wine is considered to have greater microbiological stability due to the loss of malic acid as a substrate for growth. The effect of the process on the wine varies, but it has a reputation as a destroyer of fruit character, red wine color and acid balance in low acid wines from warm areas. On the other hand, in high acid wines of colder regions, it may help to lower high acidity, make the wine more balanced and produce complexity of bouquet (see Section 2.3.16). Malolactic fermentation contributes to the character of traditional red wines (full bodied styles from cool areas), but it may not be appropriate in New World styles of fruity red wine. Conditions that encourage MLF include pH 3.3–3.5, low levels of sulfur dioxide and a temperature of 18 to 25 °C. The fermentation may start with difficulty below 15 °C. The fermentation will often occur spontaneously in wine left on lees for an extended period, but wine can also be inoculated with a commercial starter culture (usually *Oenococcus oeni*) at the same time as the yeast is added (coinoculation) or after fermentation. Lactic acid is not as intense in taste as malic acid, so the wine loses its hard, acidic character and becomes more supple, mellow and full bodied, and softer and rounder. The aroma becomes richer due to the contribution of malolactic fermentation by-products like diacetyl, glycerol, ethanol, succinic acid and mannitol. Color may decrease due to the effect of the increased pH on anthocyanins and other pigment compounds in solution. The malolactic fermentation may take from a few days to several weeks, or even months if the conditions are cold. Temperature is the most easily monitored and controlled of the factors influencing the fermentation, and winemakers are starting to realize the benefits of coinoculation, or adding bacteria during alcoholic fermentation, which takes advantage of the higher temperatures and good nutritional state of the must/wine. If the wine is run off to barrel (sometimes to finish fermenting), the malolactic fermentation will often happen spontaneously if it has not already occurred, encouraged by yeast autolysis products. In the event that malolactic fermentation is to be prevented (for example when making lighter fruity red styles for early drinking), the immediate addition of relatively high levels of sulfur dioxide at completion of the alcoholic fermentation is required. Early filtration of the wine and early bottling with sterile filtration are also good preventative measures.

Postfermentation operations like filtration and fining are less crucial during the making of red wine when compared with white wine due to the presence of phenols in red wines, which makes them naturally more stable and less prone to oxidative or microbial spoilage. Some winemakers will do an egg white (albumin) fining either in the tank, postfermentation or in the barrel, in order to remove unstable tannins and soften the wine, and gelatine can be used for the same purpose. Filtration will usually only be carried out on less robust styles, or styles that contain some residual sugar. Most well made red wine is perfectly capable of withstanding bottle ageing without being filtered, and some winemakers even claim that filtration damages the phenolic structure of the wine and removes color and flavor. Fining and filtration of wines are discussed in more detail in Section 2.9.4.

Barrel Maturation of Red Wines

Red wines are oak aged after fermentation (after skin separation), as opposed to white wines, which will be barrel fermented and then aged in the same oak, if this is the style that is required. Red wines may be aged for several years in barrels, but it is a time consuming, labor intensive and expensive process, which will increase the final price of the product significantly. The effects of barrel maturation on red wine should include a slow change in color progressing from cherry red to a deeper red to brick red, with an associated

softening of the grape (and oak) tannins as they polymerize and eventually precipitate as sediment. It is generally accepted that tannins become less aggressive and the color more stable as a result of a slow ingress of oxygen into the wine, but there is very little scientific evidence to demonstrate that atmospheric oxygen actually permeates through oakwood. The slow oxygen supplementation (around a few milliliters a month) is more likely to happen during topping up and through absorption from the porous cavities and cells of the wood itself. Certainly wine that has been stored in new barrels shows as much color density as wines that have had microoxygenation treatments. Wines in second and third fill barrels (i.e. older barrels in their second and third year of use), where the internal surfaces have been covered in a fine layer of tartrate and phenolic deposits, show reduced oxidative effects and impart less flavor than new barrels. Wines kept in small barrels have been shown to mature (soften and stabilise) more quickly than those kept in tanks or larger barrels due to the larger surface to volume ratio, and therefore a relatively higher exposure to oxygen. Even in wines that are high in alcohol, full bodied and tannic, with great ageing potential, it is prudent to avoid too much oxygen contact as the wine may become oxidized, prematurely aged and be susceptible to oxidative bacterial spoilage.

The stabilizing effects of barrel ageing also relate to the process of natural clarification (sedimentation) at cool cellar temperatures and the reactions of wine and oak tannins with colloidal proteins. The composition and balance of the wine are important determining factors for the time of oak maturation. A period of two years in a second or third fill barrel is quite normal for a full bodied style, with a lesser period of time for lighter bodied wines or wines in new barrels.

Maturing wine in oak barrels also imparts oak flavor, which adds to the complexity and quality of the product. In order to bestow the benefits of oak flavor on early release wines, producers may add products to the wine ranging from oak powder to full staves, for a prescribed period. However, many winemakers are not concerned with flavor extraction into the wine from the oak; indeed heavily oaked wines are no longer as popular as they used to be and overoaking is seen as a fault by many consumers.

The effects of oak treatment relate to the soluble flavor components inherent in the various types of oak. Geographical origin and species have a considerable influence on the aromatic and polyphenol content in oak. *Quercus robur* (French pedunculate oak) has a low odoriferous content, but a fairly high concentration of extractable ellagitannins. *Quercus sessilis* (French sessile oak) has a tighter grain and less extractable ellagitannins, but relatively high concentrations of lactones, volatile phenols, and phenolic aldehydes. *Quercus alba* (American white oak) has low concentrations of phenols, but is known to be highly aromatic, particularly in methyloctalactones (coconut aroma) and vanillin.

A discussion of the effects of oxygen, temperature, pH and time on anthocyanins and other wine components during wine ageing can be found in Section 2.9.5.

The implications of reactions during maturing of red wine for the winemaker are that the wine must be held under conditions appropriate for the structure and pH of the style of wine being made, i.e. wine that is released soon after harvest will undergo different winemaking procedures to one that is to be matured for years before drinking. If maturation reactions are unwanted, as during the production of a light or medium bodied fruity red style, the wine will not undergo barrel ageing, will be protected from oxygen andwill be held at low temperatures.

Bottle Ageing

A detailed discussion of the effects of bottle ageing on wine components can be found in Section 2.9.5. During bottle ageing the wine has virtually no exposure to air, and a measurement of redox potential in the bottle will usually show that it is at a minimum some months after bottling, particularly under screw cap. The length of time a wine will benefit from bottle ageing is dependant on the structure and flavor of the wine, as stated, and wines which were made to drink early do not benefit from bottle ageing. Because of their

ability to act as 'electron sinks' in wine, phenols help maintain the low redox potential in the bottle, and any residual oxygen in solution after bottling will be absorbed in reactions involving phenols rather than other wine components. This is considered beneficial in the development of an aged bouquet during long in bottle maturation, as the development of acetaldehyde and acetic acid will be limited. Many of the great French wines, due to their excellent phenolic concentrations, do not display their full potential until they have spent a decade or more in the bottle. The low phenolic content of white wines makes them particularly susceptible to oxidative browning, and less suitable for extended ageing.

Rosé Wine

Rosé wines are those of a color somewhere between red and white wines, and are usually chosen by consumers on the basis of their color and sugar content. There is no strict legal definition of a rosé wine, but they are usually made from red grapes with colorless juice by a process involving limited skin contact and phenolic extraction. It is illegal to make rosé wines by blending red and white wines (except for pink Champagnes), but blending red and white grapes or musts is permitted.

The qualities usually sought in a good quality rosé wine are an attractive appearance, freshness and crisp acidity, good fruit flavors and fresh, fruity or floral aromas. Although some rosé wines may have a higher alcoholic content than red wines from the same vintage, as less alcohol is absorbed by the marc, the trend nowadays is to make lighter styles in line with healthier lifestyle choices by consumers. A high alcohol concentration does not always suit the fresh, fruit driven style that winemakers aim for in a good rosé, and the wines are usually drunk within 24 h of purchase, so they are not made for prolonged ageing. In the past, rosé wines were not always produced with as much care as whites or reds and sometimes unripe or rotten grapes from mediocre varietals were used. This led to the perception that rosé wines were an inferior style, but this is changing with the emergence of examples of excellent quality, varietal rosés and a wide range of red and pink sparkling wines.

The color of rosé wines is a key quality indicator for the style and has a range of descriptions which are not legally defined, such as blush, '*pelure d'oignon'* (onion skin) or 'clairet.' Most consumers dislike orange tints, and see the wine as faulty, even if it tastes and smells excellent. The level of extraction of anthocyanins (red color) depends on the cultivar, the ripeness level of the grapes, the skin contact time and temperature, and the dose of sulfur dioxide used. As sulfur tends to bleach the anthocyanins, winemakers may try to avoid its use during the initial skin contact phase so as not to overextract and end up with too dark a color later on.

The two main methods of making rosés are direct pressing, and prefermentation skin contact. When direct pressing is used the vintage is crushed, often drained, then pressed immediately. Color is extracted with the first pressings, and later pressings are more yellow and higher in tannins and so are not usually blended into high quality rosés. The juice is then treated as a white juice, with SO_2 addition and prefermentation settling. As it is a fruit driven style, appropriate steps will be taken to avoid oxidation. Malolactic fermentation may or may not be carried out, depending on the type of wine desired and the acid level of the juice. The fermentation is carried out as for a fruity aromatic white style wine, using an aroma enhancing yeast and low temperatures.

After fermentation, a moderate dose of sulfur dioxide is added to preserve fruit aroma and flavor. Clarification and stabilization is as for white wines, but it is more easily carried out due to the tannin content. Sweet rosés are produced by ending the fermentation prematurely by racking, cooling, filtering and adding sulfur dioxide. The wine may also be fermented dry then sweet reserve, or rectified concentrated grape must added to the desired sugar level.

For short skin contact rosés, the berries are destemmed, crushed, sulfured and left to steep in their own must for up to 24 h, depending on the state of ripeness and health of the vintage. The level of color extraction must be monitored frequently, taking into account that there will be some color loss later. This is an aqueous maceration rather than an alcoholic one, so that color and fruit flavor are extracted rather than tannins. A

small dose of sulfur dioxide and low temperatures help protect against oxidation and the onset of a wild fermentation.

After skin contact the must is drained and the marc pressed. Often only the free run juice is used for rosé. Blending white and red grapes was the traditional way of producing Bordeaux claret, and although it is no longer carried out for this purpose, it is a permitted method of rosé production under the EU regulations. A proportion of the vintage is direct pressed (usually half), and the rest is crushed. The two portions are then mixed, and the vinification is carried out as in red wine production. Successful rosés have also been produced by short carbonic macerations. Wine may also be bled off a red wine ferment early in the fermentation to produce a robust rosé (the *Saignée* method), which increases the skin to juice ratio of the remaining red fermentation, resulting in a better color and higher tannin level in the red wine. It is important to do this early in the fermentation before too much tannin is extracted.

2.9.3 Sparkling Wines

A sparkling wine is one that contains a significant concentration of dissolved carbon dioxide, which is, of course, released when the bottle is opened. There is a wide range of such wines, which vary according to the grape varieties used, the method of production and the extent of carbonation. The latter can vary from very light ('pétillant') through medium ('crémant') to heavy. The emphasis here is on Champagne and its method of production ('Methode traditionelle'), but methods of producing other sparkling wines are discussed in the later paragraphs.

Champagne and Traditional Method Sparkling Wines

Champagne, arguably the most famous style of wine in the world, is named for the 35 000 ha region under vine in northern France from which the style originates. The region is one of the most northerly wine production areas in the world, with yearly heat summation for the growing season (total degree days) averaging only 75% of that for the Napa Valley in the United States (Zoecklein, 2002). Heat summation is defined here as (mean daily temp in °C – 10 °C) × number of days.

The conditions in Champagne and the methods of production have combined to produce a range of wines that are associated with luxury and celebrations, and which have been copied in many other wine regions with varying degrees of success. Over time, the name 'champagne,' to the chagrin of the c*hampenois*, became synonymous with any effervescent wine in which a second alcoholic fermentation was carried out in a bottle, no matter what sort of quality it showed. In order to protect the reputation of the true product and region, since 1994, it has no longer been possible to use the term 'champagne' unless it is the 'real thing.' Robinson (2006a) noted that at the time of publication of her *Oxford Companion to Wine*, champagne accounted for less than one bottle in 12 of total production of sparkling wine. The production of champagne is controlled by the CICV (Comité Interprofessional du Vin de Champagne) and wines which are produced in the approved way to required standards are awarded the AOC (Appellation d'Origine Controlée) (Ribereau-Gayon *et al.*, 2000). The term allowed to describe other sparkling wines in which the second fermentation has happened in the bottle is 'Méthode traditionelle' or the 'Traditional Method,' also known as 'Méthode champenoise' in the Champagne region. Each production region makes use of its own adaption of the term, for example, in South Africa, the traditional method is known as 'Méthode Cap Classique.'

Sparkling wines are those containing a significant level of carbon dioxide and are classified on the basis of production or the source of this CO_2. The excess CO_2 can be formed by the fermentation of the residual sugar from the primary fermentation (e.g. Asti Spumante). It may be formed during malolactic fermentation

e.g. Vinho Verde from northern Portugal and Frizzante from northern Italy, or by the fermentation of sugar added in the bottle after the primary fermentation, as in Champagne.

Carbon dioxide can also be added through carbonation in a large tank designed to withstand the pressures involved (as in the Charmat process – see Other Methods of Sparkling Wine Production) or as a result of carbon dioxide injection. By definition, sparkling wines are those that contain a visible excess of CO_2, with a minimum pressure of around 1 bar (0.392 g/100 ml) in bottle at 20 °C from the first or second fermentation (Amerine and Singleton, 1977). The carbon dioxide pressure in champagne and similar style sparkling wines is frequently far higher than this, about 4 atm ('mousseaux'), and wines with less than 2 atm are usually marketed as 'perlé' or semi-sparkling, under various names including 'crémant.' In addition to their major house wine, many producers in Champagne will make single vintage champagnes, typically around three or four a decade (Robinson, 2006a).

There are numerous rules attached to the production of Champagne, some of which have interesting relevance for other sparkling wine producers. For example, only three cultivars: Chardonnay, Pinot Noir and Pinot Meunier are permitted in the blend, and grapes should be hand harvested. Since 1992, the maximum permitted yield in Champagne is 65 hl/ha, with high vine densities and dates for picking fixed village by village (Robinson, 2006a).

Due to overproduction, and steady reduction in sales during the two preceding years, some champagne houses were calling for this to be reduced by half during the 2009 vintage (Fallowfield, 2009). However, balancing reductions in sales in Europe with increasing demands from emerging economies such as China, India and Russia is a challenge for the *champenois*. Expanding the volume produced by increasing the amount of land registered for production in the Champagne AOC would lower property values of vineyards throughout the region. The legal wranglings associated with attempts to change classifications in Bordeaux in recent years prove what an unpopular move this might be with present landowners and producers. Another possible solution might be to increase the output of existing vineyards. At higher crop loads, (for example 80 hl/ha), the grapes take far longer to achieve physiological maturity and are perfect for the neutral, slightly acidic style needed for a base wine, whereas at 50 hl/ha, the ripe fruit flavors become too concentrated to make good champagne (Fallowfield, 2009).

Alcohol concentrations in sparkling base wine are usually lower than in table wine, but in quality sparkling wine (EU legislation) 10% v:v is a minimum (Morgan *et al.,* 2006). Information on the label must include the vintage year, cultivar, reference to geographical area of production and whether the product was fermented in the bottle in which it is being sold. Preparation of base wine must be in accordance with local regulations for still wine. In Europe, the *cuvée* (particular blend to be used for sparkling wine) can be acidified by up to 1.5 g/l tartaric, and there is no limit on deacidification. Alcohol level in the base wine should be a minimum 8.5% v:v (Morgan *et al.,* 2006). Second fermentation can take place only in the bottle or in a closed (pressurized) tank. The wines must be aged in the producing winery for a certain minimum period starting from the onset of secondary fermentation ('prise de mousse') before release: 15 months for nonvintage, three years for vintage champagne. Liger-Belair (2004) noted that the best and most expensive champagnes will be aged for five years or more before being released.

Terms like 'crémant' and 'petillant' have been developed to describe the various sensations of the mousse (froth, foam or sparkle) in the mouth. Crémants have a slightly greater effervescence than petillants, but less than mousseaux, which should have an excess carbon dioxide pressure of around 3 atm at 20 °C. The term crémant was originally used in France to define those wines with a carbon dioxide pressure lower than 2.5 bar, but since 1992, has become the generic term for any sparkling wine made in France in an AOC outside Champagne, for example: crémant d'Alsace, crémant de Bourgogne, etc. The word is also associated with the sensation produced by lower carbon dioxide pressure on the palate. Petillants are generally made in two regions (the Loire valley and Bugey-Cerdon) and have a lower alcohol concentration than other sparkling wines in France, as well as carbon dioxide pressures that are lower (between 1.5 and 2 atm).

The size and duration of bubbles in a sparkling wine can also suggest which process was used in its production. The quality (persistence and fullness) of the mousse is tied to conditions during the second fermentation, concentration of nitrogenous compounds in the cuvee, and yeast autolysis products, such as proteins and high weight polysaccharides in solution. Champagne and sparkling wine made according the *Méthode Traditionelle* process has a small 'bead' (bubble size), which rises vertically through the liquid in a steady continuous stream ('bubble train'), rising to the surface and radiating out to the edge of the glass to form the 'collar'. Sparkling wines made by the transfer process, the Charmat method, or by carbon dioxide impregnation will have coarser (large) bead, which is less persistent. An older sparkling wine made in the traditional way may lose a lot of its gas through cork drying or shrinkage, and will present a much slower bubble train with a very fine bead. Older champagnes may be served very cold in chilled glasses to preserve the bubbles as long as possible.

Interesting studies on the nucleation, formation and distribution of bubbles using high speed video photography has been carried out at the University of Reims Champagne-Ardenne. According to Liger-Belair (2004), bubble nucleation sites are not scratches in the glass, as popularly believed, but particles such as cylindrical cellulose fibers, which contain small gas pockets and adhere to the glass or float in solution. The gas pockets act as 'bubble guns' or nucleation points for bubble formation. Liger-Belair observed that in a completely clean container, carbon dioxide escapes only through the surface of the liquid and the wine appears still. Very few bubbles nucleate in a bottle of freshly opened sparkling wine because, over several years of storage, most of the as pockets within the liquid have become wet, and therefore no longer function as nucleation sites. Yeast autolysis products affect bubble stability and flavor. Proteins act as surfactants and lipids reduce surface tension (Jackson, 2000c). Gauging optimum levels of protein in the wine is a matter of experience. It has been determined that many of the premium champagnes have a higher protein level than other sparkling wines, probably as a result of time spent sur lie as well as possible cuvée nitrogen constituents. It is also recognized that champagne with a high proportion of Chardonnay in their composition have better foaming properties than those with high levels of Pinot Noir or Pinot Meunier (Zoecklein, 2002). Research at the University of Reims has observed a link between infection of the grapes with *Botrytis cinerea*, and the foaming properties in champagne, with a 40% infection of grapes reducing 'foamability' by as much as 70% (Jeandet, 2006; Cilindre *et al.*, 2007).

The release of carbon dioxide significantly magnifies the odorous components of the wine. An overview of sparkling wine production using the traditional method is shown in Figure 2.9.19.

Cultivars for Champagne Production

As previously noted, grapes used in the Champagne region are almost exclusively Pinot Noir, Chardonnay and Pinot Meunier (Ribereau-Gayon *et al*., 2000), with yields. Different styles require different cultivars, for example, Blanc de blanc is made from white grapes (usually pure Chardonnay), and blanc de noir is usually made from Pinot Noir. Rosé champagne may be made by allowing some skin contact prior to pressing, and then 'bleeding off' (saignée) free run juice from the crushed red grapes, or by adding a small amount of red wine to the white base wine later (Robinson, 2006a). Champagne is the only Appellation d'Origine Contrôlée where such a practice is authorized. Each of the cultivars adds an essential component to a blend, and the origin of grapes will affect the quality and style of the final product. This is usually as much of a secret as the carefully guarded information concerning the blends and additions for each house. Chardonnay is reputed to give finesse, freshness, floral notes and ageing potential to the cuvée. Originally planted on the east facing slopes of the Côtes des Blancs, a region south of Epernay, Chardonnay is now far more widely planted. Robinson (2006a) suggests that Chardonnay imparts austerity and elegance to the young wine, but is long lived, and gives good fruitiness in older champagne. Zoecklein (2002) noted that great care should be taken to avoid using mature (ripe) Chardonnay grapes for premium sparkling wine production in warmer climates, as

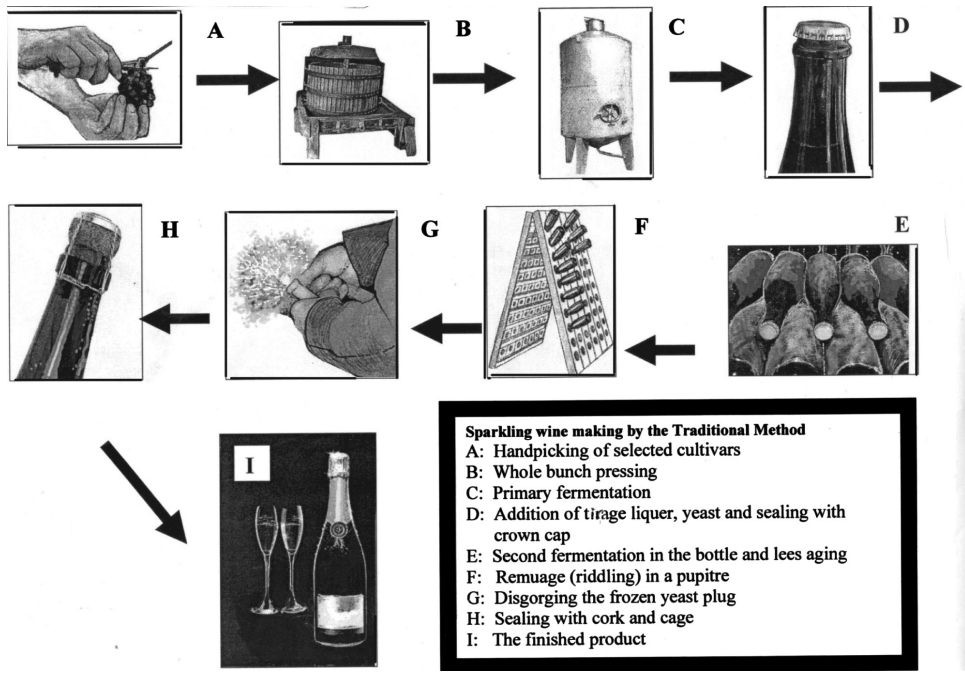


Figure 2.9.19 *An overview of sparkling wine production by the traditional method*

these can produce an aggressively varietal character. Pinot Noir adds body, complexity and structure, and is planted in the Montagne de Reims mainly on north facing slopes and in the Aube in the south. Pinot Meunier is found mainly in the Vallée de la Marne and is generally neutral, but also has spiciness and roundness (Jackson, 2000). The latter cultivar ages more quickly than Chardonnay, but helps to add fullness, body and length to the finish (Zoecklein, 2002). It usually has a lower titratable acidity than Chardonnay, and is harvested earlier in the season. Other varietals that may be suitable for base wine production include Chenin de la Loire, Ugni Blanc and Mauzax de Limoux, as long as the grapes are at the right acidity level (Ribereau-Gayon *et al.*, 2000) Outside of Champagne, AOC regulations will determine proportions and blending of cultivars within each appellation.

Harvest Procedures

Grapes for sparkling wine production are picked earlier in the season than those destined for still wine (Jackson, 2000c) in order to avoid the stronger varietal aroma associated with ripe fruit. Robinson (2006a) noted that traditionally champagne was made from grapes from a number of different vineyards within the appellation (major firms can use the produce of between 50 and 200 communes in their blend), but nowadays a fair number of producers make wines from a single vineyard.

In the finished product, the fermentation and autolysis bouquet are regarded as primary indicators of quality rather than fruit character, and therefore the Comité Interprofessionnel du vin de Champagne (CIVC) encourages harvesting when sugar to acid ratios are between 15 and 20. This means grapes reach optimum maturity for base wine production at only 14.5 to 18 °Brix, for a target alcohol from the first fermentation of between 10.5 and 11.5% v:v. Titratable acidity is high, around 12 to 18 g/l, with malic acid making up as much

as 50–65% of the total acid content (Zoecklein, 2002), which has implications for the microbial stability of the base wine. Harvesting is done by hand into small trays, selecting out rotten grapes. Selecting out rotten bunches and berries by hand, although very labor intensive and expensive, is done by some houses in order to avoid the physicochemical effects of fungal metabolites on foaming properties and yeast activity in the wine. Low levels of sulfur dioxide are used in base wine production. Low flavonoid phenol content is desirable and so grapes are harvested cool to avoid excessive phenolic extraction, which may introduce astringency, color and bitterness to the base wine. High temperatures may also encourage microbial or oxidative spoilage of fruit which reduce ageing potential, and form off flavors such as aldehydes and volatile acidity.

Pressing the Grapes

Whole bunch pressing in traditional basket presses (sometimes known as Coquard presses after the original manufacturer) is usually carried out for the production of champagne or other premium sparkling wine (Robinson, 2006a). Membrane and tank presses can also be used. The advantages of the more modern presses are that they are more efficient, fairly gentle and give protection against oxidation. The traditional press held 4000 kg of grapes, a quantity that became known as '*marc*,' and a standard unit of measurement in Champagne. Nowadays, the word is more usually associated with the fermentation cap or press cake from red wine macerations.

Skin contact is usually minimized in order to avoid extraction of color, tannin and fruit character into the base wine. Pressing of whole bunches of grapes is carried out, a slow process which involves a high level of manual labor, and exposes the juice to oxygen if carried out in traditional vertical basket presses. No destemming occurs before pressing, as stems give a better pressing efficiency at lower pressures, forming channels within the press cake along which the juice can escape. By AOC law, 4000 kg batch of grapes, pressed at around 1 bar, are only allowed to yield around 2600 l of juice if the juice is to be used in champagne production. The first 512 l/tonne (2050 l in total) constitute the *cuvée* portion. This is the least fruity, highest in acidity and sweetest of the fractions and is usually reserved for the *vin de cuvée* (top quality base wine of a specific batch) (Robinson, 2006a). The next 410 l are pressed at less than 1.2 bar and are known as the 'premiere taille' (51 l/tonne – fruitier, less fresh and less elegant than the cuvée), and 206 l of 'deuxieme taille', extracted by pressing up to 1.4 bar, which may be discarded (Jeandet, 2006). Later press fractions are not allowed to be included in the cuvée due to high pH, excess color, high total phenolic content, excessive varietal character, harshness and higher insoluble solids. The different fractions are usually kept separate until assemblage (blending).

Combining press fractions can affect such things as the fullness and flavor of the *cuvée*, as press fractions can be separated on the basis of their titratable acidities (later fractions have lower acidity). For red varieties such as Pinot Noir and Pinot Meunier, care is taken to avoid excessive color extraction and astringency by pressing fruit cold and separating pressing fractions. Short, cold skin contact periods of crushed grapes or carbonic maceration may be used to enhance color without adding to astringency and tannins. In some cases the juice is deliberately exposed to oxygen in order to remove color through polymerization and subsequent precipitation.

Additions

Sulfur dioxide is never added directly into the press in order to avoid extraction of phenols from the skins. Jackson (2000c) noted that an addition of 40 to 60 mg/l sulfur dioxide added to juice, is normal in Champagne, but the dose will depend on the condition and levels of rot in the grapes. Up to 80 mg/l may be added to press fractions if there is grey rot present.

A standard addition of 5–10 g/hl of diammonium phosphate is widely used for nitrogen adjustment, and between 10 and 25 g/hl of bentonite can be added during the primary fermentation of the cuvee (Zoecklein, 2002). Additions of up to 150 g/hl of a bentonite/casein mixture are often added to the '*tailles*' when a significant amount (greater than 15% of the berries) of rot is present, and the first taille may also receive 60–70 mg/l SO_2. Press juice fractions with turbidities of between 200 and 400 NTU (Nephelometric Turbidity Units) (Section 4.6.3) may be treated with pectolytic enzymes and are usually cold settled for 18 to 24 h at cold temperatures (10 °C) or centrifuged to reach a solids level of between 0.5 and 2.5% prior to fermentation. Settling decreases levels of proteins, pectins, and cations including iron (Jeandet, 2006).

The base wine should be relatively low in free sulfur dioxide content or the yeast may struggle during the second fermentation, and malolactic fermentation may not start.

Yeast (for First Fermentation)

The yeast employed maybe the same for the primary and secondary fermentation, frequently strains of S*accharomyces bayanus* (Jackson, 2000c), as this has a slightly better tolerance than *S. cerevisiae*. Sparkling wine yeasts are selected for their ability, among other things, to produce esters, but care needs to be taken if seeking a traditional Champagne style to avoid overproduction of fruity, floral characters. The same yeast can be used for both primary and secondary fermentations, but, as noted by Zoecklein (2002), care is taken to avoid the selection of a strain that produces high levels of esters in order to avoid a product that is too fruity and floral. A producer may choose to use an appropriate dried commercial starter culture developed by the (CIVC), or a strain selected from the yeast collections that large Champagne houses maintain. Winemakers will usually do some in house testing to determine the merits and deficiencies of the strains within their own blends. Traditionally the *champenois* used small wooden barrels to ferment all or part of the cuvée, which added structure ('carpentry') to the wine, but nowadays the primary fermentation is frequently carried out in stainless steel tanks with a capacity of 50–1200 hl (Robinson, 2006a). Krug and Bollinger ferment the entire vintage slowly at low temperature in oak vats, which is consistent with their full flavored, mature style (Jeandet, 2006). For product consistency, and to achieve the house style, a producer may choose to add a proportion of the previous year's cuvée (reserve wine) to the fermenting juice. Reserve wine can also be added during *assemblage* or blending and may be a component of the *dosage*, based upon production and vintage dating considerations. Some producers choose to ferment their *cuvées* warm to reduce the floral intensity, thus making a more austere product. Higher fermentation temperatures are desirable if malolactic fermentation is sought, and 25–30 °C is not uncommon.

According to Ribéreau-Gayon and coworkers (2000) higher crop yields and pHs after the 1950s meant that during the 1960s a number of wine losses occurred in Champagne as a result of bacterial contamination during the bottle fermentation. Wines became 'ropy,' riddling was difficult, there were numerous stuck fermentations and high volatile acidities in the wine. With the exception of Lanson and a number of other Champagne houses, most producers now prefer to 'play it safe,' and induce a malolactic fermentation before the second fermentation. This helps to reduce varietal character and ensures microbial stability. Some c*hampenois* use malolactic fermentation of all or part of the *cuvée* to improve the finish and flavor through raising the pH and increasing complexity. Producers will keep their sulfur dioxide levels low in their base wine in order to encourage the malolactic fermentation and thereby prevent it happening in the bottle, as bacteria are almost impossible to remove by riddling. If malolactic fermentation is desired, it will be induced with an inoculation of malolactic bacteria (*Oenococcus oeni*) at a concentration of around 10^7 cells/ml. Because of the difficulty of initiating the malolactic fermentation, especially in highly acidic wines such as Champagne wines, studies have focused on the nutritional requirements (namely amino acids) and the metabolism of lactic acid bacteria under wine conditions. In particular, studies performed at the University

of Reims Champagne-Ardennes demonstrated that aspartic acid inhibits the growth of lactic acid bacteria by modifying the transport of glutamic acid (an amino acid essential for bacterial growth) into the bacterial cell (Jeandet, 2006).

Protein based fining agents such as isinglass and gelatin can be used to lower the phenolic content of the base wines. A mixture of bentonite and casein may be added to the *taille* fractions to assist in precipitating tannins. The base wine will be racked off the lees after the fermentation. The clarified wine after malolactic fermentation is sometimes referred to as '*vin clair*.'

Gauging optimum *cuvée* protein is a matter of experience, and producers of sparkling wine must manage their protein levels so that there is minimal protein precipitation in the bottle, but enough retained to ensure good bubble stability. While sugar diminishes CO_2 solubility and may therefore decrease bubble stability, free amino acids and proteins can help to retain dissolved carbon dioxide in the medium (Descoins *et al*., 2006). Those using bentonite as a riddling aid should avoid further fining of the base wine, as additional protein will be removed during disgorging.

Assemblage

Blending or 'assemblage' is the blending of wines from different vineyards, vintages or cultivars after the first racking and before the second fermentation, and is one of the most crucial steps in the making of champagne. A number of important decisions are made during the assemblage, for example, whether the cuvee is vintage or not, and if it is of sufficient quality for second fermentation in the bottle, as the carbon dioxide produced during the process will enhance any flaws in the aroma. Nonvintage champagnes rely completely on product consistency and usually require vin de reserve (cuvees from previous years) to be added to the blend in order to minimize any distinctive characteristics. Rosé wines can be obtained by mixing a white wine with a red wine (Côteaux champenois) (e.g. in the case of the Dom Pérignon Rosé; Vintage of Veuve Clicquot) in a proportion of 10 to 15% red wine (Jeandet, 2006). Rosés de Saignée can be obtained by a short period of maceration of the fermenting grape juice with the grape skins, where anthocyanins (the red pigments of grapes) are extracted (e.g. in the case of the Champagne Rosé from Krug). The brut nonvintage and the demi-sec champagnes are usually a blend of wines from several years, from different grape varieties and a number of crus. The brut style, especially, is a product that is very typical of the wine producer. Vintage champagnes are produced exclusively from the wines of a single harvest.

As stated previously, producers may use a number of different wines from different vineyards (as many as 200 communes may be included in the blend) within an appellation, as well as between 10 and 50% of reserve wine during *assemblage* (Robinson, 2006a). According to one source, Champagne is the only AOC where red and white wines may be blended. The goal is to produce a *cuvée* that is clean and neutral, but has enough structure to support the additional alcohol and bouquet from the second fermentation. *Assemblage* is also carried out to increase the complexity, balance and subtlety of the base wine, as it is rare for a single wine from a single vintage to be perfectly composed for premium sparkling wine production. The selection of the base wine components also needs to ensure the production of a sparkling wine of consistent flavor and style (the house brand) in sufficient quantity to be economically viable, so another important consideration is the amount of press wine to include in the *cuvée.* The inclusion of later press fractions makes economic sense, but may have implications for the quality of the final wine. The winemaker must blend a wine that produces a reliable bouquet during second fermentation, that withstands ageing well, and that may only be consumed (even decades) in the future. Some producers prepare *cuvée* blends prior to stabilization, because as the base wine is blended with other wines of different vintages, cultivars and origins, potassium bitartrate and protein precipitation can occur. Cellar treatments such as fining and filtration can also affect potassium bitartrate stability, and it is normal for protein and bitartrate stability be evaluated just prior to bottling.

Preparation for the Bottle Fermentation

Prior to the introduction of the yeast for the second fermentation, many producers cold stabilize their base wine at –4 °C in either a static contact process, or a continuous process, to precipitate potassium bitartrate. The wines are then filtered with diatomaceous earth in order to remove residual crystals and other particles that may cause gushing during degorgement, undesirable microorganisms and to present a clean 'canvas' to the yeast. If malolactic fermentation has been completed, it is sufficient to carry out a pad or diatomaceous earth filtration, but if not, a membrane filtration will is necessary. Some producers, for example, Krug, do not filter, but clarify with isinglass (Zoecklein, 2002). Barrel-fermented wines are generally not filtered, as they are naturally clarified by the combination of oak tannins and yeast mannoproteins during and after fermentation. Work has been done to research whether the very expensive treatment of wines with artificial cooling could be advantageously replaced by the addition of inhibitors of the potassium hydrogen tartrate (KHT) crystallization process such as metatartaric acid or carboxymethylcellulose (CMC). Such inhibitors increase the width of the supersaturation field of KHT in the wine, thus delaying tartrate salt precipitation in the bottle (Jeandet, 2006). Inhibitors for tartrate crystallization have not yet been authorized for winemaking. The later compound is an unstable ester that hydrolyzes with time and increasing storage temperature, and may actually pose more of a problem to the stability of the wine in the long term.

Tirage

At the bottling line, a mixture of selected, rehydrated wine yeast, water, dissolved sugar, sulfur dioxide, riddling aids and nutrients known as the 'liqueur de triage' is added to each bottle in order to produce the second fermentation.

The temperature of the second fermentation is said to affect the quality of the mousse, as well as yeast autolysis, and is kept fairly low (between 10 and 15 °C). In many of the older production houses, this is not monitored, but the second fermentation takes place in *caves* (underground cellars) where it is likely the temperature remains a constant 12 °C. Wine will be aerated slightly prior to the second fermentation to encourage growth of the yeast (Amerine and Singleton, 1977).

Yeast (for the tirage)

The second fermentation places extraordinary physical demands on yeast cells. A species needs to be tolerant of low temperatures, tolerant of high pressure and should possess the ability to ferment to dryness without producing foam, in the presence of high concentrations of alcohol. The yeast should not produce any off odors or sulfur dioxide, and should also die and flocculate after fermentation. Many firms use their own 'in house' yeast strains, and new producers will experiment to assess the performance of different yeasts under their own conditions. The yeast should also impart a positive fermentation and autolysis character to the wine. 'Epernay' is a good example of a highly flocculent yeast with good riddling ability, but it has an assertive character and is therefore usually not employed to carry out both the primary and secondary fermentation. 'Pasteur Champagne' (UCD 595) is also a popular yeast for secondary fermentation and is available in dehydrated form (Zoecklein, 2002), as are DV10, 10 C and Levuline CHP strains (Jeandet, 2006). Some sparkling wine producers use cultures containing a mix of yeast strains for the secondary fermentation believing that such a procedure adds complexity. Generally, 2 to 3% by volume of active starter culture is used (Amerine and Singleton, 1977), or around 15 g/hl dry yeast, which is then rehydrated giving a cell concentration of around 10^6/ml.

One of the important characteristics of yeast used for the second fermentation is the ability to form aggregates, which can be easily dislodged during riddling and removed by disgorging. There is significant

variation in the agglutination ability of yeast. Yokotsuka *et al*. (1997) produced bottle-fermented sparkling wines using *S. cerevisiae* immobilized within a double layer of calcium alginate beads and strands. Beads contained greater numbers of immobilized yeast cells, and were also considered by the authors to be more useful for commercial sparkling wine production as they were more easily removed from the wine than the strands during disgorging. A study by the same workers compared wines made with the immobilized yeast and traditional freely suspended yeast, which showed that there were no chemical differences between the wines. The authors also investigated factors affecting leakage of viable cells from the gel into the wine during fermentation and ageing. Fewer free yeast cells appeared in the wines with higher initial numbers of immobilized cells and those wines with higher ethanol concentrations.

Riddling aids in the tirage

In the traditional bottle fermentation of sparkling wines, the riddling (remuage) and disgorging (dégorgement) process are time consuming, labor intensive and can be accompanied by significant loss of wine, thus adding considerably to the costs of production. Most modern producers add riddling aids (usually fining agents) before the second fermentation to assist in sedimenting the yeast into a compact, granular deposit that is relatively easy to shake down the bottleneck prior to removal. The development of smoother glass in bottles also helps to make the process easier than previously. Riddling aids include sodium and calcium bentonite, commercial bentonite preparations like Clarifying Agent C, Adjuvant H, isinglass (e.g. Colvite) tannin (e.g. Botane), gelatine and diatomaceous earth (Zoecklein, 2002), with some producers using alginates (proteins derived from wheat, pea or alfalfa). The choice of riddling aids is based upon the expected time sur lie, and the age and composition of the wine. The effects of riddling aids on the yeast deposition are not predictable, and may in some cases be detrimental to the wine composition. Overdosing bentonite, for example, can adversely affect bubble formation and retention by stripping out protein, and using too much of any of these agents can actually make riddling and degorgement more difficult (Zoecklein, 2002).

Other components of the tirage

The amount of sugar is regulated to give the required increase in the alcohol concentration and CO_2 level, and the individual recipes used depend on the individual house. Sucrose, sugar syrups of various compositions and beet sugar are used, with different producers using various sugar sources for the second fermentation. This information is key to the 'house style' and is privileged. If there is residual sugar in the base wine, the amount of sugar added will need to be adjusted or excessive pressure may result as it ferments. Amerine and Singleton (1977) note that 4 g of sugar per liter of wine will produce one atmosphere of carbon dioxide pressure, and that for a total of 6 atm, around 24 g/l of sugar will need to be present at the start of refermentation. This equates to between 6 and 8 l of carbon dioxide per liter of wine, which is also the amount that can be safely contained by a crown cap or cork with a wire cage (Robinson, 2006a). The actual yield of gas is less than this due to production of small amounts of aldehydes, volatile and fixed acids, glycerol and other yeast by-products. The refermentation will also produce between 1 and 2% (v:v) additional alcohol. A small percentage of citric acid may be added with the sugar. Some producers add around 15 to 20 mg/l of sulfur dioxide to the tirage liqueur, which helps to protect the wine from oxidation and activity by microorganisms other than yeast during the ageing period. As stated previously, the free sulfur dioxide level is kept low (preferably $<$20 mg/l) in order to avoid yeast inhibition.

Yeast assimilable nitrogen compounds are essential for the growth and development of yeast, especially under the difficult conditions of refermentation. Diammonium phosphate additions of between 50 and 200 mg/l are not uncommon, and apparently favor the production of esters and diminish the production of fusel oils

and sulfites. The yeast growth stimulator biotin may also be added at a concentration of around 0.5 mg/l (Zoecklein, 2002).

After the cuvée has been mixed well, it is transferred to heavy glass bottles that are specially designed to withstand the pressures produced by carbon dioxide released during the second fermentation. The bottles will often have a push up or punt at the bottom for added strength (Amerine and Singleton, 1977). However, if the fermentation temperature is too high, the dosage of sugar incorrect or the glass is defective in any way, the bottle may break during fermentation, and the wine will be lost. In the event that a batch of bottles suffer from a production fault, this can be significant. The fill level will be based on estimations of loss during disgorgement, and the desired dosage volume. A 'bedule' (hollow polyethylene cup) is inserted into the neck, which helps to seal the bottle and prevent the metal crown from making contact with the wine. Following this, a stainless steel or aluminium crown cap closure is placed on the bottle.

Bottle Fermentation (Prise de Mousse)

Following crown capping, the bottles are placed horizontally on laths ('sur lattes') or into crates on their sides in a cool (10 to 15 °C), dark area such as in the traditional limestone *caves* underground in Champagne, for the second fermentation. The rate of the second fermentation is a function of yeast strain as well as temperature, pH, phenolic content and sulfur dioxide concentration. Complete fermentation and pressure generation can take 6 to 8 weeks, after which the bottles may be moved to a cooler location (around 10 °C) where they will remain undisturbed for the maturation period. The period between the initiation of the second fermentation and disgorging is usually around 15 months.

The rate of the secondary fermentation is a function of the yeast strain, yeast numbers, the ambient temperature and cuvée chemistry, but generally the process takes around two to three months. The rate is increased by high pH, high yeast nutrients, a low phenolic content, a low alcohol concentration, low sulfur dioxide levels and low carbon dioxide pressure. If the secondary fermentation temperature is too high, it is believed to result in coarse bubbles that less persistence and finesse (Ribéreau-Gayon *et al.,* 2000). Other factors affecting bubble retention include the yeast strain, whether the wine has been fined with bentonite (which will affect the levels of protein in the cuvée) and the length of time under pressure in contact with yeast. The pressure due to the carbon dioxide produced by the yeast builds steadily during the fermentation, and has an inhibitory effect on the fermentation, which can be counteracted by nutrient supplements in the triage and by using a high yeast dose. After the fermentation has finished, the wine must stay on the lees for a minimum of 15 months for nonvintage champagne and a minimum of three years for vintage champagne, according to the appellation rules. Some wines will age sur lie even longer than this.

On lees bottle maturation (sur lie)

Méthode traditionelle bouquet is a result of yeast autolysis and ageing. Sur lie (on lees) ageing takes place in the period after the second fermentation, and can vary, depending on the type of champagne, between one and a half, and eight years. Immersed in the wine, the internal membranes of the yeast give way and release enzymes that gradually break the cells down from within (autolysis). After a few months, the cell walls collapse and the cell contents are released into the wine. As a result of storing wine in contact with autolyzing yeast, there is an enrichment of the wine with amino acids through execretion or diffusion (Ribéreau-Gayon *et al*., 2000) as well as esters, proteins, fatty acids, peptides and terpenoids. The yeast layer protects the wine from oxidation phenomena as it is a source of reducing compounds such as glutathione. Products of yeast autolysis and ageing not only improve flavor, bouquet, complexity and depth, but perhaps also CO_2 retention and bubble size. The concentration of amino acids increases significantly between the 12th and the 43rd month sur lie (Zoecklein, 2002). Autolysis is dependent upon such parameters as pH, ethanol concentration,

and temperature. Higher pH values significantly increase the rate of autolysis, as does ageing bottles at elevated temperatures although this may a detrimental effect on both bubble retention and flavor of the wine. During sur lie, the wine may develop a yeasty smell and taste, the result of photodegradation of cysteine and methionine conjugates to methional, methanthiol and dimethyldisulfide (see Faults in Sparkling Wine, below). The difference in amino acid constituents of the *cuvée* as a result of sur lie ageing contributes to the character and complexity of Méthode Traditionelle wines and explains the sensory differences between these and sparkling wines produced by other methods. The development of what some call a 'yeasty' character ('le goût champenoise') does not refer to yeasty fermentation aromas, but to a toasty or biscuit note that is the result of ageing and yeast autolysis (Zoecklein, 2002).

Wines that are designed for long-term ageing undergo 'poignetage' or shaking once a year before riddling. This helps dislodge sediments and avoid crusting and staining of the bottle with the yeast lees.

Riddling (Remuage)

When the winemaker considers that his wine has matured for a significant length of time sur lie, the process of removing the sediment is started. Remuage is the process of encouraging all the particulate material (yeast cells, protein material, bitartrate crystals and riddling aids) in the wine to move into the neck of the bottle, eventually ending up as a compact plug against the bedule. The bedule helps to insure that the yeast pellet will be ejected uniformly during disgorging and that no yeast residue will be left in the bottle.

The riddling process is started by shaking the bottles to dislodge the lees layer, and then the bottles are transferred to a special A-shaped sloping rack known as a 'pupitre' (Figure 2.9.20) where they twisted several

Figure 2.9.20 *Sparkling wine in pupitres at the start of the remuage process at J.C. Le Roux, South Africa.* Photograph by courtesy of Paul Gerber

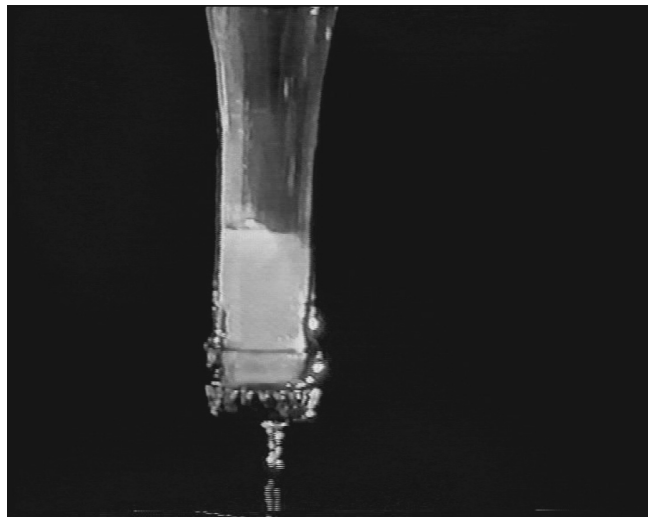


Figure 2.9.21 *The yeast plug compacted in the neck of the bottles*. Photograph by courtesy of J.C. Le Roux, South Africa

times a month. The bottle is placed in a more vertical position each time it is rotated so the yeast deposit gradually moves down the neck.

Eventually the bottles in a particular pupitre will all be upside down (sur point), ready for disgorging. The difficult work of riddling was traditionally done by hand, and a skilled riddler ('remueur') may do tens of thousands of bottles per day. However, riddling racks occupy large amounts of space in the cellar, and the process was highly labor intensive, so nowadays, in both Europe and the USA, the whole process is often done mechanically, on large, programmable 'gyropalletes.' Using this technology, riddling time is shortened to around a week, but most producers will still employ a riddler to attend to difficult wines, as it is widely acknowledged that hand riddling is more effective at compacting lees. Many wines appear to be easier to riddle shortly after fermentation and again after about 12–14 months sur lie (Zoecklein, 2002). The longer the yeast has been in contact with the wine, the more homogeneous is the sediment. Nowadays, economic pressures require shortened remuage times (two to four days at most) so immobilized or agglomerating cells may be used. Agglomerating yeasts or yeast immobilized into gels assist in the riddling process by being easier to dislodge and move into the bottleneck (see above). The disadvantage of shortening the riddling time is that the wines obtained sometimes still contain particles in suspension termed voltigeurs, which have been shown to be of mineral origin (probably bentonite added in the liqueur de tirage) (Jeandet, 2006).

When the sediment is compacted in the neck of each bottle (Figure 2.9.21), and the wine is clear, the bottles are ready to be disgorged.

Disgorging ('Dégorgement')

Dégorgement is the removal of the plug of sediment from the neck of the bottle, a process Robinson (2006a) rightly refers to as 'cumbersome.' Prior to this step, the wine is usually chilled to about 5 °C in order to prevent significant loss of carbon dioxide or wine during disgorging. The process should not cause loss of more than 2% of the volume of the bottle, but this can only be achieved if the wine is very cold, and the carbon dioxide better contained. If disgorging is carried out properly, only about 0.5 to 0.8 bar of carbon dioxide pressure

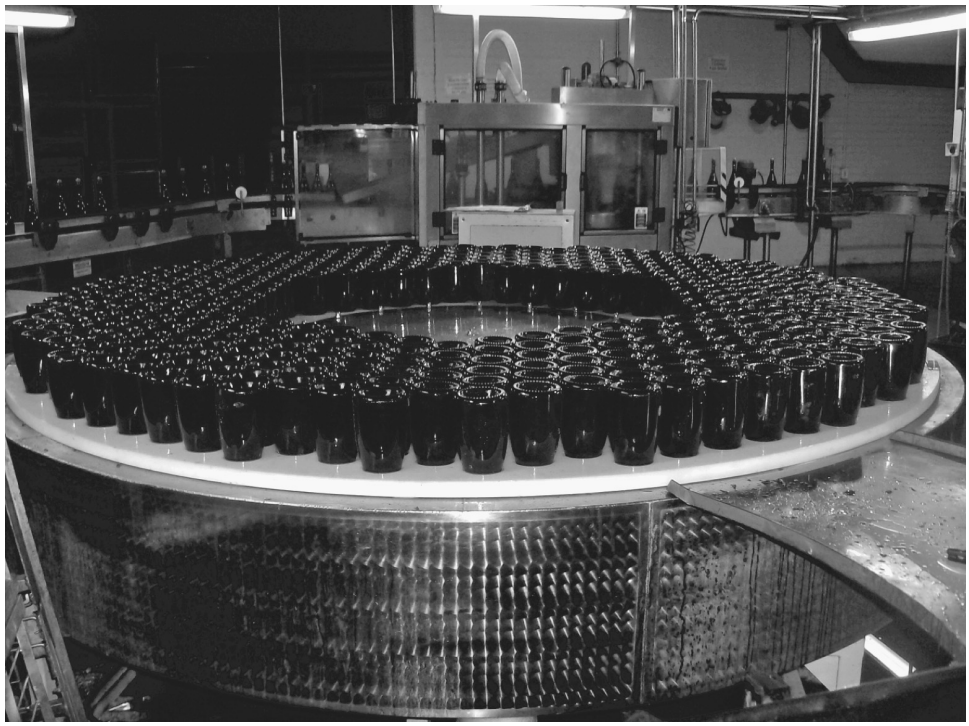


Figure 2.9.22 *The glycol brine bath at J.C. Le Roux, South Africa*. Photograph by courtesy of J.C. Le Roux

should be lost (Jeandet, 2006). One person disgorging manually may be able to complete around 2000 bottles per day, and an automated system will handle the same amount in an hour, so many larger concerns have automated.

Bottles of higher capacity such as magnums, jeroboams and methusalems, are still disgorged manually.

After cooling, but while still 'sur point.' the bottlenecks are carefully placed to a depth of about two to three inches into a very low temperature ($-15\,^{\circ}C$ to $-30\,^{\circ}C$) brine of calcium chloride or a glycol solution (Figure 2.9.22), which freezes the sediment and a small portion of the liquid in the bottle neck.

The frozen portion (around an inch) of liquid in the neck helps to form a barrier to the wine and carbon dioxide beneath it, which assists in preventing loss of product. If too much liquid is frozen, disgorging will be very difficult, as some carbon dioxide pressure is needed in order to eject the plug.

Just prior to disgorging, the bottle is rinsed and held upright, at about 45° off the vertical. The crown cap is removed briskly, and the bedule, yeast plug and a little frozen wine are ejected (Figure 2.9.23). The disgorger then places his thumb over the bottle mouth to avoid any further loss of pressure or liquid, and wine is evaluated for clarity and off odors. If too many yeast cells are left in the bottle, refermentation may occur after the sugar containing 'liqueur d'expédition' is added during dosage. Wines that are not yeast free, and tainted wines, are usually discarded, but clean, clear bottles will placed on a carousel apparatus ready for the dosage. Only a few bottles are ever opened at a time, and if there is any sort of delay in the process, they may be temporarily capped to minimize gas loss.

At this point, the final manipulations to the composition of the wine must be made by adding the liqueur d' expédition before the bottle is corked. The main adjustment is usually the sweetness, as the base wine

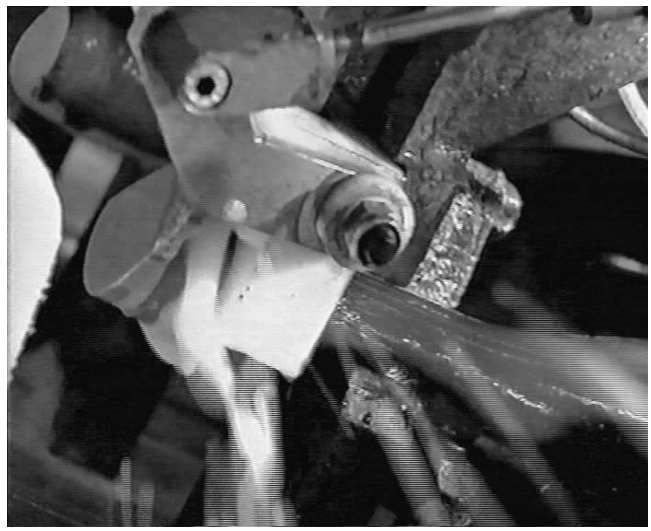


Figure 2.9.23 *Disgorging machine in action.* Photograph by courtesy of J.C. Le Roux, South Africa

has high acidity and champagne with no added sugar may be unpleasantly tart. Robinson (2006a) noted that sur lie ageing and the accompanying yeast autolysis can have a mellowing effect, and may be an 'excellent substitute' for dosage, with older wines having progressively less need for sugar. Some champagnes are made with zero sweetness (see Table 2.9.1), but generally speaking, the liqueur d'expédition will contain sugar, invert sugar, or sugar syrup, some vin de reserve or deionized water, sulfur dioxide, and possibly brandy to mimic age in young wine (up to 3% Cognac may be used) (Zoecklein, 2002). The liqueur is prepared according to a closely guarded secret recipe used by each house, and may have been aged for some time before use. Levels of sweetness are adjusted according to the style of wine, and the liqueur will be filtered brilliantly clear before use to avoid gushing.

The addition of recent reserve wine or low levels of citric acid (up to 500 mg/l may be added in the dosage to provide freshness in older wines, a little red wine would contribute depth and brightness to the color of sparkling rosés, and oak aged wine can be used for depth and complexity if these are needed (Zoecklein, 2002). Some producers add ascorbic acid (up to 90 mg/l), in conjunction with sulfur dioxide as an antioxidant, and copper sulfate may be included in very low doses ($<$0.4 mg/l) to counteract reductive aroma development.

Refermentation of the sugar in the dosage is unusual. Residual carbon dioxide pressure in the bottle is normally too high (up to 4 atm) for yeast metabolism, even after disgorging losses, and the alcohol concentration will have increased due to the second fermentation. Sulfur dioxide is thought to reduce the quality of the product, but will be added if the bottle ageing period is under nine months, and there is a chance there may be viable yeast cells present.

A manual dosage machine adds the liqueur and will then top up each bottle to the required fill level with a measure of the same wine from another bottle set aside for the purpose. Following the dosage, corking and caging of the corks, bottles are shaken to distribute the dosage liqueur.

After dosage and corking, many producers lay wines down for a time in order that the wine and dosage 'marry' prior to release, in a process known as empilage. During this time, the corks will assume their characteristic shape, and leaks can also be detected. At the time of disgorging, the reductive, protective effect of the lees layer is no longer present and oxidation of the wine begins. Zoecklein (2002) noted that by the time champagne is exported, distributed and finally consumed, it may be excessively oxidized, and prove

Figure 2.9.24 *The release of foam and carbon dioxide on opening.* Photograph by courtesy of J.C. Le Roux, South Africa

quite disappointing, as most sparkling wine is released for immediate consumption (Figure 2.9.24), and does not benefit from ageing.

Faults in Sparkling Wines

The appearance of sparkling wine, especially the presence and quality of bubbles, is a very important quality feature. Bubble trains are ideally small and continuous, with great persistence. The collar of the wine (the collection of bubbles or foam around the rim of the glass) should last, ideally, until the glass is almost empty. The liquid should be brilliantly clear (other than the bubble trains), and color should be typical of the age and style of the wine (pale straw in young wines, deep yellow and gold in older wines, pink, blush or onion skin in blanc de noir or rosé styles).

Gushing, the excessive frothing on opening the bottle, as discussed previously, is a sporadic problem due to particles (yeast cells, potassium bitartrate crystals, cork or packing dust) within the wine acting as nucleation sites. If an entire cuvée has the problem, it may be due to excessive CO_2, a lack of cold stabilization or excessive tannins (Ribéreau-Gayon *et al.,* 2000a).

D'Auria *et al*., (2003) conducted an experiment in which champagne was analyzed using solid phase microextraction (SPE) (Section 4.2.4) before and after UV irradiation. They noted that significant modifications in the composition of the flavor were observed, and ester profile of the wine after the irradiation was completely modified. Ethyl acetate, ethyl butanoate, ethyl 2-hydroxypropanoate, 3-methyl-1-butanol acetate, ethyl hexanoate and ethyl octanoate were significantly reduced. Cheese, vegetable and/or shrimp-like aromas in sparkling wine may be caused by the breakdown of methionine or cysteine in the presence of ultraviolet light at around 370 nm to methional and methanethiol, with subsequent dimerization to dimethyl disulfide (Jeandet, 2006). These components are responsible for a fault known as *goût de lumière* ('light struck' taint). Using dark green glass (which does not transmit light at wavelengths below 450 nm) should help with preventing this problem (Ribéreau-Gayon *et al.,* 2000a), but even limited exposure to light can result in the production of unwanted aroma, so it is important to store bottles in the dark.

Cork taint (2,4,6-trichloroanisole) is less usual in champagne than it is in still wines due to high positive pressure within the bottle. The CIVC tests for the fault regularly, and figures for affected bottles are put at between 0.5 and 2% (Jeandet, 2006), which is still very high, and involves considerable financial loss for producers. Presumably in response to these unacceptably high figures for cork taint, in 2009 entrepreneurial producers capped their bottles with aluminium tops for the first time in Champagne's 350 year history (Wallop, 2009). Until recently, this was not thought possible due to the carbon dioxide pressures involved. Duval-Leroy, a Champagne house, which produced more than 6 million of the 320 million bottles made in 2008, capped a limited number of bottles of Duval-Leroy's Clos des Bouveries range with aluminium to test market reaction. As screw caps on still white wine bottles have only recently been embraced by consumers, it is foreseen that the new technology will take decades to penetrate markets worldwide, and producers will have to live with losses due to taint for a while yet.

In conclusion, the process of champagne production is complex, labor intensive and prolonged, and the resulting product is usually expensive. Amerine and Singleton (1977) pointed out that up to 120 hand operations may go into the production of one bottle of champagne, and the production process involves a high measure of skill, risk and higher taxation on the wine, justifying its sometimes hefty price tag.

Other Methods of Sparkling Wine Production

The sparkling wine market is growing rapidly worldwide, particularly among young adult consumers. An estimated 2150 million bottles were produced in 2008, of which around 25% were produced in France (Theron, 2009a).

Sparkling wines, by definition, are wines that contain a visible excess of carbon dioxide on pouring, and they are usually classified on the basis of production or the source of the gas within them. There are four basic methods of preparation. The first is carbonation of the wine by injection of 'artificial' carbon dioxide (CO_2) under pressure. The other methods of sparkling wine production (bottle fermentation, tank fermentation and the transfer method) rely on 'trapped' carbon dioxide generated through fermentation of sugar by yeast. According to Theron (2009a), yet another method, the continuous process, has been developed in Russia, and is now used in Germany and Portugal.

Despite their differences, all the methods of sparkling wine production require a base wine of relatively good quality, as gas release in the final product will exaggerate any faults. In most cases of sparkling wine production, the alcohol content of the base wine should be between 10% and 12% (from an original sugar concentration in the grapes of between 18 and 19.5 °Brix), a limited amount of SO_2 should have been used during production, the pH and polyphenol content should be fairly low and flavor should be neutral, unless the style specifies a particular character. Theron notes that an important point in any sparkling wine production, over and above the method of production of carbon dioxide within the wine, is that the grapes should be selected or harvested with the style of wine in mind.

Carbon Dioxide Impregnation (Carbonation)

The simplest and cheapest method of adding bubbles is carbonation. Carbonated wines are generally recognized as being of a different quality to bottle-fermented sparkling wines and are usually priced more cheaply. The base wines will have fresh fruity styles with moderate alcohol concentrations, and there will be no increase in the alcohol content from a secondary fermentation as in the bottle-fermented products. The choice of base wine is important as it directly affects the character and quality of the final product. Impregnated sparkling wines may be dry, but are often semi-sweet or fully sweet, in which case the fermentation will be terminated prematurely by yeast removal or cooling in order to retain sugar. Base wines are often made from cultivars that have higher levels of terpenes, such as the muscats, which suit the sweeter styles of wine. The

yeasty, toasty character due to autolysis in the bottle is usually absent, unless it was present in the original base wine. Nowadays, a number of producers are avoiding malolactic fermentation as it changes the character of the wine, and destroys fruit flavor and varietal character. Instead, they will dose their wine with carbon dioxide after sulfuring.

There are a number of different ways of impregnating wine with gas. A suitable still wine is exposed to high pressure carbon dioxide either in a tank or in line at very low temperature (−4 °C) to enhance dissolution of the gas within the solution. When the temperature of the wine is allowed to increase to ambient, the gas pressure in the bottle will increase to the required level, and bubbles will result when the bottle is opened. With tank carbonation the wine is stirred under pressure in a CO_2 atmosphere, and there is little control over the quantity of carbon dioxide, which dissolves in solution. In line carbonation is more precise. The wine is passed through a column in a thin stream, and the gas flows in the opposite direction through it. The temperature, flow rate of the liquid and gas pressure can be controlled to give a known amount of carbon dioxide in the wine.

A characteristic feature of carbonated wines is that the gas bubbles are inclined to be larger than those of the other types of sparkling wines and that they lose CO_2 more rapidly once the bottle has been opened. The retention of gas bubbles is influenced by various factors, including a lack of particles in the wine, which can act as a core for bubble formation, and protein content. As carbonated wines are usually filtered before gas impregnation, and seldom experience any time on the lees, it seems self-evident that bubble formation and retention would be affected. Although no scientific evidence correlates the quality of a sparkling wine with bubble size, people nevertheless believe that the two are inversely proportional, i.e. the smaller the bubble, the higher the quality of sparkling. It is usually given that the transfer and Charmat wines have larger bubbles that do not last as long as those of Méthode Traditionelle. In work carried out by Liger-Belair *et al.*, (2003), the experimental diffusion coefficient of CO_2 molecules in five carbonated beverages (a high quality champagne well known for the fineness of its bubbles, a low quality sparkling wine, a beer, a soda and a fizzy water) were accurately determined by using the nuclear magnetic resonance (NMR) technique. The author concluded that the differences in the bubble size between the beverages could not be explained by differences in their diffusion coefficients, which were very similiar. The differences were more likely to be due to differing CO_2 concentrations between them, or significant differences in the pool of surface active macromolecules, which adsorb on the surface of a rising bubble, modifying its surface state.

Impregnated wines make up the highest volume of the sparkling wine market, and include a wide range of styles from Vinho Verde, to an ever increasing variety of flavored sparkling wines and fruit flavored coolers. In the Vinho Verde (meaning ‘green wine’), Portugal’s largest wine-producing region, wines are characterized by fruit flavors, good acidity, astringency and low alcohol, as well as a slight pétillance originally derived from the malolactic fermentation, but now added by most producers by means of bulk carbonation. The wines are designed to be drunk young (‘green’). Other aromatic types of sparkling wine include Asti style Muscat based wines, Spumante and Crackling.

As long as the base wine is of a sufficient quality and a pure source of carbon dioxide is used, the product need not be inferior. The wine can be produced at any time of the year on demand as the production process is far quicker and cheaper than the labor intensive bottle-fermented methods. The consumer is assured of an affordable product of consistent quality. Carbonated wines are not designed to age in the bottle after impregnation, and are best drunk fairly soon after purchase.

Tank Fermentation (The Charmat Process)

The origins of the bulk method for making sparkling wine using ‘natural’ carbon dioxide generated by yeast during fermentation are controversial. Some texts state that it was originally developed in the early twentieth century by Frenchman Eugène Charmat, but others note that Charmat copied an earlier method by the Italian,

Frederico Martinotti, who carried out controlled fermentations in pressurized stainless steel 'autoclaves.' There may be substance to the latter claim, as Martinotti used his pressurized tanks in 1895 in order to produce Asti Spumante, a *Denominazione di Origine Controllata e Garantita (DOCG*) sparkling white wine from Piedmont. Present day tank fermentations are modifications of the original method in which the interconnecting tanks retained the pressure created by the production of carbon dioxide during fermentation. Nowadays, around 7–8 atm is maintained throughout the production process.

The fermentation tanks for bulk sparkling production represent a significant investment for a winery, as they are fairly 'high tech' pieces of equipment, able to withstand very high pressures. They are fitted with temperature gauges, pressure release valves and 'jackets' in which to circulate cooling water for temperature control. Capacities are relatively small (less than 100 000 l) due to the need for pressure and temperature control. Since excess gas can escape from the tank, the regulation of sugar in the dosage is not as critical.

Depending on the style of wine being made in the tank fermentation process, the primary fermentation in the base wine may continue to dryness or be terminated to keep residual sugar if the alcohol levels warrant it. The wine will have sugar added to it if necessary, followed by yeast and nutrients. The tirage liqueur will not contain bentonite or other riddling agents as these are not necessary – the yeast lees will be filtered off under pressure rather than removed through riddling. In Asti Spumante production, 'refermentation' is probably more accurate term to use than 'second fermentation,' as the wine goes through one fermentation, which is 'paused' and then restarted. The first phase finishes at around 7% alcohol, whereupon the wine is transferred to a second autoclave in which it referments for about two to four weeks to form 8–9% alcohol. The wine is cooled to –4 °C and transferred to a third autoclave for bottling. In recent times, a derivation of the Charmat method known as *charmat lungo* has been devised for sparkling wines made from Chardonnay and Pinot Noir. This is a longer process which gives a drier product that has contact with the lees or '*fecce*' in the tank for three months to a year to duplicate some of the effects of the classic method. When refermentation is complete, the wine is cold stabilized under pressure to precipitate tartaric acid crystals, which are removed. The wine is then clarified by centrifugation or filtration under pressure, and this is maintained as the wine is bottled and corked. Wines made by the tank fermentation method are usually sold soon after bottling, though some *charmat lungo* wines seem to benefit from a few months of storage.

Tank fermentation enables winemakers to produce inexpensive sparkling wines within three months of harvest, compared to the much longer and more expensive bottle fermentation. This process works well with sweeter styles as it suits wines that rely on varietal rather than ageing character, like those produced from aromatic cultivars (in Italy good quality grapes of the cultivars Moscato, Prosecco, Malvasia and Brachetto are used to produce Spumante). In the sparkling style of Lambrusco production (from northern central Italy), the grapes are crushed, then moved into tanks where they are allowed to ferment with the skins to extract color and aroma compounds. During fermentation, wine is circulated through the marc (or cap of skins) in a process that is quite oxidative. The wine is then pressed under pressure, before the end of fermentation, and the rest of the winemaking process is conducted under pressure. Prosecco, a dry white sparkling wine from the Veneto region of Italy, also produced using the Charmat method, has recently gained enormous popularity globally as one of the less expensive alternatives to Champagne.

In the USA, wines will be labelled 'charmat process,' in France 'cuve close' and in Italy 'metodo charmat' or 'autoclave.' This process is used in Germany for sekt wines, which must stay in the tank on yeast lees for at least nine months to allow yeast autolysis to occur. This autolysis and dissolution of the yeast contents are assisted by the tanks being agitated periodically. Tank fermentation is also used in New Zealand and Australia to make spumante type sparkling wines, and wines of reduced carbon pressure like 'Cold Duck' (Jackson, 2000c).

Although wines produced this way are generally regarded as superior to those made through carbonation, they are not as highly esteemed as those produced by Méthode Traditionelle.

The Transfer Process

The transfer process (which does not have a proprietary name, and is known as *méthode transfert* in France) was developed in the 1940s in Germany as a means of avoiding the low quality associated with the bulk process and the expense of manual riddling. Since the development of automated riddling and yeast encapsulation, some of the advantages of the transfer method have been negated (Jackson, 2000c), and as the equipment required is expensive and there is a large requirement for refrigeration this process is only used by large producers nowadays. The preparation of wine up to the riddling is essentially identical to that prescribed for the traditional method. The bottles are stored neck down in cartons, and after ageing on the lees, the wines are chilled to 0 °C, the bottles are opened by a transfer machine and the wine is poured into pressurized receiving tanks to retain the dissolved CO_2. The wine is sterile filtered and then sweetened and sulfured with the dosage prior to rebottling with a counterpressure filler.

An important point is that although sparkling wine made by the transfer process follows the same procedure as Méthode Traditionelle, the secondary fermentation does not take place in the actual bottle sold to the customer. This can be confusing for consumers, especially as labeling can be deceptive. For example, of wines sold in the USA, the label statement 'Fermented in this bottle' means Méthode Traditionelle, whereas the statement 'Fermented in the bottle' refers to the transfer process.

There are advantages to the process, as the complicated rémuage, riddling and dégorgement steps of the Méthode Traditionelle are done away with. As well as being less expensive, the transfer method is also far less time consuming than the traditional method. The period from harvest to bottling can be as little as three months, and as long as a year, which is still a great deal faster than the conventional alternative. The mixing of the wine allows for a more consistent product, which is a very important consideration for consumers' brand loyalty. Although some would say the transfer process strips flavor and aroma, many Champagne makers commonly use this method to produce any size bottle smaller than 750 ml standard or larger than 1.5 l, as their facilities are not set up to produce these volumes in the traditional manner. The future of the transfer process is questionable, however, as it is still more labor intensive than the Charmat method, and does not really provide a better product.

Continuous Production

Another method for producing sparkling wine, the continuous process, was developed in Russia, but the first wines made by the continuous fermentation of grape juice on a commercial scale were produced in California and in Argentina. Since that time both red and sparkling table wines have been made commercially by continuous fermentation in South America, the USA and Europe in a variety of proprietary fermentors. A considerable number of continuous single stage red wine fermentors are in operation, particularly in Southern Europe. In some incarnations of this process, grape juice is fed through a series of five or more multi (or reticulated) tanks of identical volumes and carbon dioxide pressures and the contents are gradually changed from juice, to base wine and finally to sparkling wine. In others, base wine is added to the first tank and undergoes second fermentation, with autolysis taking place in later tanks, and the wine emerging clear in the last tank, having spent three to four weeks in the system. Early studies by workers like Cahill *et al.* (1980) showed that Montrachet wine yeast (usually used in batch fermentations) would grow under continuous fermentation conditions. The onset of fermentation was delayed by carbon dioxide pressure of 0.6 atm (gauge) and there was a fourfold inhibition of growth rate with the final maximum cell concentration about half of the control, but the yeast were able to maintain steady state growth at each of the carbon dioxide pressures tested, in spite of the inhibitory effects. It was found that if the second fermentation begins when the concentration of alcohol is around 4%, undesirable microbial activity was virtually suppressed, and the retained carbon dioxide could be used to control the rate of growth of the cultured yeast thereafter. The second

and third tanks may also contain materials like wood shavings on which dead yeast cells can accumulate and stay in suspension in the wine, adding a limited autolysis character.

Continuous fermentations have advantages in commercial operations, which include the avoidance of lag periods in the starting of the fermentation, shortening of the time needed for 'shut-down' at the end of the fermentation, and the possibility of automation of the production. The new process is obviously much faster than the traditional one and the sparkling vines obtained are perfectly clear without showing quality faults. A large fermentor could produce about 2000 bottles per day if consumption required it, but higher concentrations of sugar mean the production of a much sweeter wine than would be acceptable to the most sparkling wine consumers.

2.9.4 Fining, Filtration and Clarification

The quality and haze stability (resistance to haze formation) of any commercially produced beverage are very important to consumers and there are many options available to the winemaker to enhance the natural color, flavor and aromas, while also improving the long-term stability of a product. For beer, cider and fruit wine clarification treatments see Sections 2.6.9, 2.8.5/6 and 2.11.3, respectively, and for a general discussion of filtration, see Freeman and McKechnie (2003).

Sources of Instability and Hazes in Wine

The particles left in solution after fermentation fall into three groups in terms of their size. Simple molecules such as alcohols, sugars and acids are less than 2 nm in size. These dissolve in the fermented product, are a necessary part of its chemical composition and remain through all treatments. Unless they are involved in a reaction or interaction, which causes a larger molecule to form a haze or precipitate, they are unlikely to cause problems. Large particles (>1000 nm) such as grape debris in wine, yeasts, bacteria and proteins, may contribute to the cloudiness of the fermented product. If required, these can be removed by sedimentation or filtration.

Certain substances known as colloids, exist neither in solution or suspension, but in a dispersion. These molecules range in size from 2 nm to 1 micron (or micrometer, 1 μm), and although they are considered large, they are not sufficiently large to be visible or to be removed by a filter. Indeed, filtration of wines containing high levels of colloids may lead to filter blockages and breakthrough, for example glucans (polysaccharides produced by yeast and *Botrytis cinerea*) are notorious for their ability to block filters.

Some colloids carry a charge and as long as conditions remain the same, they are kept in suspension by interactions with other charged particles in the wine. Examples of naturally occurring colloids in wine that are negatively charged are tannins, pectins, dextrans and glucans. Positively charged colloids in wine include colored phenolics and proteins. Changes in the redox state of the solution or conditions of temperature or light may lead to the molecules losing their charge, enabling them to clump together (flocculate). Flocculated material is harmless and tasteless, but can form an unattractive haze or deposit in the bottle. Even a product that has been sterile filtered will not remain clear for long if it contains a high level of colloids, as sooner or later these will flocculate, producing a haze.

Spontaneous Clarification: Initial Sedimentation

Cold settling of juice before (*debourbage)*, and after alcoholic fermentation is a batch process and is often used in small and medium sized wineries. Under the influence of gravity alone, turbid juice separates into

two phases: an opalescent juice and a layer of deposits (lees) varying in thickness. Clarification consists of separating (usually by racking) the clear juice or wine from the lees.

The factors affecting settling include the density difference between solids and juice, particle size, the attachment of bubbles to solids, temperature differentials in the holding tank, juice viscosity and colloid content. Tall, narrow tanks minimize the natural convection currents and provide better settling conditions, but a larger lees layer. Containers that are poor conducters of heat, like barrels, are also suited to sedimentation. Settling takes time, and if a wild fermentation starts during the settling period, it is often it is impossible to clarify the fermenting juice, which may lead to poor quality of final product.

In order to prevent spontaneous fermentation during the initial settling period, the juice should have an effective level of free SO_2, low pH and low temperature. Settling times can be up to 10 days (Morgan *et al.*, 2006), and both the yield of clear juice and degree of clarity depend on the settling conditions. If conditions are not ideal or the fruit was not healthy, the settling time should be reduced, the minimum settling period being around 12 h (overnight), and bentonite may assist in reducing oxidative enzymes (Jackson, 2000e). Settling overnight, below 15 °C, should reduce suspended solids to around 1 to 2%; clarification with pectolytic enzymes, followed by centrifugation or earth filtering will reduce solids to 0.1–0.5% (Morgan *et al*., 2006). The degree of juice clarity may be measured with a nephelometer measuring in NTU (Nephelometric Turbidity Units). Specific clarity objectives are advocated by some winemakers in order to reduce or eliminate the potential for alliaceous mercaptans and thiols associated with poor clarity (Ribereau-Gayon, *et al.,* 2000b). However, overclarification may cause yeast fermentation problems due to lack of fatty acid nutrients, giving rise to volatile acidity and hydrogen sulfide production. A turbidity range between 100 and 250 NTUs, which corresponds to 0.3–0.5% particles, is usually recommended for juice before alcoholic fermentation (Ribereau-Gayon *et al.,* 2000b).

The clear juice may be racked off the top of the solids, with a racking plate or through a racking valve, or lees may be run off from the bottom of the tank until the juice is clear. In all cases it helps to have a sight glass in the line. If necessary, juice may be recovered from the lees layer through filtration, or by centrifugation.

Fermented products may also be turbid after fermentation and should be clarified before bottling. The initial postfermentation clarification is usually carried out by gravity settling, and the gross lees layer (mostly dead yeast cells) should settle fairly quickly. Some winemakers claim that any other form of clarification, such as centrifugation and filtration, is detrimental to the quality of wine, particularly red wines aged in barrels, which are often clarified through sedimentation only. The big disadvantage of sedimentation is that it is a slow process, does not guarantee absolute clarity, and the lees produced are considerable. Once settling has occurred, the clear wine is racked into another container, leaving the lees behind. Racking can also be used to homogenize the solution, aerate it and remove carbon dioxide. Racking off the gross lees is normally carried out straight after the alcoholic fermentation unless yeast autolysis characters are a desired part of the bouquet. Further racking can be done three weeks later, and then every two months for wine in tanks, and every three months for wines in barrels (Morgan *et al.,* 2006), until the wine is either bottled or stored without racking. Traditionally, racking was carried out before the spring, to remove tartrate and protein deposits which had precipitated during the cold winter months. Racking is usually accompanied by a free sulfur dioxide level assessment and adjustment, and may be preceded by a stability test to see if the wine will stand aeration.

Centrifugation

Centrifuges work by speeding up the sedimentation of particles. An apparatus consisting essentially of a compartment spun about a central axis to separate contained materials of different specific gravities, or to separate colloidal particles suspended in a liquid. Gravitational acceleration is replaced by the centrifugal force generated by the very high speed spin of the centrifuge (often up to 10 000 rpm). Most centrifuges are now self-decanting (automatic extraction of lees), and those used for sparkling wines are

completely hermetically sealed. Their uses include rapid clarification of musts, removal of yeasts during fermentation to slow down the alcoholic fermentation, postfermentation clarification, and immediate clarification of fined wines. Centrifuging is particularly useful if juice is heavily laden with particulate matter (Jackson, 2000f).

Foster and Cox (1984) found the decanting centrifuge to be a valuable tool in white winemaking. According to the authors, it decreased the volume of fermentation lees, and lowered processing costs and losses due to lees filtration. It gave better fermentation control by eliminating the premature onset of fermentation due to wild yeast. It decreased the need for fining agents such as PVPP and Cufex, lowering labor and supply costs. The authors also stated that the method increased the overall quality of the white wines, through lowering the flavonoid phenol and iron contents, thus decreasing browning capacity.

Centrifuges are excellent for removing dense particles, such as bits of grape skin, but will only remove a proportion of them; they never lead to their complete elimination. They are also expensive and very noisy, but they are often used as they have a very high flow rate, and can handle quite high solid levels. If wine is not blanketed with inert gas in the centrifuge chamber, oxygen may be forced into the wine during the process, and lead to oxidative problems. The use of a centrifuge usually requires that a specific area is set aside in the winery, and trained personnel carry out the operation. Despite these disadvantages, automation combined with continuous centrifugation has improved efficiency and economy of the process to such an extent that in larger concerns, this is often a preferred clarification technique (Jackson, 2000f). Jackson, (2000e, g) also notes that by removing only suspended particles, centrifugation affects the chemical composition of wine very little, and this, together with the minimal juice loss, and speed of operation, have made it a fairly popular technique. The juices of Chardonnay and Colombard may show nitrogen limitation leading to a retarded fermentation if juice is overcentrifuged.

Fining

Fining is an ancient process of clarification that is used to prevent hazes and improve the balance of the wine (Bird, 2005). Fining can involve complex interactions, but the principle is simple. An introduced agent reacts with the specific wine component either chemically or physically, to form a new component (usually larger or heavier) that can be separated from the wine by precipitation and racking, or filtration (Margalit, 2004).

As previously stated, the colloids in wine usually remain in solution due to their electrostatic charge, but under certain conditions and over time, flocculation will occur due to changes in charge state, or through interaction with oppositely charged molecules. This will lead to haze formation, followed by precipitation. If this happens during bulk storage, the wines can be racked and filtered, but if it happens in the bottle, it is a problem. Winemakers will therefore add fining agents in order to 'force' reactions that would naturally happen over time. An example of a naturally occurring interaction in red wine is that between tannins and proteins (Figure 2.9.25), which has the beneficial effect of removing excessive tannins from solution and precipitating unstable proteins, resulting in a better balanced, softer and more stable wine.

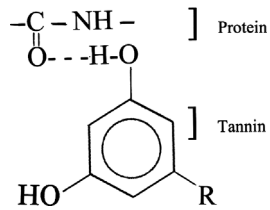


Figure 2.9.25 *General scheme for interaction between proteins and phenols*

Table 2.9.2 *Dosage and efficacy of fining agents (Morgan* et al.*, 2006)*

Wine	Agent	Dose rate (mg/l)	Properties	Clarity
White	Bentonite	250–500	Reduces protein, and associated copper complexes	++
	Gelatine	15–150	Reduces bitterness, astringency and off tastes	+++
	Casein	50–500	Reduces colour and oxidation	++
	PVPP	200–600	Reduces bitterness and browning precursors	+
	Milk	2–4 ml/l	Deodorizing, removes color	++
	Carbon	max. 1 g/l	Reduces color and off odours	+
	Silica sol	30–300	Flocculates proteins	+
Red	Gelatine	30–300	Reduces astringency, off tastes and color	+++
	Isinglass	10–100	Leaves wine brilliantly clear	++++
	Egg albumin	60–100	Reduces astringency	++

Bentonite, gum arabic and liquid silicon dioxide (also known as *kieselsol*) are negatively charged at wine pH. Proteinaceous fining agents are invariably obtained from natural sources, and at wine pH, carry a positive charge. When a fining agent is added to a wine it usually adheres to oppositely charged colloidal matter in solution and the colloidal groups agglomerate, flocculate and drop to the bottom of the vat as sediment. The flocculation of finings is faster at warm temperatures, but sedimentation is better at low temperatures.

To speed up naturally occurring processes and improve stability in wine, negatively charged fining agents can be added to remove unstable proteins or, alternatively, positively charged protein based fining agents can be added to remove tannins.

Fining agents are often used as blends, and the correct fining to use depends on the wine's chemical composition, and the nature of particles in suspension. Usually, comparative trials using small volumes of wine will be carried out in order to establish which fining agent, at which dosage, gives the best clarity for a particular wine. The selected fining should be the lowest dose to give the best quality of wine aroma and flavor, clarity, rapid sedimentation and result in the least lees volume. Table 2.9.2 gives some of the important agents used in fining in winemaking. Overfining is when some of the fining agent remains in solution. This may flocculate later with temperature changes, time or with tannins from barrels or corks, and so should be avoided. Overfining with protein fining agents is unlikely to occur in red wines due to their high tannin content, but can happen in whites, especially with gelatine.

Proteinaceous Fining Agents

Gelatin, a widely used fining agent, is essentially denatured collagen; it is made from collagen, the major structural protein in skin, tendon and bone, and has been used as a fining agent for the selective removal of phenols from wines for many years (Yokotsuka and Singleton, 1995). It is a polypeptide consisting of a number of different amino acids, with the main ones being glycine, proline and hydroxyproline. The concentration of these three amino acids, which can be as high as 56%, makes gelatine a good protein fining agent because their presence favors a flexible noncrystalline peptide chain with a high availability of hydrogen bond accepting carbonyl groups (Yokotsuka and Singleton, 1987). The industrial production of gelatine dates from the early eighteenth century and several different types are now available, produced by acid, alkaline and enzymic hydrolysis (Ribéreau-Gayon *et al.*, 2000c). Gelatine is usually added to wines to reduce astringency and bitterness, but also off flavors resulting from poor quality fruit. Fining with this protein eliminates tannin molecules that react most readily and are most astringent, and also removes molecules that contribute to the

impression of body and volume on the palate (Ribéreau-Gayon *et al.*, 2000c). To ensure the fining of the wine is not detrimental to the overall quality, fining trials must always be carried out prior to additions made in the winery.

Gelatine is available in various forms for its use with wine. It can be purchased in sheet, powder or liquid form and the choice is purely dependent on the preference of the producer, however powders and sheets may have the disadvantage that dissolution requires both heat and time and solutions can form gels on cooling, (Cole, 1986). Gelatine viscosities are grouped according to their bloom strength, i.e. the resistance to deformation of the gel made under specific conditions), which range in wine gelatines from 80–150 (Margalit, 2004). There are already various grades of gelatine available on the market for the use of fining wines from whites to heavily structured reds and with the ever improving technology and understanding of tannin–protein interactions the choice can only increase, with the specificity of the tannins removed a great possibility. As gelatine is extracted from animal bones or hides by boiling, there are ethical issues concerning its use, and winemakers should bear this in mind when labeling. The preparation should be odorless and colorless and is often sold as a powder, which requires dissolution in warm water (30 °C) into a 8–10% solution before use. In white wines, gelatine should be used in conjunction with enotannin (derived from grape pips and skins) or Kieselsol at a ration between 1:5 and 1:10 (Ribereau-Gayon *et al.*, 2000c). Thorough and immediate mixing is very important. In red wines fining with gelatine will also increase suppleness, but can also strip out some of the colored phenolics. There is no legal limit for gelatine addition to wine (Margalit, 2004), but usual additions range from 1.5–15 g/hl in white wines and 3–10 g/hl in red wines (Ribéreau-Gayon *et al.*, 2000c). Maury and coworkers (2001) found that protein molecular weight (MW) may be of importance in a gelatine fining treatment, as it correlates with the nature of precipitated tannins. Their experiments used a range of different gelatines to fine four different wines, and it was found that the protein having the lower molecular weight was more selective, probably due to its small size, which makes it more flexible and therefore more accessible to the tannins (Maury *et al.,* 2001) Sarni-Manchado and coworkers (1999), however, concluded that the similar behavior of different grades of gelatine was because although tannins differ greatly in their affinity for proteins, and proteins likewise differ greatly in their affinity for tannins, the difference encountered is more due to the composition of proteins then their size (Sarni-Manchado *et al.*, 1999). This is supported by Yokotsuka and Singleton (1995), who found that affinity for phenols is considerably influenced by the amino acid composition and structure of peptides rather then their molecular weights.

Gelatine is very subject to overfining in white wines as it is flocculated by tannins. There is no direct relationship between the levels of fining and the levels of tannin required to flocculate it – it depends on the pH and temperature. A sample of the wine should be tested for protein stability after fining either by heating (70 °C for 30 mins), or by adding 0.5 g/l tannin and looking for cloudiness after 24 h.

Isinglass

This is a proteinaceous fining agent originally derived from protein extracted from the swim bladder of sturgeon. More recently, it is manufactured from fish cannery waste (Ribéreau-Gayon *et al.*, 2000c). Previously, the preparation of isinglass was a long, arduous task, but nowadays it is prepared as fine chips, which are mixed with water as a 1% solution without heating. Isinglass flocculates on contact with wine due to the wine's tannin content, and falls to the bottom of the vessel, removing particles by screening and electronic attraction.

It is commonly used at doses of 1–2.5 g/hl for white wines, just before bottling. It gives a better brilliance than gelatine and is less detrimental to wine color and flavor, and is less prone to overfining, although it may require a little aeration to flocculate, and too much may result in a fishy nose and palate. Its disadvantages are that it produces small amounts of light, fluffy lees, which clog filters, is comparatively expensive and can

be difficult to prepare. There are also environmental and ethical issues concerning the use of products from endangered sources, and a number of winemakers prefer to use alternatives like alginates. Isinglass is widely used in the fining of cask-conditioned and other beers (Section 2.6.9).

Albumen

Albumen is a very old fining agent, and has always been presented as the only fining agent for really great red wines (Ribéreau-Gayon *et al.*, 2000c) It is produced from the white of chicken's eggs, and can be fresh, frozen or powdered. Dosing is between 100 and 150 mg/l if using powdered egg white, and this should first be mixed with water. If using raw egg, between three and eight fresh egg whites per barrel (225 l) are used depending on the astringency of the wine. In many New World wines, due to shorter maceration periods, as little as one egg white per barrel will suffice. Egg whites must first be diluted up to 250 ml with water, and a pinch of salt is often added to help flocculation (Morgan *et al*., 2006). Egg albumen is not suitable for white wines, as high levels of tannins are required for flocculation (Ribéreau-Gayon *et al.*, 2000c). It is the best fining for red wines as, although it clarifies the wine poorly, it removes the colloidal phenolics, and renders the wine supple without removing too much color or flavor.

Casein

This is the principal protein of milk. It is a good fining agent for white wine and removes excess color, oxidized character and reduces the iron content of the wine (Ribéreau-Gayon *et al.*, 2000c). It does not overfine, as its flocculation is mostly due to wine acidity. It is often used for the fining of Sherry wines (Section 2.10.2). It is most effective at 15–20 °C, and must be mixed thoroughly and rapidly, as it coagulates as soon as it mixes with the wine. Fining with whole milk is not permitted in the EU (Ribéreau-Gayon *et al.*, 2000c), but skimmed milk can help with color removal and deodorization. The recommended dose is 2–4 ml/l. The disadvantage with milk is that it can lead to overfining at very high doses, and provides a wide range of nutrients for bacterial growth.

Powdered blood

Fresh blood, generally from cattle, was used as a fining agent for many years, but has recently been banned on health grounds. The alternative is powdered blood, which is made by treating fresh blood with sodium citrate and centrifuging. The precipitate is dried, and mixed with carbon to remove its taste, and help it flocculate. It is either brown or yellow, and must be mixed into a paste with water before addition to the wine. This is a very high quality fining for white wines, as it is good at clarification, reduces astringency greatly, reduces herbaceousness, and softens bitter, stalky young wines (Ribéreau-Gayon *et al.*, 2000c). It does not strip color, and there is no risk of overfining at normal doses. It is expensive, and ethical considerations should be taken into account when using it, in particular it may be required by wine legislation that the presence of animal proteins be declared on the label.

Nonproteinaceous Fining Agents

Ribéreau-Gayon and coworkers (2000c) note that sodium alginate is a polymer of mannuronic acid extracted from phyophaceae algae, particularly kelp, by alkaline digestion. It is an odorless, tasteless preparation which, when mixed with water and added to wine at doses of between 4 and 8 g/hl, can help to clarify wine, though not as effectively as traditional fining agents.

Carbon (charcoal)

Charcoal is derived from burnt animal or plant matter and carbon fining is often used as a last resort to remove off odors and color. It is best used in conjunction with 50 mg/l ascorbic acid, as otherwise it may provoke oxidation. Often used at low doses for final pressings.

Tannins

Tannins are extracted from various sources for fining purposes, but a typical source is the insect galls on oak leaves (Jackson, 2000f). They are usually combined with gelatine, and form a network that helps to sweep excess grape tannins and proteins out of the wine.

PVPP

Polyvinyl polypyrrolidone is a resinous polymer that acts in a similar way to proteins to bind tannin (Jackson, 2000f). It removes lower molecular weight phenolics (particularly flavans, and mono and dimeric phenolics), and so is often used to reduce bitterness in wines, particularly white wines which have undergone skin contact. It has little effect on astringency, but can help to reduce pinking and oxidative browning caused by smaller phenolics in white wine. Unfortunately, it is also good at removing resveratrol, one of the components frequently associated with the 'French Paradox,' and health benefits in red wine (see Chapter 5.1, Sections 5.6.3 and 5.8.6). It functions well in wine conditions, and precipitates quickly.

Silica sol (Kieselsol)

This is an aqueous, colloidal suspension of silicon dioxide, used to help flocculate fining agents such as gelatine, or remove mucilaginous protective colloids (Jackson, 2000f). Because it is available in both positively and negatively charged forms, it can remove colloidal material in both charge states, but is most commonly used to remove bitter polyphenols from white wine. It does not remove much color, and produces less sediment than bentonite.

Bentonite

Bentonite is a montmorillonite clay widely employed as a fining agent. It exists in a negatively charged state at wine pH, and so removes positively charged colloids such as proteins, including enzymes such as oxidases, vitamins and amino acids, thus increasing microbial and heat stability. It also increases copper stability, as it removes proteins that may be involved in copper haze production (Jackson, 2000f). However, it tends to strip and turn red wine color brown (especially in young red wines), and so is usually only used in whites and rosés. In red wines the tannins present react with the protein, and precipitate out any excess, giving the wine stability. There are two types of bentonite, calcium and sodium bentonite, of which the latter is more commonly used in the United States (Jackson, 2000f). Bentonite usually needs swelling in approximately 10 times it own weight of water for 24 h before addition.

Fining Trials

Fining trials should always be carried out before fining wine. Fining agents are generally nonspecific and remove a range of compounds. It is important to assess the results of fining on the appearance, smell and

taste of the wine. The most suitable fining agent to try in a given situation is often chosen using experience gained of the style of wine and the fining agents available. It is also important to keep detailed records of all fining trials carried out even if the wine is not fined. The least amount of the fining agent that gives the desired result should be used to fine the wine. This is facilitated by the use of graduated levels of fining agent mixed with the wine, plus, importantly, a control sample with no added fining agent. By observing the level of deposit, degree of clarity and organoleptic effects in a quantitatively graduated series of test tubes or clear glass bottles, it can be ascertained when an increased level of fining agent has no further effect on the wine. Once the required level is determined, the actual fining procedure can be carried out. It is vital that fining agents are mixed thoroughly in the wine as soon as they are added. They are then left to settle before being racked and, if required, filtered.

Filtration

This involves the separation of liquid (wine) from any sedimented deposits or suspended particles through screening and/or adsorption mechanisms. The size and nature of the particles to be removed will determine which filtration system and grade is required. The two major mechanisms involved in filtration are outlined below, and illustrated in Figure 2.9.26.

Absorption (depth filtration)

This refers to the type of filtration where particles adhere to the filtering medium because of their opposing electrical charge. For instance, cellulose fibers have an overall positive charge, and so attract yeasts, which have an overall negative charge. In this type of filtration, the pore size is not as important as the bulk, type and surface area of the fibers. The flow rate remains constant (though it may drop due to screening caused by a bridging effect of absorbed particles), but the wine comes out progressively cloudier until saturation point when the filter no longer has any effect. This is also called 'depth' filtration. (see Section 2.6.9 for further discussion, with respect to beer).

Screening (surface filtration)

This type of filtration involves retention of particles by pores in the filter material (usually a membrane), which are smaller in diameter than the particles. The flow rate of a filter that uses screening exclusively decreases in proportion with the volume of liquid passing through. Only screening filters can be rated absolutely according to pore size (10 μm, 20 μm, etc.) – adsorption filters will only remove a proportion of particles larger than their rating. Depth filters are much more sensitive to 'break through' due to increased pressure, pressure surges or pulses, than screening filters. On the other hand, depth filters have a much higher dirt holding capacity than membrane filters (Morgan *et al.,* 2006), so as a general rule, screening filtration is carried out either on wines that are clear to the naked eye or have been treated already with adsorption filtration. Figure 2.9.26 outlines both the adsorption and screening mechanisms of filtration.

Filtration may be used at a 'coarse' or 'rough' level to remove yeast lees or fining sediment and at its 'finest' or 'tightest' level to remove bacteria. The former may be required throughout various stages of the wine making process using, for example, a coarse pad or a rotary vacuum drum filter. The latter may be required at the ultimate stage, bottling, to polish and 'sterile' bottle the wine, for example with a much finer pad or membrane cartridge system (Morgan *et al.,* 2006).

The efficacy of a filter is determined by the size of the pores, or the permeability of the filtering medium. The flow rate across a filter is proportional to the surface area, pressure and permeability of the filter, and

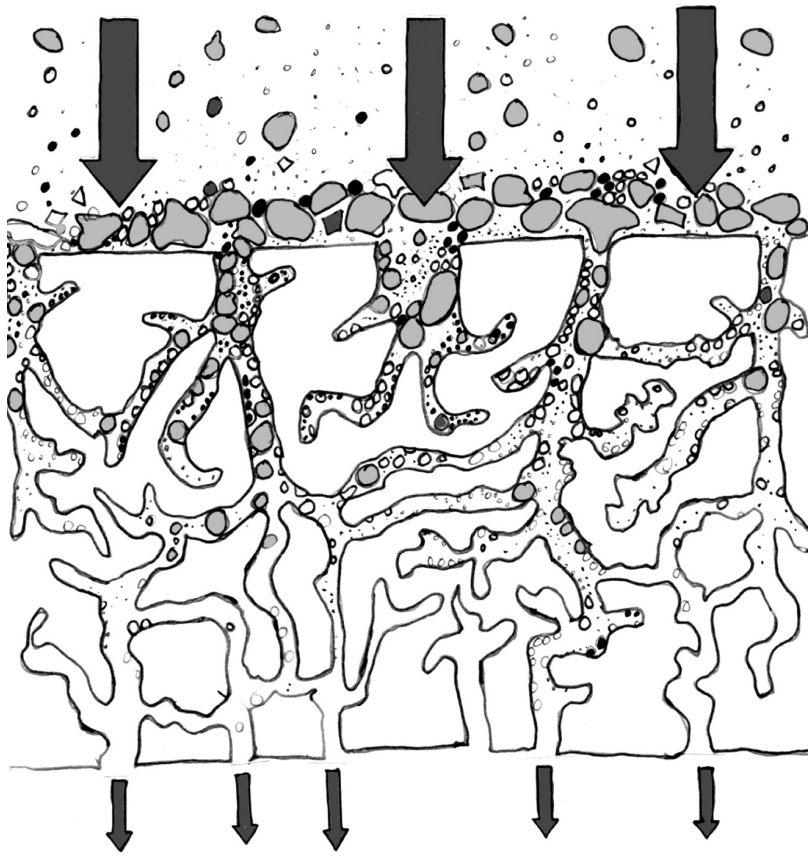


Figure 2.9.26 *Mechanisms of filtration showing screening (larger particles) and adsorption onto pore walls (smaller particles)*

inversely proportional to the viscosity and the thickness of the filtering medium. A filter is also defined by its efficiency, that is to say the volume of wine that can be filtered before the filtering surface becomes blocked. The ability of a wine to clog a filter depends not only on the percentage of solids that it contains, but on the nature of the particles, in particular, 'plastic' molecules such as gums and mucilages. Other particles that block filters easily are colloidal proteins, bentonite and isinglass. Bacteria clog more than yeasts, as they tend to agglomerate in groups, and so wines having undergone malolactic fermentation are more difficult to filter.

Filtering surfaces are defined according to their rating: nominally rated surfaces are rated according to their average pore size; absolutely rated surfaces are rated according to their maximum pore size.

Pad filtration

The wine is passed across a filter pad made of cellulose, cotton, diatomaceous earth (DE) or synthetic fibers such as polyethylene. This method uses adsorption and screening and also sedimentation, inertia and turbulence.

Figure 2.9.27 *Earth filtration system. Commonly used to filter cider and beer as well as wine.* Photograph by courtesy of Vigo Ltd., UK

Plate and frame filtration

This consists of a series of specially designed plates held in a frame. The sheets are fibrous material, which are packed between the plates and screwed tightly together. This type of filtration is known as depth filtration as the wine passes through a large depth of fibrous material. Different grades of filters are available, up to sterilizing grade. These filters are versatile and widely used. They can be expensive to install, but cheap to run. The operator needs expertise in operation of the filter as exceeding the limits of pressure and flow rate can cause breakthrough of the pads. An example of a depth (earth) filtration system for use in beer, wine and cider applications can be seen in Figure 2.9.27.

Diatomaceous earth filtration

Diatomaceous earth (DE, kieselguhr) is a filtration aid made from a sedimentary rock composed of the silicate skeletons of diatoms (microscopic algae) that lived 60–100 million years ago. The most important deposits are in Santa Barbara, California, and fine and coarser grades can be specified. Diatomaceous earth filtration is used extensively in winemaking for initial rough filtrations as it removes large quantities of mucilaginous material very effectively. Earth filtration is mostly by screening, though some adsorption takes place. Earth filters either have supporting plates or a rotating drum through which the wine is sucked by a vacuum pump. Normally, earth filtration takes place in two stages: firstly a layer of coarse grade earth (precoat) is deposited on a supporting screen made of nylon, stainless steel, ceramic or cardboard; then powdered, purified

diatomaceous earth is made into a thin slurry with water and fed continuously into the wine stream, where it builds up in a layer on the filter plate or pad, continuously providing a new filtration surface until the distance between plates is filled (Morgan *et al.*, 2006). With a rotary drum vacuum filter (RDV) the diatomaceous earth bed is laid down on the drum before filtration starts. The drum rotates through the wine, which dries on to the surface as a result of the internal vacuum and the dirty layer is automatically removed as the drum rotates.

Perlite

This filtration aid consists of the pulverized remains of heat-treated volcanic glass (vitreous rock, like pumice), and has a very fine structure (Jackson, 2000f). It is used using the same support and equipment as diatomaceous earth for musts and cloudy wines, but is coarser and more abrasive to pumps and metal pipes. It can also be incorporated into pads and sheets for use in different *filter housings.*

Cellulose filtration

Cellulose is the basic component of all cell walls. This filtration aid is prepared from pine, birch or beech wood which is powdered, chemically purified, washed and dried, and then mechanically broken down to different grades. It can be used as a powder during depth filtration or in pads to be incorporated into appropriate filtration housings. The cellulose used in filtration is relatively pure, but it will give a papery taste to the wine and is usually rinsed through with 1% citric acid first.

Membrane filtration

This most modern type of filtration uses a cartridge made of cellulose esters, polysulfonate, nylon, polypropylene or glass fiber inside a filtration unit. Although there are several versions of this technique, it works mostly by screening, with a little depth filtration. This method is used more for microbial stabilization than clarification, and is commonly the last filtration before bottling wine. A membrane filter consists of a thin membrane of perforated material with holes of a controlled size mounted in a housing. This is known as absolute or surface filtration and particles of more than a certain size are removed. The normal grade used in wineries is 0.45 μm, which removes most bacteria, but the pore size can go down to 0.2 μm. The equipment is relatively cheap, but cartridges are expensive and easily blocked. The wine must be clarified (preferably given an initial filtration) before reaching the membrane as it is the final guard filter immediately prior to bottling.

Cross flow filtration is an adaptation of membrane filtration, where the flow of liquid is across the face of the membrane rather than through it. The flow of liquid constantly removes deposits from the face of the membrane and the wine flows in a closed circuit.

The wine is passed along the surface of a porous membrane with sufficient pressure to force liquid through the pores. This parallel feed prevents clogging, but as the wine is being pumped around a partially closed circuit, the particle concentration increases. This method can be used for musts or wines with very high particle concentrations, and has also been developed for ultrafiltration (dealcoholization), and reverse osmosis (concentration) of wines and other alcoholic beverages (Section 2.13.3).

Ultrafiltration is a cross flow system using a membrane with a nominal molecular weight cut off (NMWC) of 10 000 Da. This will remove large phenolic polymers and proteins, thus increasing the stability of the wine, but unfortunately may also affect structure and color. It is therefore seldom used in red winemaking (Jackson, 2000f).

Osmotic distillation is used for the production of high quality grape juice concentrates. Juice is passed on one side of a polytetrafluoroethylene (PTFE or Teflon) membrane with high strength sodium chloride

solution on the other side. The membrane is gas permeable and so vapor can pass through. The differences in osmotic and hydraulic pressures cause water vapor to pass from the juice through the membrane and into the brine. The salt solution is being continually reconcentrated by a desalinator. The method is characterized by low operating temperature and pressure, long membrane life and no browning or oxidation of the product (Morgan *et al.*, 2006)

Reverse osmosis (RO) is used for dealcoholising wine (Section 2.13.3). Wine is passed along a membrane (cross flow) with very small pores, which will only allow small uncharged molecules such as water, methanol, ethanol, acetic acid, ethyl acetate and acetaldehyde. Any dissolved ions or molecules with a relative molecular mass of more than 200 cannot pass through. This pressure must be high enough to counteract the force of osmosis, which acts in the opposite direction. Dry wine has an osmotic pressure of 14 bar, and so RO systems can pump wines at up to 40 bar (Morgan *et al*., 2006). As the wine concentrates, its osmotic pressure increases, and so it is diluted with water so that as much alcohol as necessary can be extracted. This water should come from the original wine (extracted from the filtrate by distillation) for the wine to retain its designation of origin, grape variety and vintage. Total inert gas blanketing and low temperatures are used so the wine retains flavors. Systems used are quiet and fully automated.

Flotation

Flotation is used for clarifying musts, including some cider must (Section 2.8.5). Gas and fining agents such as gelatine, bentonite and silica sol are injected into the medium under pressure, gas is dissolved into the must under pressure, and then the pressure is released. The gas comes out of solution and adheres to the solid particles, thus making them rise to the top of the vessel. These solid particles can then be removed physically (e.g. by a vacuum) or decanted from the juice and passed through a press filter or RDV.

Practical Implications for Clarification

Fining before filtering improves the efficiency of a filtration, but some fining agents can cause filtration problems. Fining clarified wines often gives better results, and so fining is often carried out after an initial 'rough' filtering. The advantages of fining over filtering are that it requires little equipment, it improves wine stability; and it can have desirable quality effects (e.g. reducing bitterness) (Morgan *et al.*, 2006). However, filtration is more efficient in clarifying, can remove microorganisms, has few side effects, and if the right equipment is available, is quick and easy to carry out. In practice, however, the processes of fining and filtering are complementary and invariably carried out in succession (usually fining followed by filtering). The winemaker must have a clear overview of the processes he or she plans to consider for each wine. For example, it is obviously wise to protein 'fine' before bentonite additions, since these would require a second bentonite addition if subsequent tests showed up any 'overfining.'

2.9.5 Wine Maturation and Ageing

This section is divided into four parts: an introduction, a discussion of the factors affecting wine maturation and ageing, a discussion of the effects of ageing on wine, and a brief description of the process of accelerated ageing.

Introduction: The Need for Maturation

Maturation and ageing are two different concepts when applied to wine, although they are frequently used interchangeably. Maturation in winemaking terms is the time period, and associated changes, that occur in

a wine between alcoholic fermentation and bottling, while the wine is still in bulk storage in the production facility. This period may be anything from two to 24 months, or longer, depending on the style of wine being made, and may include processes such as malolactic fermentation, oak coopering, racking or sur lie (on lees) ageing in tanks or barrels, fining and filtration.

Chemical processes during maturation include the oxidation of phenolics and other substances, formation of aldehydes and esters and hydrolysis of glycosides and other components. Physical effects include salt precipitation, loss of carbon dioxide, evaporation of volatile substance and dissolution of oak components. Effects may include loss of brightness, changes to the color of the wine and character of the bouquet, and rounding and softening of tannins.

The effects of microorganisms during the maturation period may lead to the metabolism of malic and other acids and concomitant increases in by-products of these fermentations such as acetic acid and diacetyl. Yeast autolysis during the maturation period will release cell contents into the medium and yeast cell walls (hulls) can be involved in binding and removing substances (for example, α-ionone) from solution. Other physicochemical effects of maturation are linked to changes in the redox (oxidation–reduction) potential of the wine, and polymerization and flocculation of colloids during or after fining.

The term 'ageing' when related to wine, is often used negatively to denote a product that has been too long in the bottle, and is past its best. Strictly speaking, the period after bottling and before consumption in the life of a wine should be referred to as 'bottle ageing,' but for the purposes of this discussion, it shall just be termed 'ageing.' This is when the maturation of the wine continues with no, or very little, oxygen present, and includes the period when the wine is at its peak, and the time during which it loses quality and becomes unacceptable. The length of time that this process takes depends on the cultivar, vintage, wine style and storage conditions.

A successful ageing period will depend on the specific composition of the wine being matured, and will vary from weeks, (in the case of fresh, fruit driven white wines) to decades, for highly structured, tannic reds. It is generally recognized that the quality of a wine will increase during a period of maturation up to a certain point, after which it starts ageing and loses quality. Changes over time may be very subtle, or extremely obvious and unpleasant. Although some styles (usually red wine) are robust enough to withstand, and can even benefit from the associated volatile acidity and deepening color, obviously oxidized wine is normally perceived as overaged.

The wine ageing process is complex and there are very few rules that can be applied to maximize success. It is generally recognized that a slower maturation process at cool temperatures is correlated with higher quality for most wine (see Figure 2.9.28). It is also an advantage for ageing to take place in a dark, vibration free environment, with a relatively high humidity. This is why winemakers in chalk or limestone districts such, as Champagne, Burgundy and Vouvray/Montlouis in Touraine (Loire), often use cellars hewn out of the local rock for ageing their wines prior to sale.

The shelf life of wine is a primary concern of the wine industry, and wine will be 'engineered' by the winemaker to be consumed within a specified time period. Aside from problems caused by spoilage organisms, the shelf life of a wine is directly related to its resistance to oxidation, which in turn depends on the concentration of polyphenols in solution, the reliability of the bottle closure, dissolved oxygen levels and sulfur dioxide concentrations on bottling.

Factors Affecting the Maturation and Ageing Process

Oxygen

Oxygen plays a fundamental role in wine maturation and ageing. It is necessary for the maturation of young red wines, but detrimental to older reds and most white wines with the exception of ranciostyle

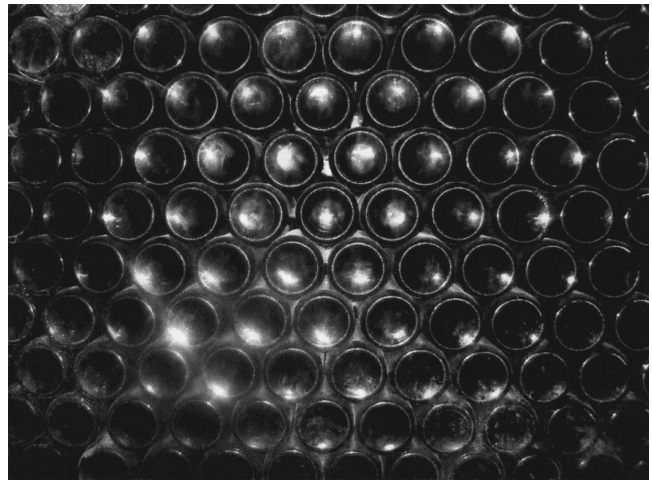


Figure 2.9.28 *Red wine bottles ageing in cool conditions, packed horizontally in a South African winery cellar*

wines (Sherries, Madeiras, some Vins Doux Naturels – see Chapter 2.10) where it is essential for flavor development. The effect of oxygen on the composition of wine has warranted many studies, a review of which was covered by Du Toit *et al.* (2006). Oxygen is involved in the transformation of components in the wine, and is also necessary for the growth and spoilage caused by acetic acid bacteria. At various stages in the maturation and ageing of the wine, the effects of oxygen can mask fruity and varietal character, prevent the development of a reductive bottle bouquet, and/or generate acetaldehyde and other odors associated with oxidation.

Conventional winemaking recognizes the effect of oxygen on wine quality, and accepts that the amount of oxygen that comes into contact with the processed wine at any given time can be managed, but seldom rigorously controlled. The solubility of oxygen in wine increases with increasing alcohol content, but decreases with increasing temperature. For instance, saturation is approximately 5.8 ml of oxygen per liter of wine at 20 °C and around 6.5 ml/l at 15 °C. When chilling wine (e.g. during cold stabilization), care should be taken as the cold liquid can absorb up to 12 ml/l at –5 °C, which may then go on to do all sorts of damage to sensitive components once the wine warms up to room temperature. If the wine contains carbon dioxide, oxygen dissolution is decreased, so freshly fermented and sparkling wines are less vulnerable. The free sulfur dioxide level has no effect on the oxygen dissolution process, but will affect the consumption rates of oxygen once dissolved, and will help to counteract negative effects of any reaction products of oxidation. After fermentation, due to the presence of alcohol and additions of sulfur dioxide, enzymic oxidation is reduced to virtually nothing, and chemical oxidation is responsible for any oxygen related changes. Even during bottle ageing, when the wine is totally protected from the air, there are reduction and oxidation reactions occurring in the wine, which cause the oxidation of certain components. As these reactions take place, the state of oxidation in the wine increases.

Metal ions, particularly copper and iron, are necessary to catalyze oxidation in wine, and along with other components, form intermediates in reactions. Danilewicz (2003) suggests that sulfite does not react with oxygen directly, but with products that result from catechol oxidation (i.e. quinones and hydrogen peroxide). During maturation and ageing, tartaric acid forms dioxymaleic acid (among other substances) which can be further oxidized to oxalic acid if exposed to too much oxygen, for instance in a bottle left open for too long. Some of the other compounds that have been associated with oxygen contact are products of the oxidative

degradation of unsaturated fatty acids, such as hexanal, *trans*-2-octenal, *trans*-2-nonenal and 1-octen-3-ol. Aromatic products of oxidation and ageing are further discussed in a later section in this chapter.

Oxygen can be added to wine 'by accident,' or deliberately, during the winemaking process. Dissolving oxygen in wine through aeration or sparging of a thin stream of gas, is a technique that is used frequently in modern wineries in order to reduce the susceptibility of white wine to later oxidation by deliberately oxidizing sensitive components in a controlled fashion. It may also help to reduce 'pinking' of susceptible wines like Sauvignon Blanc later on in the bottle. This process, known as microoxygenation, can also help to make wines better able to withstand the highly reductive conditions under screw cap, and avoid the reductive or vegetal taints associated with the development of disulfides and mercaptans later in the life of the wine. Microoxygenation can also help to soften harsh tannins and improve color stability in red wines, somewhat mimicking the effects of a lengthy barrel maturation period.

The normal (less controlled) exposure of wine to oxygen during the winemaking process has similar effects to microoxygenation, but may easily lead to oxidative taints in the wine if the amount of oxygen dissolved is too high. As previously noted, oxygen can dissolve in wine quite rapidly and the rate at which it is then used up in chemical reactions depends on a number of factors including phenolic content of the wine, its pH and temperature, and the rate of oxygen ingress. The total oxygen consumed by wines under normal acidic conditions ranges from about 30 to 100 mg/l, but phenols can have substrate, catalyst and buffer roles during oxidation, and their concentration will greatly influence consumption. Danilewicz *et al.* (2008) found that sulfur dioxide actually accelerated the reaction of oxygen in model wine conditions and in red wine, and proposed the increased rate was due to the reaction of bisulfite with quinones. The accelerating effect of SO_2 on oxygen consumption was also observed by these workers in red wine. The free SO_2 concentration was increased to 51.2 mg/l in one portion of the Beaujolais wine and removed entirely with hydrogen peroxide in a second. Both were saturated with aerial oxygen, and the consumption of oxygen was markedly slowed by the removal of sulfur dioxide.

de Beer *et al.* (2005) studied the effects of bottle ageing on the antioxidant activity of Pinotage, Cabernet Sauvignon, Chardonnay and Chenin Blanc wines, by storing wine at 0 °C, 15 °C or 30 °C for a period of a year. They found a significant decrease in both the total antioxidant activity and the total phenol content of the wines. The total anthocyanins in the red wines decreased significantly over the year at all temperatures except for storage at 0 °C, while the flavanol content of the Pinotage, Cabernet Sauvignon and Chardonnay wines increased up to nine months storage with a subsequent decrease to 12 months. The stabilization of flavonols and anthocyanins in wines seemed important in protecting the wine against loss of total antioxidant activity. As wines are not normally consumed directly after production and a decrease of between 18% and 50% in total antioxidant activity could occur even under favorable storage conditions (15 °C) during one year, the use of total antioxidant activity values to market wines should be treated with caution. The authors noted that understanding the complexity of these reactions may provide clues for preserving the antioxidant ability of a wine without losing the beneficial effects of bottle ageing.

Wine is capable of absorbing the effects of very small quantities of oxygen over longer periods of time with few negative effects, but rapid oxygen ingress for even short phases will lead to a loss of aromas and the flat taste associated with the presence of acetaldehyde and ethyl acetate. Slow, light aerations (e.g. barrel ageing) mean that the beneficial oxidation intermediates formed are weakly reactive and perform best at low redox potentials. Although it is generally agreed that postbottling oxidation is usually detrimental to wine, Jackson (2005) highlighted the fact that a little in bottle oxygen ingress may have some benefits. Some wines, such as Sauvignon Blanc and Shiraz, can suffer from the rotten egg smell of hydrogen sulfide, and circumstantial evidence indicates that oxygen slowly negates this, as well as helping to remove the burnt rubber smell of 2-mercaptoethanol in affected wines. The author added that whether slow or intermittent oxygen ingress aids, or is essential to, the formation of a desirable aged character in older red wines is unknown from a purely scientific perspective at this stage.

Free sulfur dioxide

The phenomenon known in the wine trade as 'bottle shock' or 'bottle sickness' is a temporary condition in which there is a loss of varietal aroma and fruit flavor, and an increase in harsh, flat characters due to the adjustment of the chemical composition of the wine to the bottling process. In the past, bottle shock was attributed to the production of a small amount of acetaldehyde by oxygen contact at bottling, but as most commercial wines are bottled under anaerobic conditions, it is more generally associated these days with the dose of sulfur dioxide that a wine is given just before bottling, and usually affects lighter, delicate styles of wine. It lasts for a few weeks after bottling, during the transport of wines between producer and point of sale, and goes unnoticed by most consumers. In the event that the loss of primary aroma and flavor is obvious to people other than the winemaker and wine experts, or continues for a longer period than a few months, it should be considered that the problem is more severe than bottle shock, and may due to excessive doses of sulfur dioxide, or higher levels of dissolved oxygen (depending on the bottling conditions).

The antioxidant effect of sulfur dioxide on wine is well known, but a full understanding of this mechanism of action has yet to be achieved. Concerns over health issues related to sulfur dioxide during recent years have led to the maximum levels of sulfur dioxide permitted by law being systematically reduced by producers, with knock on effects on the longevity of their wines.

The multitude of reactions in which sulfur dioxide can be involved in wine include antimicrobial, antioxidasic, carbonyl binding (e.g. binding of acetaldehyde formed by the oxidation of ethanol) and antioxidant mechanisms. These are discussed in more detail in Sections 2.5.2 and 2.5.3. It had been previously suggested that sulfur dioxide protects wine from oxidation by reacting directly with molecular oxygen dissolved in the wine, but most authors now agree that although this reaction occurs in industry in the presence of metal catalysts, it is not particularly relevant at wine pH, as the reaction with molecular oxygen is not favored by the electron configuration of the oxygen in its ground (triplet) state. Studies have provided evidence as to the catalytic importance of iron and copper in the autoxidation, not only of polyphenols, but also of sulfur dioxide in a wine model system (Danilewicz, 2007). The studies indicated that nonmetal catalyzed pathways were so slow that they were unlikely to play a significant part in oxidative processes in wine, and that the autoxidation of sulfur dioxide is a radical chain reaction in which powerful intermediate oxidizing radicals (including hydrogen peroxide) are produced.

Hydrogen peroxide, by way of the Fenton reaction, will oxidize ethanol, but is intercepted by radical scavenging polyphenols, which break the radical chain process. The sulfate radical is another powerful oxidant that can oxidize bisulfite to regenerate the sulfite radical. This regeneration of the sulfite ion means that the oxidation of bisulfite will continue as long as there is oxygen available, and for this reason, bisulfite appears to consume oxygen dissolved in wine. Radicals formed during the process are obviously capable of oxidizing other wine constituents, so sulfur dioxide exerts its main protective effect through scavenging these reactive species, and protecting sensitive wine components from their effects. Waterhouse and Laurie (2006) stated that SO_2 acts in other important ways to limit oxidation. These include the ability to bind reversibly to acetaldehyde, as well as other aldehydes and ketones, and to reduce reactive quinones back to phenols.

Within the European Union, the maximum permitted level of total sulfur dioxide in dry red wines is 160 mg/l. In dry white, dry rosé and sweet red wines, 210 mg/l is permitted, and in sweet white and rosé wines where higher levels of sulfur dioxide are needed to inhibit possible fermentation of the residual sugar, the permitted level goes up to 260 mg/l. Certain styles with a high sugar content like the sweet white Loire wines, Trockenbeerenauslese and Ausbruch from Germany and Austria are allowed up to 400 mg/l sulfur dioxide, which goes some way to explaining the extreme longevity of such wines. The maximum level permitted in Australia was reduced to 250 mg/l in the 1990s, except for wines with 35 g/l or more residual sugar for which up to 300 mg/l sulfur dioxide is permitted (Robinson, 2006b).

Certainly the search has been going on for a number of years to find a replacement for sulfur dioxide as a preservative for wine, but so far alternatives have been elusive. From permitted levels of up to 500 parts per million (ppm) in 1910, which again may help to explain the excellent preservation of some of the wines made around this time, legal maxima had dropped to half this amount around the world by the end of the twentieth century, largely in response to public health concerns about the effect of high doses of sulfur on asthmatics and other sulfur sensitive individuals.

Researchers continue to try to find alternatives to sulfur based winemaking preservatives which have antibacterial and antioxidant properties, but it is rare to find both in one compound. Lower levels of sulfur can be used in combination with other antioxidants like ascorbic acid for protecting wine during ageing, but no other single substance manages to combine all the attributes that sulfur dioxide does. In a recent study, sulfur dioxide and mixtures of glutathione and caffeic acid or gallic acid were tested as inhibitors of the decrease of isoamyl acetate, ethyl hexanoate and linalool (positive wine aroma contributors) during storage of a model wine medium (Roussis and Sergianitis, 2008). Model wine samples containing 0, 20, 40 or 60 mg/l free sulfur dioxide were analyzed and it was found that isoamyl acetate, ethyl hexanoate and linalool generally decreased during storage of the model wine medium, but sulfur dioxide protected the three volatiles at 20 (isoamyl acetate), 40 (ethyl hexanoate) and 60 mg/l (linalool) respectively. Mixtures of glutathione with caffeic acid or gallic acid were also protective – more so at higher than lower levels. A combination of glutathione and caffeic acid was more effective than glutathione and gallic acid. Unsurprisingly, the presence of sulfur dioxide at 40 mg/l increased the protective effect of the mixtures. The results indicated that higher concentrations of sulfur dioxide protected wine aroma volatiles, but that mixtures of glutathione and caffeic or gallic acid were also effective, and their efficacy was boosted by sulfur dioxide.

Some attempts to produce wines without any addition of sulfur dioxide have been reasonably successful (especially in the still red wine category), but sweet wines are particularly prone to oxidation and attack by wild yeast and bacteria, and usually require pasteurization if they are to 'survive' without SO_2. Preserving wines with alternatives like natamycin leads to complications when shipping to countries where this preservative is forbidden – even adding small amounts of sweet reserve in the final stages of winemaking can elevate the natamycin concentration to unacceptable levels.

Sulfates are natural components of fermentable substances and it is normal to encounter small amounts of sulfur dioxide in fermented products such as bread and wine as by-products of yeast metabolism (Robinson, 2006b). Indeed, anecdotal evidence suggests that the levels of sulfur dioxide found in salad bars in restaurants, as well as in packaged foods, far exceeds that found in wine.

Compulsory labeling information is now required on wine bottle labels in various countries, usually just a small legend to the effect that the wine 'Contains sulfites,' but Australian labels must state that sulfur dioxide, or 'Preservative (220),' has been added. Within the European Union (EU), sulfur dioxide is known as E220, but it does not need to be specified on wine labels.

pH and acidity

Cilliers and Singleton (1990) also observed that the rate of autooxidative reactions in *o*-dihydroxyphenols like caffeic acid and caftaric acid can be slowed substantially by operating at low pH and low oxygen concentration. Any increase in pH (i.e. decrease in hydrogen ion concentration), the authors noted, will greatly increase the rate of this oxidative reaction, and that high pH during ageing is the reason why so many 'hot-country' wines 'fall apart' too soon. In his earlier review of oxygen with phenols, Singleton (1987) also suggested that lowering the pH as early as possible would improve the maturation rate and staying power of wines from over-ripe grapes from warmer seasons and regions and improve otherwise flat tasting wines. Certainly winemakers are starting to take cognisance of the fact that a lower pH will help to preserve a wine for a number of different reasons (better sulfur dioxide efficacy not least amongst them). Methods of increasing

acidity have come full circle in recent times, with winemakers now practicing differential harvesting (picking at staggered times during the ripening period) and fermentation, with subsequent blending of the wines to achieve the desired pH and titratable acidity, as used to be done in the times before relatively cheap tartaric acid was available on demand.

Singleton (1987) noted that the negative charge of the phenolate ion is delocalized through the benzene ring, lending about 8 kcal extra resonance stabilization to the anion compared to the protonated phenol, and therefore phenolic oxidation in wine is faster at higher pH as the removal of an electron is much easier from the phenolate anion than from the protonated phenol. The phenolate ion concentration at pH 3.5 is very low (about 0.004%) of the total, but this ion is so reactive that oxidative reactions will still take place as equilibrium will replace any phenolate ion consumed. du Toit, *et al.* (2006) in a review of the role of oxygen in wine, stated that the oxidation rate of ascorbate decreases as pH decreases, becoming very low at pH 2, as at lower pH phenolic molecules tend to be protonated and less easy to oxidize. Danilewicz (2003) stated that the pH of the solution determines the position of equilibria involving ionizable species such as phenols, and hence their reduction potentials. The protonated species of the quinone radical anion is remarkably acidic as it is an electron deficient system. It has a dissociation constant pK = 5.0, so that at wine pH (3.0–3.6), the semiquinone will be protonated. At wine pH, electron transfer rates were lowered, but still remained appreciable, and it was possible that equilibria would still exist for the more easily oxidizable components, depending on the stability of the phenoxy radicals generated. As pH rises, the proportion of phenols in the reactive phenolate (negative) state increases, enhancing potential oxidation and decreasing the ability of the wine to withstand the ageing process.

During his studies on pH effects, Singleton also observed that wines that are made alkaline will rapidly consume a lot more oxygen, reaching saturation quickly. The total amount of oxygen consumed, however, under 'slow' acidic conditions was much higher – and ranged from 1.4 to 18 times as much in the same wine under alkaline conditions. From a practical viewpoint, slow oxidation did not exhaust the original oxidizable substrates of wine as rapidly as fast oxidation, and suggested to the author that additional, slower reactions occur which augment the pool of substrates. He also stated that fast oxidation does not lead to the same 'good' result in wine ageing, but that there is not a lot of data available to substantiate this statement.

In apparent contrast to received wisdom, Silva Ferreira *et al.* (2002) found that bottled wines subjected to extreme conditions (45 °C temperature, and oxygen saturation) for three months seemed to experience more off lavor formation at pH 3 than at pH 4. They showed that synergistic effects of increasing temperature and oxygen levels at lower pH were significant, both on the decrease in levels of terpene alcohols and norisoprenoids (which impart floral aromas), and on the development of off flavors such as 'honey-like,' 'boiled potato' and 'farmfeed' associated with the presence of phenylacetaldehyde, methional and 1,1,6-trimethyl-1,2- dihydronaphthalene (TDN).

As wine is such a complex medium, and the equilibria in solution dependent on so many factors, it is unsurprising that seemingly conflicting reports are written on the sensory effects of various treatments. Wine pH, so fundamental to the chemistry of the solution and therefore the flavor and aroma of the product, is certainly an area that deserves far more attention than it has so far received in enological laboratories and wine cellars.

Phenolic compounds

Because of the different techniques used to produce white and red wines, the concentrations of phenolic compounds vary greatly between them. Phenolic compounds play a major role in red wine quality, since they contribute to sensory characteristics like color and astringency, and exert a strongly protective influence on the wine during processing and maturation due to their antioxidant and antimicrobial properties. As white wines contain few phenolics to protect them, they require higher levels of free SO_2, and are usually made in a

way that minimizes exposure to oxygen unless the alcoholic fermentation needs to be stimulated, or hydrogen sulfide needs to be removed.

As discussed in Section 2.9.2, there are two major classifications of phenolic compounds in grapes: flavonoids and nonflavonoids. Nonflavonoids (smaller, less complex phenols) are concentrated in the pulp cell vacuoles and are present in all juices. White wines contain relatively low levels of total phenols and the majority of these are nonflavonoids like caftaric acid (an ester of caffeic and tartaric acids). The flavonoids (complex, larger phenols including anthocyanins and tannins) are usually concentrated in the skin, seeds and stems of red grapes. During the maturation and ageing of red wines, the concentration of grape anthocyanins, initially responsible for color, decreases because they progressively react with other phenolic compounds, particularly flavanols (Singleton, 1987). This phenomenon results in the color change from the purple tone of young wines towards the brick red color of matured wines, as well as in the decrease of wine astringency observed during ageing.

At wine pH values, anthocyanins exist as two forms in equilibrium, namely the red flavylium cation and the colorless hydrated hemiketal form, the latter being predominant (Fulcrand and Cheynier, 2006). Various pathways involving both anthocyanin forms have been proposed to explain the conversion of anthocyanins to new, more stable pigments in mature wines. The first process is a direct reaction between flavanols (F) and anthocyanins (A), leading to two kinds of products, denoted A-F and F-A, according to the position of the anthocyanin moiety. Recent work has revealed the presence in wine of both types of compounds, which respectively arise from addition of tannins onto A and from addition of AOH to the carbocation formed by acid catalyzed cleavage of proanthocyanin interflavanic bonds.

The second pathway concerns the reactions between anthocyanins and flavanols mediated by acetaldehyde, a product of the oxidation of ethanol during ageing. This mechanism starts with condensation between acetaldehyde and a flavanol leading to a carbocation intermediate, which reacts in turn with either another flavanol or the hydrated form of an anthocyanidin. The effect of unlimited exposure to oxygen and the associated acetaldehyde addition reactions is an initial increase in color density and polymeric pigments, followed later by a decrease in color and formation of a colored precipitate. Eventually most of the color in the wine will be in the form of dark brown, black or brick red precipitated aggregates (often combined with potassium bitartrate crystals), and the wine itself will be thin, oxidized and pale brown in hue.

The third type of reaction established in wines is a cycloaddition process between anthocyanins and some yeast metabolites possessing a polarizable double bond, for example, vinyl phenol and pyruvic acid, which leads to more stable pigments, structurally similar to pyranoanthocyanins, with excellent stability and resistance to sulfite bleaching.

As stated previously, oxidation in wine occurs faster at higher pH levels because electron removal is easier from phenolate anions than it is from the neutral, or positively charged forms of phenols. Phenols are very weak acids with pK_as between 9 and 10, so the higher the pH, the greater the extent of dissociation, and the more phenolate ions there are. The fact that the hydroquinones formed from oxidation have a lower redox potential than the phenols, and therefore are more oxidized, means that they serve to protect some of the original wine phenols from oxidation, so providing a buffering effect from oxidation (Singleton, 1987). As noted in the previous section, the slower the rate of oxidation, the more the pool of oxidizable phenols is augmented by regenerative polymerization. If oxidation occurs rapidly and most available phenols are oxidized to quinones, there will be less consumption of oxygen because less regenerative polymerization can take place, but greater production of oxidized products. Rapid oxidation of a wine may therefore be deleterious to its potential longevity, but slow oxidation, such as that which occurs during intermittent racking of barrels, has far fewer negative effects. The way in which a wine of a particular phenolic composition responds to oxygen depends on the redox potential of the wine, which is influenced by pH, its phenolic composition, the presence of ascorbic acid and sulfur dioxide concentration.

Phenolics are discussed further later under the heading ‘effects of ageing on color.’

Temperature

In recent work, it has been found that out of a range of possible factors affecting the ageing potential of wine, temperature has the greatest affect on the consumption of oxygen, decrease in sulfur dioxide concentrations and oxidation of phenols (McKay *et al*., 2008).

It is well recognized that prolonged exposure to higher temperature will degrade quality due to the increased rate of reactions within the bottle, and associated reaction products. In the case of oxidation this is especially relevant; the same amount of dissolved oxygen is consumed by reactions in three months at 3 °C as in three days at 30 °C. The effect of storage temperature on oxidation processes of fino wines was investigated by Macias *et al.* (2001). The degree of oxidation was found to be much greater at high (27–45 °C) than at lower temperatures (20 °C).

If oxygen is completely excluded (for example if the wine is bottled under screw cap, with a very small headspace), low storage temperature will help to preserve aroma and prevent other degradation reactions – for example, acetate esters will hydrolyze very slowly at 10 °C but rapidly at 30 °C. Temperature also has a marked effect on age induced changes in terpenes. Conversion of carbohydrates to furfural and pyrroles will be favored by increased temperature, and they may also undergo Maillard and thermal degradation reactions leading to baked flavor ('madeirized') and brown color. Color is also adversely affected by high storage temperatures during maturation and ageing. Dallas and Laureano (1994) investigated the effects of various parameters on the storage of red table wine and found that there were significant decreases in concentrations of individual and total anthocyanins at higher temperatures (30–40 °C) during ageing, which effect was ameliorated by sulfur dioxide additions. Malvidin-3-glycoside was the only phenolic under study that was not affected by temperature, but was more influenced by the time of storage.

In the event the wine is under cork, it becomes even more crucial that storage temperatures are kept stable or the seal between bottle and cork will loosen due to continuous expansion and contraction. Rapid changes in temperature also affect wine volume, and freezing should be avoided as the expansion of the volume in the bottle may even force the cork out. Mayen *et al.* (1996) subjected wines to accelerated browning at 50 °C under aerobic and anaerobic conditions, and found decreases in procyanidin contents with simultaneous increases in flavonol concentrations, due to the hydrolysis of oligomeric derivatives at this temperature. Villamor *et al.* (2009) studied storage conditions on the chemical and sensory properties of young bottled Cabernet Sauvignon and Merlot wines. Low and high tannin wines were stored at 23 °C, 27 °C and 32 °C for 0, 40, 55 and 70 days. In both low and high tannin wines, storage at 32 °C resulted in significant increase in small polymeric pigments with a corresponding decrease in anthocyanin concentrations over time, which was most pronounced in Cabernet Sauvignon. In both varieties, high tannin wines contained more large polymeric pigment (LPP) than the low tannin wines. Generally, titratable acidity and pH were not affected by storage treatments. A trained sensory panel gave higher astringency ratings to high tannin wines than low tannin wines and there was an increased perception of bitterness associated with storage at 32 °C storage for 70 days. The authors stated that results indicated that storage temperature and storage time contributed to changes in the chemical composition of typically ageing red wines, but did not impact perceived astringency.

Vivas de Gauljac *et al.* (2001) showed that concentrations of quinones formed through oxidation of (+)catechin and (–)epicatechin were higher at 40 °C than 4 °C, but the kinetics of quinone formation did not change: levels were close to their maximum values after 30 minutes, indicating that even short exposure times at high temperature can have negative effects for wine. de Beer *et al.* (2005) showed that white wines exhibited a decrease of between 5.7 and 17.2% in their total phenolics during storage at different temperatures. Storage at higher temperatures caused a faster decrease in phenolics with significant differences between wines stored at 0 °C and 30 °C. Decreases for Chenin Blanc wines were faster than those observed for Chardonnay, presumably as a result of Chardonnay's more robust phenolic profile. Chardonnay is also often given barrel

fermentation or maturation treatment, which enhances the phenolic profile of the wine and gives it better ageing potential.

Although sulfur dioxide offers some protection against the ravages caused by high temperatures, it can only do so in a limited fashion. In an early study carried out by Ough (1985), white and red table wine samples were subjected to simulated transit or storage conditions, and held at five different temperatures between 28 and 47 °C with low and medium sulfur dioxide levels for 21 days. The problems caused by minimal SO_2 usage increased dramatically with increasing temperature. SO_2 and color changes were related to temperature, with greater changes and subsequent losses of quality found at higher storage temperatures. Red pigment was lost for the red wine at about the same rate the white wine browned for the low SO_2 wines. The loss in SO_2 was more rapid with increasing temperature for the red wine than for the white.

The result of all the observations around the effects of heat on wine during ageing emphasize the importance of keeping wine in low (10–15 °C), stable temperature conditions if it is to be stored for significant periods. Even in the event that wine is subjected to high temperatures for short periods, damage may be done to the aroma and color. Wine in transit, particularly on large container ships, may experience temperatures up to 50 °C for hours at a time, and this has large implications for the quality of the product being delivered. Even refrigerated containers are not immune to temperature fluctuations of between 10 and 30 °C over a 24 h period, and winemakers need to be aware that the product that left the winery may be entirely changed by the time it reaches its destination due to the effects of heat during transport.

Light

It has always been known that storing wine in light, bright places will alter its color and chemistry over time. For hundreds of years wines have been stored away from light and heat in cool, dark cellars. The scientific rationale behind doing this only been investigated relatively recently. Timberlake and Bridle (1976) examined reactions that occurred between phenols in tartrate buffer solution at pH 3.5 at room temperature in air under darkness and light. Several reactions occurred simultaneously. The anthocyanins and phenolic compounds reacted very slowly, with eventual formation of yellow xanthylium salts. Procyanidins were partly transformed to epicatechin and polymers, the reactions being catalyzed by daylight. The addition of acetaldehyde to mixtures of anthocyanins and phenolics caused rapid and spectacular color augmentation with shifts toward violet, with extent of the shift varying with the type of component. Later, Singleton (1987) observed that exposure of wine to light, especially sunlight where there may also be heat involved, increases the rate of oxygen consumption and the establishment of a low 'rest potential' (or ageing capacity) in bottled wine. Carbon dioxide is generated as the wine is oxidized (under certain conditions) – possibly from the condensation reaction between catechol and pyrogallol derivatives, and the proportion of CO_2 generated to O_2 consumed is far higher in light than in dark conditions.

Concentrations of highly hydroxylated compounds such as gallocatechin also increase if wines are oxidized in light. The author noted that oxygen can be activated to two singlet forms which are then able to react with most organic molecules (which are also singlets). The most active form of oxygen has a very short half life and decays to form the more stable delta activated singlet oxygen, which is 97.8 kJ/mol (23.4 kcal/mol) in energy above the ground state triplet. The delta form has a 2 μs half life in water, which is an adequate time for many reactions to take place. This form can be reduced to yield the superoxide ion. Singlet oxygen is produced by UV light in the presence of photosynthesizing pigments and by certain chemical and enzymic reactions.

Mattivi and coworkers (2000) noted that exposure to ultraviolet and blue light (wavelengths 300–500 nm) will cause heating, and the radiation itself may activate some oxidation reactions, one of which is the well known shrimp-like, skunky fault of champagne called 'light struck' ('goût de lumière'). According to these workers, the photogeneration of thiols (methylmercaptan and hydrogen sulfide) and dimethyldisulfide in wine

exposed to light of wavelengths below 450 nm is caused by photodegradation of methionine and cysteine in the presence of free riboflavin, which acts as a photosensitizer and oxidising agent in wine. The higher alcohol 2-methylpropanol has also been implicated in goût de lumière, as well a number of esters. Attempts to control light struck flavor in wine include the use of copper sulfate, tannin and bentonite. It has been reported that free riboflavin concentration can be a good index of the likely degree of deterioration in the flavor of still and sparkling wines on exposure of bottles to light.

Vivas de Gaulejac *et al.* (2001) showed that the effects of temperature and UV light on the oxidation of phenolic compounds caused the concentration of quinones to increase with increasing temperature. More quinones were formed in the presence of UV light than in its absence, but UV light did not affect the kinetics of the reaction. The authors concluded that while temperature and UV light affected the quantity of quinones formed, they did not affect the rate at which they appeared. The authors stated that wine storage management greatly influences wine ageing and cited temperature and light as being the two best known storage conditions affecting the success of maturation. The authors emphasized the importance of (an absence of) heat and light in the conservation of wines as these factors lead to higher quinone formation with concomitant impact on sensory qualities in white and red wines.

Grape derived aroma components are also affected by light. The alkyl methoxypyrazines (MPs) are grape and insectderived odor compounds responsible for 'green,' 'capsicum' and 'vegetative' aroma in wine. At higher concentrations, MPs are detrimental to wine quality, but at lower concentrations, they are responsible for 'grassy,' 'fresh' smells that are better suited to the style and aroma profiles of white rather than red wines. Blake and coworkers (2010) examined the influence of light and temperature during cellaring on MPs and other impact odorants. Riesling and Cabernet Franc wines were stored at ambient temperature (22 °C) under fluorescent lighting, in the dark at ambient temperature, or in the dark at 12 °C. Additionally, the wine exposed to light was stored in clear, green or amber bottles, the most common glass for wine, which transmit 95%, 50%, and 10% of 350–550 nm light, respectively. Isobutylmethoxypyrazine decreased over a 12 month period by around 30% under all conditions in both cultivars. Acetate esters also decreased with time, regardless of light or temperature conditions, while phenethyl acetate and isoamyl acetate decreased faster at 22 °C than they did at 12 °C. Retention of free and bound sulfur dioxide was higher in light excluded conditions and influenced by bottle hue. Browning in white wine was inhibited by SO_2 and reduced in light exposed conditions and suggested to the authors that the combination of clear bottles and elevated storage temperature was not optimal for protecting against premature browning and other negative quality indicators in white wine. Practices such as bottling in dark colored glass containers and protecting from bright light help to protect the wine in the short, as well as the long term. These results should assist winemakers in selecting bottle hues, and assist in selection of optimum storage conditions for preserving wine quality in both retail and cellar environments.

Vibration

From the anecdotal evidence of the changes in wine that happen during periods of transit (particularly by road), it has long been suspected that wine 'does not like' vibration, and reacts with differences to the flavor and bouquet. According to Chung *et al.* (2008), vibration disturbs the delicate sediments in fine wine and the process of biochemical evolution, which can cause undesirable flavor and taste development. There is very little scientific research on the subject, so Chung and coworkers studied the effect of vibration on some physicochemical characteristics of a commercial red wine. The wine bottles were placed in a specially designed vibration rack, and aged for 18 months under controlled dark conditions with a constant temperature of 20 °C. There were four different 'degrees' of vibration (1, 5, 10 and 20 cm/s^2 or 'Gal'). The pH of the wine was 3.4 before storage, and it did not change significantly during storage. The authors found that although the total acidity increased slightly with ageing up to nine months, especially at the highest vibration level

(20 Gal), the amounts of tartaric and succinic acids, and levels of tannins, decreased during storage. The refractive index increased during the first three months and thereafter it remained constant. The propanol and isoamyl alcohol contents were higher in the wine stored at low levels of vibration. In the initial period up to three months, the lightness significantly decreased (the wine became darker in hue), and then remained relatively constant afterwards. The authors did note that various reactions including oxidation, condensation and polymerization may have resulted in the color changes observed, rather than the vibration.

Closures

Goode (2006) cited cork as the 'prime culprit' in an overview of premature oxidation among white Burgundies as this was the only factor that varied amongst the wines that were studied. He suggested a number of reasons why corks might be responsible, which includes the use of peroxide as an alternative sterilising agent to chlorine containing bleaches associated with trichloroanisole and cork taint. Residual traces of the peroxide in some corks could have led to the random postbottling oxidation observed. However, the scale of the problem and the fact that so many cork suppliers were involved seemed to negate this as the only contributing factor. Goode's suggestion is probably based on work done by Fabre (1989), who demonstrated the presence of peroxide in corks treated with oxygenated water, peracetic acid or sodium peroxide, and showed that peroxide was associated with premature ageing, a drop in SO_2 content, darker coloration and loss of organoleptic quality in wines. The author noted that corks treated with peroxide did not necessarily lead to the presence of oxidizing residues within corks, but use of corks containing peroxide residues was not recommended. Later work by Waters *et al.* (1996) at the Australian Wine Research Institute (AWRI) demonstrated that oxygen permeation through cork was just one mechanism of oxidation affecting bottled wines, but that it was a significant defect for a cork to possess. They cited the example of a wine held for three years under a permeable cork, and noted that up to 36 mg/l of sulfur dioxide was consumed by oxygen ingress and that unlike residual oxidant activity which decreases with time, permeation will continue for the life of a bottled wine. Data gathered in the study also indicated that oxidative damage could result from cork containing oxidants, which might result in losses of sulfur dioxide of up to 73 mg/l in six months. The authors also stated that effects observed for oxidant activity and permeability were highly variable across the corks examined in this study, and that the samples used in the investigation were not representative of corks in the wine industry as a whole, as the wines were from batches that had already shown a sporadic oxidation problem and the corks were thus defective.

Goode suggested that there was a decrease in cork quality in line with large demands for cork during the 1990s, leading to corks with lower integrity and more physical faults being used. Low humidity levels during storage may also cause drying and deforming, exacerbating any physical defects in the corks. If the cork shrinks, cracks or loosens, air will enter the bottle and accelerate oxidative changes. On the other hand, a high humidity level (above 80%) can create the risk of mildew formation on the cork, and possible moldy odors being transferred to the wine.

Goode noted that the theoretical maximum of 11.36 mg of oxygen incorporated on bottling can react with 45.5 mg of SO_2 assuming a reaction ratio of one oxygen molecule to four sulfur dioxide molecules. Danilewicz (2006) suggests a ratio of 1:1 or 1:2). Goode goes on to say that if total loss of SO_2 in a 750 ml bottle exceeds 45 mg then the loss cannot be explained by oxygen entrained at bottling, and must be due to oxygen ingress through the closure. Lopes *et al.* (2006) also stated that bottle ageing of wine (presumably successful bottle ageing) is extremely dependent on the oxygen barrier properties of closures. These workers measured the oxygen ingress through different types of closures using a nondestructive colorimetric method and found that rates of oxygen ingress were dependent on closure type and independent of bottle storage position. Closures that performed best were screw caps and technical corks, with synthetic corks showing the highest levels of oxygen entering the bottle, and conventional natural corks an intermediate level. In another study by the AWRI carried out on wine closures, including cork, cork substitutes and screw caps, it was found that screw

capped wines retained their delicate characters and showed no oxidation or corky off odors. However, screw caps did show rubbery or other sulfur related off odors (Jackson, 2005). Generally speaking, closure type will not dictate whether a wine will receive a gold medal for quality in an international competition. However, it has been regularly observed that closure type affects the types of defects observed in faulty wines: typical defects for wines under cork include fungal, musty, oxidative and 'atypical ageing' notes, while for screw caps defects include sulfide, 'stinky' and vegetative notes. Cork and screw caps therefore have their detractors and fans in equal portions. Jackson (2005) noted that those favoring cork closures argue that limited oxygen ingress (associated with cork use) neutralizes hydrogen sulfide and other reduced sulfur compounds which exist in the wine before bottling. Cork critics might state that the presence of reduced sulfur odors in the wine indicates a lack of understanding of the conditions in the bottle on the part of the winemaker and that the problem is not the 'fault' of the screw cap. It should be noted that reduced sulfur odors can certainly occur (though they are less common) in bottles sealed with a cork. There are certainly broad rules that can be applied to winemaking for either closure. Independent studies on screw caps have demonstrated that Riesling wines can retain their fruity character for upwards of 30 years, but whether the same can be said for red wines (with their greater phenolic content and lower redox potential) remains to be seen.

Effects of Ageing on Wine

Color changes

For white wines, ageing can contribute to quality defects such as browning and to a lesser extent the pinking phenomenon. In young red wines, as described in the section on phenols earlier in this chapter, both anthocyanins and tannins participate in the coloration of the wine. As the wine ages, the anthocyanins polymerize with themselves or copolymerize with proteins, polysaccharides, tannins, ethanol and tartaric acid. Anthocyanin/protein and anthocyanin/polysaccharide copolymers are poorly soluble, and tend to flocculate and precipitate out. Anthocyanin polymers are moderately stable, but flocculate and precipitate out during maturation as a deposit in the bottle. Anthocyanin/tannin copolymers are the most stable. It is well known that barrel ageing a red wine will help to stabilize its color. This is due to the production of a limited amount of acetaldehyde, which activates the formation of the latter small complexes. Not only do these complexes protect the anthocyanin chromophore (part of the anthocyanin molecule that generates color), but they also increase color depth and reduce color loss by enhancing solubility. The yellow-orange xanthylium compounds also significantly contribute to the color shift in red wines towards a tawny red. These reactions help to explain why red wines, which are high in anthocyanins and low in tannins (e.g. from thermal extraction) are poorly color stable, and why sulfur dioxide does not decolorize old wines, as tannin/anthocyanin copolymers are not bleached by sulfur dioxide.

Some of these reactions are accelerated by the presence of oxygen, but they will still occur in a wine protected from oxygen due to the presence of other catalysts like metals and acetaldehyde. The speed of these reactions increases with temperature.

While browning has already been explained as the result of condensation products from quinones, pinking is believed to be caused by the fast conversion of flavenes to red flavylium salts in the presence of oxygen. When white wines (particularly Sauvignon Blanc) are made under highly reductive conditions in order to preserve their aromas, oxidation sensitive phenols are never exposed to oxygen and either precipitated out through polymerization, or preventative treatments included the addition of active carbon, casein, milk powder or polyvinylpolypyrrolidone (PVPP) for the removal of the phenolic compounds, but due to the nonselective nature of these fining agents, they were always regarded as a last resort. In the past years, the use of oxygen in a controlled manner for the removal of phenolic compounds has been received attention. In studies concerning the sensory effects of hyperoxidation on musts and wines, there is general agreement upon improved color stability of wines treated. Although initial color intensity (determining the brown hue at A420) is often

slightly higher than in the controls obtained by conventional winemaking practices, browning capacity during oxidative ageing is significantly reduced or eliminated.

Browning under accelerating conditions is only the visual indication of further profound flavor and odor alterations to expect during oxidative ageing. Development of bitterness and astringency can be avoided or at least drastically reduced by must oxygenation. Kallithraka *et al.* (2009) studied the effects accelerated browning on the polyphenol content, antioxidant activity, reducing power and color changes during storage over nine months in bottles in selected white varietal wines. The results showed that the contents of most of the individual phenols diminished with time, with the exception of caffeic, ferulic and *p*-coumaric acids, the concentrations of which probably increased due to hydrolysis reactions. Antioxidant activity did not show any significant change, but reducing power decreased during the browning test. Absorbance at 420 nm was significantly increased after accelerated browning, as could be expected.

It has also been found that the decrease of wine color during the ageing period is less in red wines that have been made in a more oxidative way (in open fermenters, with punching down and pumping over during fermentation, for example). Anaerobically vinified samples (for example, those made in enclosed fermenters), have a lower free anthocyanin degradation rate initially and a greater percentage of the colorless hydrated form (AOH), but more of the color will precipitate out in the long term. It is fairly common knowledge now that oxidative winemaking generates more stable pigments than nonoxidative winemaking.

Red wines initially deepen in color after fermentation, but subsequently take on a brick red hue. Vivas *et al.* (2008) described the formation and diversity of new compounds resulting from nucleophilic reactions in wine, and which included dimers, trimers and polymers. The research focused particularly on the color properties of the released products and their potential impact on the color of white wine. After 50 to 60 days, the color of the solution was mainly due to soluble polymeric forms, and there was a color shift towards brown. Temperature directly affected the rate of polymerization, and the authors suggested that mild heating might be used to stabilize color. White wines took on yellow gold and eventually brown shades, generally darkening in color.

Color is also an issue for rosé wines. Most rosés are designed to be drunk soon after release, and they do not age well, losing color and fragrance within a few years (Jackson, 2005). In a study conducted by Blanchard *et al.* (2004), results indicated that phenolic fractions derived from grapes (anthocyanin and catechin) influenced the evolution of 3-mercaptohexanol (an important fruity, 'rhubarb,' 'grapefruit' aroma in rosé wines) content in a model solution in the presence of oxygen. The disappearance of 3-mercaptohexanol with catechin in the presence of oxygen was demonstrated in the study, as well as the fact that this mechanism can be inhibited by adding sulfur dioxide. A synergistic effect of sulfur dioxide and anthocyanins in the stabilization of the compound was found. In a model wine, the combination of anthocyanins and sulfur dioxide reduced the oxidative decrease of this aroma constituent significantly. The effect is, however, limited and large doses of oxygen would negate it completely. The protection provided by anthocyanins results from their antioxidant properties, and unfortunately oxygen consumption by the molecules results in their color changing, and gradually the pink color of the wine will go orange. As outlined in the next section, the presence of certain polyphenols (see tannins, below) may actually speed up degradation of odor. Jackson suggests that the use of screw caps may help in limiting the color degradation.

All the reactions and processes listed above may influence the color and color stability of wine, as well as astringency, but their relative importance, as well as the structure of the end products, will depend on the initial wine composition, and also on the presence of yeast metabolites and on oxygen exposure.

The development of tertiary aromas

The oxidation of juice is much more complex than the simple elimination of readily oxidizable phenols, since oxygen can affect other wine constituents, and as such, reports on olfactory changes generated by

hyperoxidation and other oxygen exposure studies in wine are occasionally conflicting. Generally speaking, the oxidative degradation of white wines leads to a loss of their sensorial qualities as primary and secondary aromas degrade, and tertiary aroma compounds increase in concentration. Primary aroma constituents (e.g. terpenes, damascenone and mercaptopentanones) are often changed by oxidation, and so winestyles in which these components are critical will seldom benefit from the effects of time and oxygen, even if well protected by ascorbic acid and sulfur dioxide. Monoterpenes, important in the aroma of Muscat and aromatic cultivars like Gewürztraminer) evolve in the bottle to form products with quite different characters. For example, nerol (with a citrus aroma) will degrade to hydroxynerol with a more resinous odor. The decreases in geraniol (fruity, berry aroma), linalool (sweet, floral) and citronellol (citrus) are usually significant over time. The oxides that form from these terpenes are generally waxy and eucalyptus-like in their odors, so the fresh, floral bouquet of the young wine will be replaced by a 'heavier,' more complex aroma profile in the older wine. Some terpene glycosides can hydrolyze slowly under bottle conditions and offset the loss, lending further complexity.

Flavor loss in white wines is also associated with changes in ester content, although the importance of these compounds in a wine's long-term bouquet is questionable. The esters originating from the berry are highly volatile and unstable, those resulting from microbial activity are often unfavorable, and those resulting from ageing are nonvolatile. There is no direct relationship between the total concentration of a wine in esters and the quality of its bouquet. Esters are formed by the condensation of an acid and an alcohol, are part of the primary aroma, and it cannot be argued that a great number are produced during fermentation and generate the fresh, fruity bouquet of young whites, for example, the ripe banana quality of Pinotage is undoubtedly due to the formation of isoamyl acetate during fermentation. Once yeast is removed, the equilibrium will slowly shift back to normal and esters hydrolyze back to their acids and alcohols. Yeast hydrolysis during lees contact increases the wine's esterase content, which may explain why wines undergoing lees contact have a more pronounced bouquet. Certain ethyl esters such as ethyl tartrate and ethyl succinate are synthesized in the bottle in a slow, nonenzymic reaction between ethanol and the primary fixed acids in wine. They participate only very slightly in the wine aroma, as they are poorly volatile, but can cause a decrease in total acidity of up to 10%, thus 'softening' the wine.

The transformation of norisoprenoid precursors and diterpenes into vitispirane and trimethyl dihydronaphthalene (TDN) will also lead to resiny, kerosene-like characters, particularly in Riesling from warmer regions and vintages. The aroma of oxidized white wine is largely due to the Sherry-like, nutty odor of acetaldehyde, the primary compound produced on oxidation of ethanol. With further time, and oxidation, the acetaldehyde will itself be oxidized to acetic acid, and will also react with other compounds in solution. The acid catalyzed dehydration of residual carbohydrates in the wine may occur rapidly at higher temperatures leading to 'baked' notes. Even at low temperatures, other reactions involving degradation of carbohydrates occur in time, to give products like hydroxymethylfuraldehyde (from fructose) which has a 'camomile' odor, and furfural which has a 'sweet, burnt' aroma. The Maillard reaction between amino acids and residual sugars can also generate a range of melandoin products which deepen the color of white wines, and impart caramelized flavors through the production of compounds such as hydroxymethyl furfural, glyoxal, methylglyoxal and diacetyl. In addition to this, ageing will also affect any reduced sulfur compounds that happen to be present in the wine, very seldom with positive consequences for the product. At low levels, dimethyl sulfide acts as an enhancer of fruity aromas, and even at levels above detection (30–40 mg/l) it has been correlated with a desireable aged bouquet. As soon as it exceeds this level, particularly in delicate white wines that do not have the complex, aromatic 'background noise' of red wine, it becomes detrimental and its odor is sometimes described as 'shrimp-like.' Pisarnitskii (2001), in a review of minor aroma components in wine, noted a 'saprogenic, fecal, wineskin' tone in wine is usually due to the presence of low molecular weight sulfur containing compounds. Hydrogen sulfide (H_2S) is the most important of these 'reductive' sulfur compounds, formed from sulfur containing amino acids, primarily methionine. It has a characteristic rotten egg odor, easily detected and

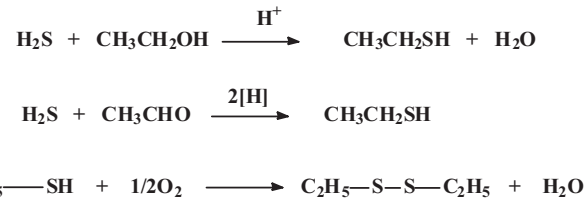


Figure 2.9.29 *Production of some odiferous organosulfur compounds from H_2S during wine maturation*

identified by most consumers. The reaction of H_2S with acetaldehyde or ethanol produces ethyl mercaptan (Figure 2.9.29), a compound also associated with 'reductive' conditions in wine, which has an 'alliaceous' or 'garlic' odor and a threshold concentration between 0.02 to 2 mg/l. In time, contact with oxygen favors condensation of ethyl mercaptan into diethyl sulfide, a foul-smelling compound, which is extremely difficult to remove or disguise.

Methyl mercaptan may similarly undergo condensation into dimethyl disulfide (Figure 2.9.29), a foul smelling compound with a threshold concentration of between 0.1 to 12 mg/l, depending on the matrix of the solution. Dimethyl sulfide may well present as the 'metallic' flavor sometimes associated with wine under screwcap, especially if it is in combination with ethyl methionate. These sulfur compounds are most commonly associated with wines under screw cap (i.e. in conditions where oxygen has been effectively excluded from the ageing process), but it is important to note that these compounds can develop in wine even in nonreductive conditions, at which stage further oxygen exposure may result in turning a smelly wine into a smelly oxidized wine (Goode, 2006b). Equating the term 'reduced' with the presence of volatile sulfur compounds is therefore an oversimplification.

The identification of the most important descriptors related to aged or oxidation affected wines include 'honey-like,' 'farmfeed,' 'hay' and 'nutty.' By gas chromatography olfactometry analysis three aromatic zones related to these descriptors in the oxidation spoiled white wines could be determined. Sotolon (4,5-dimethyl-3-hydroxy- 2(5 *H*)-furanone), was been found to have an important impact on the flavor of botrytized sweet wines (such as Auslese wines and Sauternes), Sherry-like wines and also aged white wines. Other aromatically important contributors to an aged bouquet include 3-(methylthio)propionaldehyde, phenylacetaldehyde, and TDN (1,1,6-trimethyl-1,2-dihydronaphthalene) (Jackson, 2005).

Flavonoids have been proven to induce odor degradation under conditions of accelerated oxygen consumption, although they are not volatile themselves. Schneider (1998) observed that when a low flavonoid wine was enriched with catechin or other flavanols extracted from grape seeds, negative aroma attributes like mushroom, earth and straw increased strongly within five months under normal bottle storage conditions, whereas the control had higher ratings for fruity attributes. During accelerated oxygen consumption, the flavonoid enhanced sample consumed more oxygen, lost more sulfur dioxide and produced more acetaldehyde than the control. All research conducted on this issue stresses that further research is needed to evaluate the reasons behind the differences occurring on different varieties, and to understand the effect of oxygen contact on the evolution of nonphenol flavor compounds.

A lot of recent research has been conducted on the atypical ageing phenomenon (ATA or UTA 'untypical ageing') in white wines. Bell and Henschke (2005) noted that these wines lose their varietal character and begin to exhibit atypical aromas/flavors such as 'acacia blossom,' 'floor polish,' 'naphthalene' (moth balls) and 'wet towel.' These characters are quite distinct from the kerosene-like odor, attributed to 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), that some white wines, notably Riesling, display as they age in bottle. The taint was first found in German wines in 1988 and has since been observed in South African, American and other European wines. One of the compounds implicated in atypical ageing is *o*-aminoacetophenone (*o*-AAP).

It is a nitrogen containing compound, which is of interest because it appears that it has a relationship with yeast assimilable nitrogen (YAN) concentration in the wines. It has been postulated that the formation of ATA is the result of the grape berries' response to growing under conditions of stress, for example, with insufficient water and low nitrogen supply in dry seasons. The precursor of *o*-AAP is the phytohormone indole-3-acetic acid (IAA), which, following fermentation, is oxidatively degraded in the presence of sulfur dioxide to *o*-AAP. Workers observed a significant increase in the free IAA concentration after fermentation of wines from stressed vines. Although ATA was not measured in the wines, the precursor concentrations were reportedly sufficient to form tainted wines. It is possible that yeast might play a role. Comparison of free and conjugated IAA concentrations after fermentation carried out in the presence or absence of diammonium phosphate (DAP) supplements showed that the concentration of both the free and conjugated forms of IAA were higher in the DAP supplemented fermentations. This may discourage winemakers from adding DAP as a matter of course, without first checking assimilable nitrogen levels as well as yeast requirements to see if supplementation is actually necessary.

Taste and mouthfeel

Red wines are known to substantially improve through moderate oxygen contact, such as during barrel maturation or racking. They become more harmonious and acquire greater stability and bouquet.

Many wines, especially reds, are rough and astringent in their youth. There is usually a decline in bitterness and astringency during wine ageing due to polymerization of tannins and anthocyanins, although the initial effect may be an increase in astringency associated with increase in number of medium sized tannins. Eventually the progressive association of smaller, more bitter tasting tannins into large complexes, as well as the reaction of hydrolyzable and condensed tannins with proteins will lead to loss of more bitterness and astringency. Because of the difficulty in isolating and characterizing these types of molecules, only limited studies of their structures and mouthfeel properties have been made to date. In a sensory study of red wine tannins in model wine solutions, Vidal *et al.* (2004) established that neither anthocyanins (in their glucoside or coumaroylated forms) nor pigmented tannin-like polyphenolic compounds influenced either astringency or bitterness. The authors suggested that the pigmented tannin-like polyphenolic compounds that are formed during winemaking and upon storage may decrease astringency in red wine, provided they are present in sufficient levels. This could occur through the transformation of part of the astringent material in the wine into pigmented polyphenolic compounds rather than astringent ethyl bridged flavanols. The authors emphasized that research efforts were needed for the structural characterization of compounds formed during vinification and ageing, as not much is known about the process or structures involved.

What is well known, however, is that over time, aggregated tannins and color will precipitate from red wine (forming sediment in the bottle), leaving a more refined mouthfeel. It is suggested that the formation of tannin–polysaccharide or tannin–protein complexes in the wine decreases their ability to react with taste receptors in the mouth. The stable proteins in wine (that is, those not involved with haze production) may provide winemakers with a new measure of how well a wine will age. It may also supply an additional means of regulating wine quality (Jackson, 2005).

As far as the effects of ageing on other components in wine go, there is also limited knowledge available. Although glucose and fructose react with other constituents and undergo structural rearrangement, the losses do not affect sweetness perceptibly. Ageing of wine may also induce small losses in acidity due to formation of esters and precipitation of acids in with polymers and as potassium bitartrate.

Accelerated Ageing

Commercial pressures have encouraged many winemakers to look at methods to accelerate the ageing process in wines. Ultraviolet and infrared radiation and ultrasonics have not had much success, but aeration and wide

temperature differences (to recreate summer and winter conditions) have been used with some effect. For instance, in the 'Monti' process, red wine is taken through several cycles of saturating with air at low temperatures, heating to 20 °C, cooling, then resaturating. This type of handling is never used for premium wines, and is mostly used in fortified wines, as it tends to modify color more than taste.

An accelerated ageing process may produce more unpleasant than pleasant effects, as wine is such a complex medium, so predicting the results of reactions and the outcome of such treatments is difficult and financially risky when dealing with large volumes. It is generally accepted that a slow, cool ageing without temperature fluctuation or exposure to light is best for the production of good quality wine.

2.9.6 Some Current Trends in Wine Production

Viticulture 'Going Green'

The concept of farming in an environmentally friendly, sustainable way rather than just to maximize profits is receiving increasing amounts of attention in winegrowing areas. In South Africa, to give just one example, the BWI (Biodiversity in Wine Initiative) (Biodiversity in Wine, 2010) is a pioneering partnership between over 150 wine producers, and the conservation sector. The goals are to minimize loss of threatened natural habitats in the Western Cape, and to contribute to sustainable wine production through the adoption of biodiversity guidelines by the South African wine industry. One of the strategies of the BWI is to identify and enlist interested producers as members or champions of the initiative, who will implement the biodiversity guidelines, conserve critical ecosystems and incorporate a biodiversity facet into their winery. Farmers who subscribe to BWI principles are involved in conservation projects, clearing alien vegetation, and farming with as little impact on the local biosphere as possible, with the aim of encouraging indigenous vegetation and vineyards to flourish in the same soils. Studies into 'green tourism' in the Western Cape and elsewhere indicate that visitors are looking for a restorative and recuperative experience in a rural environment which contributes in a meaningful way to communities they are visiting, and that farms that are adding value to their surroundings by subscribing to the principles of initiatives like the BWI are preferred destinations.

As discussed in Section 1.3.12, biodynamics, a 'super-charged' version of organic farming, is gaining popularity with wine farmers around the world in line with the current global trend for lower impact agriculture and environmentally sustainable methods of production. One of the common denominators amongst biodynamic practitioners is the importance of developing a healthy microbial population in the soil. Anything that is going to be added to the soil, even conventional supplements, is done via the compost heap, which will typically contain waste material from the winery (such as the grape seeds, skins and stems), plus cow manure and a range of special biodynamic preparations added to the heap (Goode, 2010). Compost is added to the soil sparingly so as not to promote vigorous growth, which is not conducive to wine quality. Working the soil in order to break up superficial roots and encourage deep root systems is also encouraged, and a return to the 'old ways' of ploughing using horses has also become part of the biodynamic landscape.

Currently biodynamics in viticulture still has an esoteric, cultish image without any real scientific underpinning of the practices so far being put forward in the literature. Rigorous research on biodynamics faces a number of obstacles, not the least of which is a lack of funding. The studies that have been carried out seem to suggest that biodynamics does enhance soil quality, with more organic matter content and microbial activity (Reganold, 1993). The results of a long-term study in Central Europe comparing organic and biodynamic farming with conventional agriculture by Mäder *et al.* (2002) showed that while biodynamic farming resulted in 20% lower crop yields, the input of fertilizer and energy was reduced by 34 to 53% and pesticide input by 97%. Enhanced soil fertility and higher biodiversity found in organic plots seemed to render these systems less dependent on external inputs.

So, while detractors of the biodynamic system of farming would highlight a lack of scientific evidence to back its claims of improving wine quality, point out the dubious advantages associated with applications of manure fermented in cow horns by moonlight and ascribe the whole business to the worst form of cynical marketing exercise, proponents can point to the improved soil structure and texture, and enhanced microbial activity in the soil. One thing that cannot be argued is that farming sensitively, with respect for local wildlife, can only benefit a region in the long run.

Other techniques that have been tested in order to reduce the application of chemical sprays include successful trials on the control of molds on fruits or vegetables by applications of natural saprophytic yeasts. However, their use is often limited because production on an industrial scale is very difficult, or even impossible. For example, a study has now examined the potential reduction of microbial diseases on damaged grape berries by spraying industrial *S. cerevisiae* yeasts (readily available in large amounts) onto the grapes (Salmon, 2010). Two species of fungal disease were selected: *Botrytis cinerea* (invasive disease fungus) and *Aspergillus carbonarius* (responsible for production of the toxin ochratoxin A), as well as an invasive bacterial species (*Gluconobacter oxydans*). Ochratoxin A (OTA) is a secondary metabolite of molds that contaminate food and feed (Section 5.11.4). OTA is nephrotoxic, hepatotoxic and immunotoxic in experimental animals, and due to its carcinogenic properties, the International Agency for Research on Cancer (IARC) has evaluated OTA as a possible carcinogen in humans (2B group).

It was demonstrated that the spreading of *S. cerevisiae* at the surface of previously damaged grape berries contaminated with different microbial species was very efficient for reducing fungus mycelium growth after 48 h of incubation. This was not the case for bacterial *G. oxydans* contamination, where no effect was observed. The authors noted that the yeast spraying should be done about 2–5 days after initial infection by the fungi in order to obtain an optimal antagonistic effect. The effect of yeast spraying on *A. carbonarius* development on the grape berries was particularly significant, indicating that yeast spraying before grape harvest could represent a biological alternative for limiting the occurrence of *A. carbonarius* in the vineyard. The reduction of the spread of *A. carbonarius* was accompanied by a significant reduction in the final level of ochratoxin A in the corresponding wines. The chemical and sensory properties of the final wines were not detrimentally affected by the yeast spraying (Salmon, 2010).

Precision Viticulture and Spectral Vineyard Imaging

High spectral and spatial resolution airborne sensors are increasingly being used for the assessment of the health and vigor of crops, including grapevine. The spatial resolutions available nowadays enable the capture of fine features in plantations such as the vine rows in a vineyard as well the means to analyze the health status of plants through characterizing the spectral reflectance of the plants at different stages of phenology. Advances in global positioning system (GPS) technology and geographic information systems (GIS) mean that input can be used for the application of fertilizers, herbicides, insecticides and seeding in general agricultural, horticultural and viticultural practices (Lamb *et al.*, 2004). Optical remote sensing can provide a synoptic view over entire vineyards offering viticulturists and winemakers a management tool of enormous potential with red grape varieties, especially if canopy architecture can be linked to production of phenolics in ripe grapes. Given the practice of differentiation in pricing between grapes based on quality attributes like color and total phenolics, accurate and reliable data that describe grape quality is particularly useful.

Atmospherically corrected high-resolution imagery is used to produce a number of image maps which can either individually or collectively help vineyard managers and winemakers alike to make more informed management decisions regarding the high value vineyard plantations (Arkun, *et al.*, 2001). Attributes that can be monitored include:

Vine vigor (greenness) for better targeting of vineyard practices such as irrigation, fertilization, spraying, pruning and harvesting

Crop condition, so that the winemaker can harvest segments of a vineyard block at optimum ripeness, which also avoids the mix of riper grapes with the less ripe ones increasing the overall value of the harvest
Differentiation between cultivars, ensuring varietal purity at crushing by avoiding the harvesting of inappropriate vines or rows
Plant species discrimination based on chlorophyll a, chlorophyll b, carotenoids and xanthophylls
Regional mapping of vine varieties, which can be used for planning or inventory purposes
Digital elevation models to map features across the topography and existing plantation areas and other features of interest (e.g. dams, vacant blocks, future plantation or improvement sites etc.)

Enology 'Going Green'

In the same way that biodynamic principles are moving into the vineyard, they are also moving into the cellar. Fermention in 'concrete eggs' or 'biodynamic fermenters' is being trialled at a number of establishments around the world According to enthusiastic converts, the shape of the fermenters create a vortex during fermentation, keeping the marc and juice moving, and maximizing contact and extraction without harsh punching down. However, a great number of winemakers have lined their old sunken concrete fermenters with fiberglass or stainless steel because of sanitation and concerns around the level of extraction of minerals from the porous concrete, and these are still valid issues inside the more modern versions. The concrete eggs are also hard to shovel out, and controlling the temperature of the fermentation inside the eggs is almost impossible, with wine needing to be pumped through heat exchangers if there are problems. Other features of biodynamic winemaking are that the use of preservatives is kept to a minimum, filtration is sometimes avoided, and cellar operations are occasionally carried out according to a lunar calendar.

Carbon Footprinting Wineries

Another aspect of 'greener' production trends is highlighted by Theron (2008) in a discussion on carbon footprinting to quantify the impact of environmental pollution by wineries. Theron noted that businesses may in future be compelled by law to reveal their carbon footprint and that the wineries will have to calculate this on a regular basis, and be shown to be making improvements over time if their footprint is too large. The method of calculation, which is very complex, consists of various sections that address specific winery usages or activities. The most important of these include fuel consumption of vehicles, consumption of electricity and other sources of energy, the use of carbon dioxide in the cellar, the use of fertilizers and chemicals, disposal of liquid and solid winery waste, packaging materials such as containers, bottle closures and wood products, and the impact of transportation of wine to the various markets. The wine industries of Australia, California, New Zealand and South Africa have collaborated to develop a method by which a winery may calculate its carbon footprint. The details may be obtained on the websites www.wfa.org.au or www.ipw. co.za. Theron noted that if cellars are expected to make use of this method, it requires serious attention to make it more user friendly, but that it was disconcerting to learn that a number of cellars in South Africa were still unaware of even the basic data such as their own fuel and energy consumption. During 2007, students at Stellenbosch University in South Africa calculated the carbon footprint of three local wineries as a project, using the International Wine Carbon Calculator (2010). The pressing capacity of the cellars ranged from 170 to 11 400 tons and the carbon footprint varied from 0.1 to 1.0 tons of carbon dioxide per ton of grapes pressed. The concept of food miles contributes to the carbon footprint of any product, as it implies that the further the required distance to transport the product, the bigger the carbon footprint. However, this idea is simplistic and studies undertaken in the United Kingdom on New Zealand dairy products, lamb and apples showed that the carbon footprint for these products is four times smaller than similar products produced in the UK, even taking into account the transport, by ship, of those products to the UK (Müller, 2007). Shipping transport should

be used more frequently than air transport for moving goods over long distances to reduce the associated impact on the carbon footprints of products. A Californian entrepreneur, for example, is developing yacht transport for wine, in view of the fact that conventional transport of wine is one of the biggest contributors to the carbon footprint of wineries. Californian Clark Beek intends to use his 40 foot yacht to transport wines from sustainable or biodynamic production from the Napa and Sonoma wine regions to the San Francisco Waterfront. Such transport, combined with his tourism activities, provides a service to ecologically minded wineries and consumers (Theron, 2009b)

Alternatives to Sulfur Dioxide

Sulfur dioxide is fairly ubiquitous in winemaking due to its effective antimicrobial, antioxidant and antioxidasic activities, but is still seen as a necessary evil because it is an additive, and certain individuals are sensitive to its effects. Based on EC Regulation 1991/2004, sulfites must be declared on the label if their concentration in the wine is higher than 10 mg/l. There are alternative practices and additives that can be used in reducing the use of sulfites in winemaking, but the complete elimination of sulfites is improbable. Winemakers have still not yet quantified by how much is it possible to reduce sulfur dioxide levels without risking taste and quality degradation, or increasing microbial contamination or oxidation during vinification or storage. Hyperoxygenation, a massive addition of oxygen or air with the purpose of completely oxidizing all the unstable substances, is another alternative, as is hyperreduction with ascorbic acid or other antioxidants to protect the must from oxidative reactions, but both these methods have associated disadvantages and the possibility of the production of off flavors that may result from their use. Conservation under inert gases such as nitrogen or argon can be useful in the management of the wine level inside steel tanks, but obviously has a cost associated. These gases (as opposed to others such as carbon dioxide) show a low solubility in the wine itself and are able to significantly reduce the concentration of oxygen in the headspace, minimizing the risk of oxidation. The recently introduced practice of coinoculating yeast and lactic acid bacteria permits an effective and simultaneous management of both alcoholic and malolactic fermentation without the need for long periods after fermentation with little sulfur present waiting for the malolactic fermentation to start. An alternative to sulfites is lysozyme (a family of enzymes which affect bacterial cell walls) Around 500 mg/l of this albumin derived protein has the same effect on unwanted lactic bacteria as 40 mg/l of sulfur dioxide. However, as lysozyme is a protein, its use should be carefully considered due to possible interactions with phenolic compounds which may lead to color loss in red wines. It may cause protein instability in white wines, and it can cause allergic reactions.

The use of recently developed electrodialysis bipolar membranes allows acidification to a required pH value, and thus potentially decreases the amount of sulfur dioxide required during winemaking. The bipolar process was successful at inhibiting *Brettanomyces* growth, notably with no or a very low sulfur dioxide addition (total about 10 to 20 mg/l) when the level of acidification was high. The use of the electrodialysis is not yet permitted in commercial wine production (Cottereau, 2010).

An appropriate combination good acidity, antioxidants and antimicrobial agents may help to reduce the amounts of sulfur dioxide necessary for wine protection.

New Methods of Monitoring Oxygen Uptake and Consumption

The importance of oxygen ingress through closures in the formation of oxidized aroma has long been known, and the reliability of closures has been the subject of innumerable studies, notably those by the Australian Wine Research Institute. Previously, oxygen ingress could only be measured through destructive techniques with oxygen sensitive electrodes (Section 4.5.2), but new technology developed by Nomacorc means that a ‘light reactive’ dot can be inserted into a bottle before closure. The dot consists of a substance that reacts

with dissolved oxygen in solution, is then illuminated, and the fluorescence measured. This correlates directly with the amount of oxygen in solution. This way, oxygen consumption or ingress in a closed bottle can be monitored almost indefinitely. The system provides rapid readings of total oxygen content in wine when measuring either high or trace amounts of oxygen and has applicability as a tool for controlling quality at every step in the winemaking process from crushing through postbottling aging. Although the method can only work if the 'dots' are inserted before the bottles are filled, and therefore assume a large measure of preplanning and organization. Research partners, wine producers and analytical laboratories have validated the accuracy and value of this system. See also Section 4.4.3 for further discussion.

Biotechnology in Winemaking

Changing wine styles and consumer demands for better quality products mean that winemakers are constantly on the lookout for new products and techniques to enhance their usual ranges. Using different yeast strains for different winemaking objectives has become routine practice in most establishments with a range of products on offer, for example researchers are currently looking at developing yeast that are able to produce lower levels of alcohol during fermentation from grapes with higher sugar content (Hart and Jolly, 2008). Other yeast producers are attempting to isolate, select and microencapsulate enological native to or indigenous yeasts from several European wine producing regions (Rioja Alavesa, Bordeaux, Chianti and Vinho Verde), in order to allow wineries to control the fermentation process and ensure production of high quality wine while maintaining the typical sensory properties and the aroma profile of each wine area. The usual method employed to improve yeast strains is yeast breeding. However, because of the generalized and uncoordinated way that the genetic material is rearranged during the mating process, many new strains will have none of the desired characteristics and some may even exhibit a few unwanted traits (Styger, 2008). The process of developing yeasts by natural selection and breeding is also time consuming. Second generation biotechnological procedures entail the generation of better biological agents to perform the reaction, through the so-called classical microbiological techniques, such as breeding, mutagenesis and directed evolution. Numerous laboratories around the world are working on the genetic modification of yeast strains with the specific aim of enhancing fermentation performance, facilitating wine processing, manipulating the physicochemical structure of wine and the enhancement of organoleptic qualities (through the intentional loss of negative or gain of positive characters). Novel traits have also been considered, such as the possibility that transgenic yeast may elevate resveratrol or antioxidant concentrations in wine. However, despite the rapid and successful development of genetically modified yeasts, the route to commercialization for transgenic yeasts has been slow. Their future use remains unclear as the negative attitudes of consumers towards GM products has remained unchanged for at least a decade in spite of the fact that research seems to suggest that GM yeasts have very little impact on the environment (Styger, 2008). The whole process of winemaking, using yeast to convert sugar in the form of grape juice into ethanol in the shape of wine, is in fact one of the world's oldest biotechnological processes. Despite this, biotechnology does not have a very good reputation in conservative winemaking circles, and it may be a very long time before they are able to accept that GM products are more beneficial than harmful.

Dealcoholization

It is generally accepted that low to moderate consumption of wine is beneficial to health (Section 5.6.3), but it is still not clear how much the alcohol and the nonalcoholic compounds in the wine contribute to these benefits. Owing to the obvious disadvantages associated with excessive alcohol intake, the partial or total removal of alcohol from wines and other fermented beverages has gained importance in recent times (Ligouri, 2007). The definition of a low alcohol beverage varies between countries. The content for consumers is declared as volume of alcohol per volume of beverage. According to the European Commission, drinks

are described as 'low alcohol' if they contain less than 0.5% alcohol, and 'alcohol free' if they contain less than 0.05% alcohol. A 'wine' needs to contain at least 9% alcohol, and if less than this, the beverage needs to be referred to as a 'wine based drink.' In contrast, the South African Wine and Spirits Board, advises that a 'light' wine should have an alcohol level of less than 10% v:v. In the United States, a low alcohol wine contains 6–7% alcohol. According to the Australian Food Standards Code, a 'low alcohol' beverage must contain no more than 1.15% alcohol by volume (Blackhurst and Marais, 2009).

In a winemaking context, dealcoholization is used for adjusting the alcohol content when grapes have been harvested very ripe, for producing lower alcoholic wines for the growing market, or during the production of nonalcoholic beverages. Winemakers will often pursue the flavor characteristics associated with a high degree of ripeness, and a small adjustment in the alcohol content, between 1 and 2%, is carried out in order to bring the wine into balance, so that high alcohol does not entirely dominate the palate. Although earlier harvesting, and cooler climates can limit the alcoholic content of wine, consumers have become increasingly concerned about health, spawning some interest in low alcohol wines. The decrease in the annual per capita wine consumption in the French, from 120 l in 1959 to 43 l in 2008 (Blackhurst and Marais, 2009), may thus be reversed if palatable, healthier beverages are available. Fermented beverages from which the alcohol has been removed after fermentation theoretically offer traditional beverage flavors without the health risks and side effects of the alcohol, but in practice are often found inferior to their higher alcohol counterpart. The perception that that lower alcohol wines are 'undrinkable' may also result from the fact that wines with higher alcohol content (some containing alcohol concentrations of as high as 16%) are often highly acclaimed by critics.

In the case of wine with moderate reductions, however, it seems that consumers were not able to differentiate between the taste of standard wines with 13–14% alcohol and those with a moderate reduction of alcohol to 10–11% (Blackhurst and Marais, 2009).

There are several methods available for removing or reducing alcohol in wines, like evaporation, cryoconcentration and vacuum distillation. Each process has its advantages and disadvantages in terms of cost and product quality. Technologies such as adsorption on zeolites and supercritical fluid extraction, as well as filtration, using specialized membranes, reduce the ethanol content under mild conditions, so the other organoleptic features of the wine are affected less.

European Union legislation precludes the use of recognized enological practices such as reverse osmosis, distillation, nanofiltration or centrifugation for lowering alcohol concentrations. These practices are allowed in South Africa, Australia and California, but wines may not be exported to the EU. Recently in the UK, the Wine Standards branch of the Food Standards Agency banned an 8% alcohol wine because an unauthorized alcohol lowering process was used**.** Paradoxically, similar beverages made in the USA using these practices may be sold in the EU owing to an EU/US bilateral agreement**.** There is a paucity of information on health benefits of low alcohol wines. The current evidence seems to indicate that ethanol is not vital for providing the health benefits in moderate wine drinking, and therefore reducing the ethanol in wine would not diminish the potential health benefits whilst enabling safer consumption. Genetic modification has the potential to create yeasts that give lower alcohol yields, but natural selection and breeding of yeast with lower sugar to alcohol conversion rates is the route currently used and therefore alcohol reduction is only in very small increments. Additional hybrid breeding and selective screening are ongoing in an attempt to decrease the sugar to alcohol conversion rate in subsequent generations (Hart and Jolly, 2008).

Dealcoholization is discussed more generally in Section 2.13.3.

Wine Packaging

For hundreds of years, glass bottles have been the container of choice for wine, and it is generally agreed by most producers that aluminium, bag in the box (BIB) or polyethylene terephthalate (or PET, thermoplastic

polymer resin) bottles are not appropriate for the presentation of premium wines. Consumers are increasingly aware that if wine is to be transported across the world, emissions of greenhouse gases will be associated with this transport, and that bulkier products involve higher emissions. Industry bodies in several countries have set goals to reduce such emissions and the use of lighter glass and recycled bottles are part of the strategy. Recycling of bottles in California, increased to 28.1% in 2007 compared to 23.5% in 2006 (Theron, 2009c). Bottling bulk wine at the distribution points in the destination country is another strategy that has been adopted widely, and the use of plastic containers for wine has recently met with some consumer approval. PET bottles weigh far less than glass bottles (120 g compared to 550 g), have the same shelf appearance as glass bottles and can be recycled. Glass bottles themselves have decreased in weight considerably, with 750 ml wine bottles closed with screwcaps weighing only 350 g becoming fairly standard in the South African wine industry, for example. The average weight of bottles sealed with corks has also reduced from 570 to 460 g.

The reduction in the weight of the bottles should reduce the carbon emissions created during the transportation and export of bottled wine bottles through lower fuel consumption, but the export of wine in bulk containers is also a fuel efficient alternative used by a number of producers.

Another adaption being taken on by wine producers is to package still wines in bottles without a 'punt' or concave base. This feature is used to strengthen the bottle in the case of sparkling wine (where it will in all likelihood remain a permanent feature), and to help with collection of sediment in red wines. If wines are not going to be under pressure, are not heavy reds likely to drop phenolics or tartrates and are not going to be aged, the feature is superfluous, and a great weight saving can be made by bottling in a flat bottomed bottle.

There is an increase in BIB packaging of wine but although the outer cardboard casing can be recycled, the inner foil bag cannot. TetraPak is an aseptic packaging container consisting of layers of pressed cardboard, an aluminium isolation layer and plastic covering. Recycling of TetraPak differs among countries and may range from 5 to 27% (Theron, 2009c).

To some wine producers, new market requirements or regulatory prescriptions can be restrictive and frustrating, and many fight against considering alternatives to their heavy glass bottles. But to proactive wineries or distributors, such requirements represent opportunities to gain a market advantage, and lighter bottles and alternative packaging ideas are embraced as part of a larger strategy to 'go green.'

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2.10
Fortified Wines and Liqueur Wines

There are as many characters of Port as of people, as many colours as races of mankind, and a good Port lives to be as old as a man.

—Antonio da Silva

2.10.1 Overview and Scope

A fortified wine is one to which an alcoholic spirit (usually grape spirit or brandy) has been added at some point in its production, so that its alcohol content is not derived solely from fermentation. Many fortified wines, such as Port (Section 2.10.7), the sweeter Bual and Malmsey Madeira wines (Section 2.10.5) and the liqueur Muscat wines of France (vins doux naturels, Section 2.10.9) and Australia, are made by adding brandy to the fermenting must, thereby arresting fermentation, increasing the alcohol content and leaving a residual sweetness from unfermented sugar. At what point in the fermentation spirit is added, for a given final ethanol content, determines the degree of sweetness of the finished wine and the volume of spirit that needs to be added: early addition requires much spirit and results in a sweet wine; late addition needs less spirit, but gives a drier wine. At one extreme of this general theme, some fortified wines are made by addition of spirit after fermentation has finished. Sherry and the drier Sercial and Verdelho Madeira wines are examples of this type. Sweeter Sherries are made by addition of 'sweetening wine' (Section 2.10.2) to the fortified dry wine. At the other extreme, fortification involves the addition of wine spirit to the unfermented grape juice. These beverages are known as vins de liqueur (Section 2.10.10), the best known being Pineau de Charentes (Cognac region) and Floc de Gascogne (Armagnac region). Some Australian Muscat Liqueur wines are made with brandy added at a very early stage in the fermentation. There is an equivalent of vin de liqueur in the world of apples and cider: pommeau (Section 2.8.5). One of the original reasons for fortifying wine with spirit was to increase the robustness of the wine, to make it less sensitive to unfavorable environmental factors, thus increasing its life span. In particular, fortification enabled the wine to withstand the rigors of a long journey while still in cask. Nowadays, transport is easier and much faster, and additionally much wine is shipped in bottle rather than in cask, so that much of the original necessity for fortification has vanished. Nevertheless, during the course of evolution of fortified wines, several features of their production gradually resulted in elevated status for many of the wines, which have not only thrived (taking into account fluctuations in the market and fashion), but are still evolving. For example, the development of flor (Section 2.10.3) on the

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
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surface of fino and manzanilla type Sherries, the heating process ('estufagem') used to produce Madeira (Section 2.10.4) and the gentle, but constant agitation of fermenting Port must before mixing with spirit (Section 2.10.5) are all essential to the production of the best fortified wines of those styles. To these can be added the solera system of ageing and blending for Sherry (Section 2.10.2), some Madeira and other wines, the use of fortified or concentrated must for sweetening and adding color to oloroso type Sherries and many other fortified wines and the use of oxidative ageing in the production of certain types of fortified wines, such as oloroso Sherry, Madeira, tawny Port, Marsala and others.

This section reviews the methods of production and salient features of the greatest fortified wines: Sherry, Madeira, Port and the liqueur wines of France, Australia and other countries. Also reviewed here (but more briefly) are a number of other wines, notably Marsala, vin jaune, Vernaccia di Oristano and the wines of Montilla-Moriles and Málaga. Many of these are technically unfortified, but they are considered alongside the fortified wines because they share many of their characteristics. Thus vin jaune and fino Montilla wines (unfortified) share some characteristics with fino Sherry and Vernaccia di Oristano (fortified), because formation of flor velum ('biological ageing') is common to them all. For a fairly recent account of fortified wine, see for example Reader and Dominguez (2003).

2.10.2 Sherry and Other Flor Wines

Sherry is produced in a small area around Jerez de la Frontera, in the province of Cadiz, which lies at the southwestern edge of Andalúcia. The area and methods of production (Denominación de Origen) are controlled by the Consejo Regulador. There are over 11 000 ha of vineyards, which can be increased, provided that stocks held by the many Sherry houses or companies (bodegas) are not diminished by more than 40% in any single year. This is a regulation that is intended to prevent the dominance of quantity over quality. Figure 2.10.1 defines roughly the area of Sherry production: between the towns of Rota, Sanlúcar de Barrameda, Trebujena, Jerez de la Frontera and Puerto Real. This area is divided into a number of subareas (pagos), some of which are shown in Figure 2.10.1, with the Zone of Superior Sherry (Zona de Jerez Superior) forming a rough triangle with Sanlúcar, Jerez and Puerto de Santa María lying at the corners. The soil in this area is recognized as being responsible for the finest Sherry. It is a white highly calcareous soil (known as 'albariza') that not only reflects much sunlight, helping the grapes to ripen, but also bakes hard in the hot summer sun and hence prevents the escape of moisture from the soil around the vine roots (Lichine, 1982a). The pagos of Añina, Balbaina, Carrascal, Macharnudo and Miraflores are mostly in the Superior Zone, having largely albariza soil. Outside the Superior Zone, barro (sand/chalk/clay) and arena (sandy soil with alumina and silica) soils tend to dominate.

The major grape variety for the making of Sherry is the Palomino, although it is known by many synonyms, even in the small area around Jerez. Pedro Ximénez and Moscatel are also grown, mostly for coloring and sweetening, and other varieties include Albillo, Cañocaza and Mantúo. The grape harvest is in September, when midday temperatures are often still around 30 °C, with very little chance of rain; the harvest around inland Jerez starts a few days before that of Sanlúcar, which is closer to the sea. The natural sugar content of Palomino grapes is often about 26 °Brix (or 110 °Oechsle), giving a wine of 14–14.5% alcohol (v:v), when fermented to dryness. Grapes destined for the more delicate fino style wines (Figure 2.10.2) are crushed and pressed soon after gathering, but those meant for the heavier oloroso types are spread out on esparto grass mats to dry in the sun, protected at night by plastic sheets, so that the grapes are not affected by the morning dew (Halliday and Johnson, 1992). Sometimes grapes that are to make fino or manzanilla are dusted with 'yeso' (gypsum, $CaSO_4.2H_2O$) in order to increase the acidity of the must and to encourage the formation of flor, so essential for the production of these Sherry styles (Section 2.10.3). Pedro Ximénez grapes are often exposed on mats to the sun until they shrivel to dark raisins. They are then macerated with brandy to give a

Figure 2.10.1 *Sketch map of Sherry country and list of some notable producers (shippers)*

very dark, sweet liquor known as 'PX,' which is used to add sweetness and color, especially in the formulation of blends for export, such as the dark cream Sherries and brown Sherries. Palomino and other grapes treated in a similar manner give a paler sweet liquor that is used for blends such as pale cream Sherries. It will be seen later that Sherry is a naturally dry wine – the Spanish like it that way and the sweetened blended Sherries are mostly exported.

The many wineries of the Sherry area (most of the big ones are in Jerez) are owned by the Bodegas (Bodegas de Crianza y Expedición, or Sherry shippers). At present (2008) there are 64 of them on the register

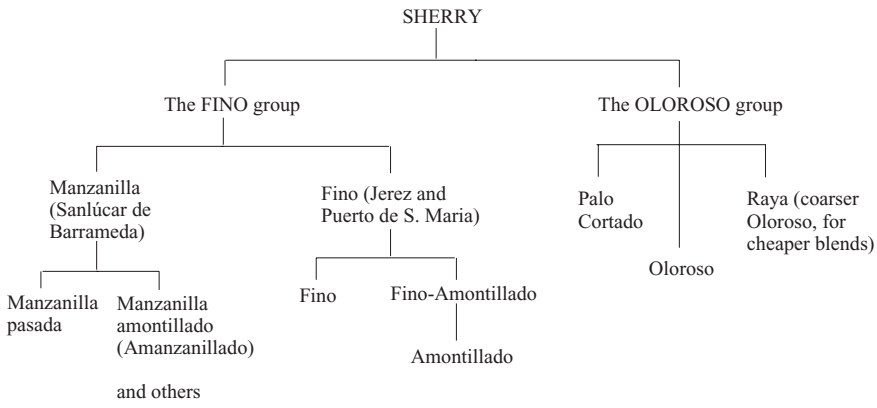


Figure 2.10.2 *The Sherry family. All sherry is naturally dry. Some are sweetened and/or colored for certain brands or styles, mainly for export*

of the Denominación de Origen's regulatory council. Depending on the shipper, the winery is used for the full winemaking process, or as a cellar (or nursery: *crianza* in Spanish) for ageing, the wines having been fermented elsewhere, often in far houses, close to the vineyards. The wineries vary markedly in style, from converted mosques and convents to tall cathedral-like buildings. The latter are preferred for the production of fino and manzanilla, because of the special microclimate and ventilation that are given by that style of architecture (see Figure 2.10.6).

The grapes are tipped onto conveyor belts on arrival at the winery, where they destemmed, gently crushed and then pressed (Figure 2.10.3). Acidity may be increased by the addition of gypsum, which reacts with potassium hydrogen tartrate in the grape must (Figure 2.10.4). Nowadays, tartaric acid is often added in place

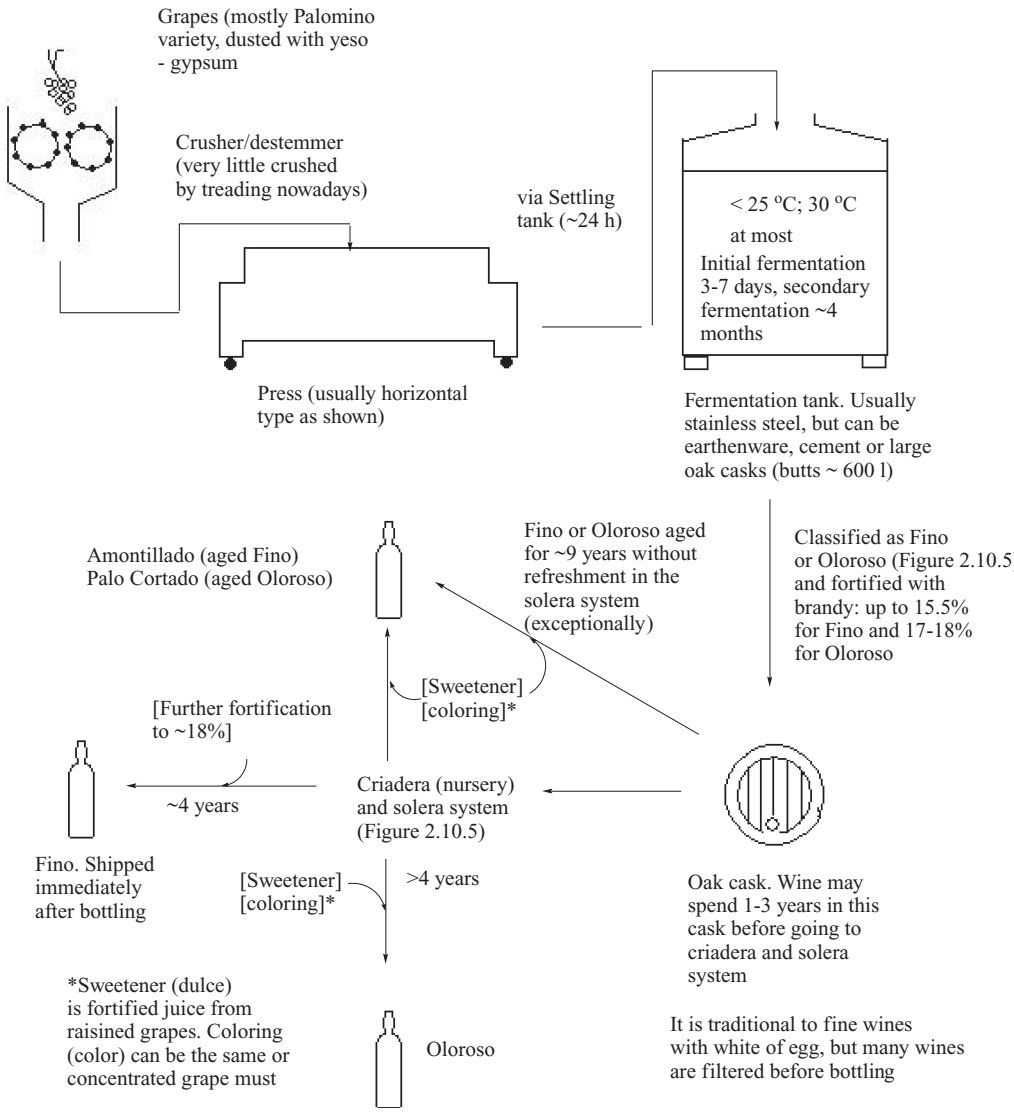


Figure 2.10.3 *Making Sherry: a summary. Square brackets indicate optional processes. Not to scale*

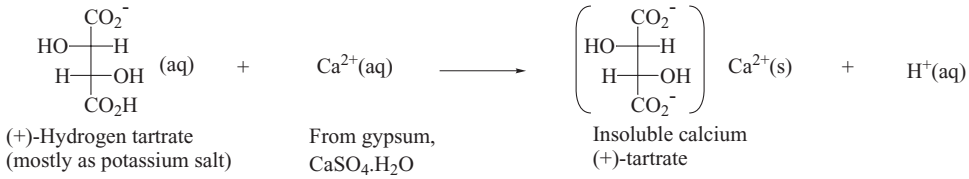


Figure 2.10.4 *Increasing the acidity of Palomino must by addition of gypsum ('yeso')*

of gypsum. Only the first pressing ('mosto de yema') is used for making Sherry (700–750 l per 1000 kg of grapes); the other pressings are used to make wine for distilling into grape spirit, an important part of Sherry. The must ferments vigorously for a few days at ~22 °C for fino and ~27 °C for oloroso. Most Sherry is fermented in stainless steel vats that can be temperature controlled, thus allowing the winemaker a chance to direct production to fino or oloroso for a particular vat. Some shippers still use the traditional method of conducting the first in new oak butts ('botas' ~600 l), the casks being used again later, in the criadera/solera system (Section 2.10.3). After the first stage of fermentation, where ~90% of the grape sugars has been used by the yeast, the wine is racked into oak butts for the gentler second stage of fermentation that lasts until December. Some winemakers allow fermentation to be carried out by wild yeasts in the bloom on the grape skins, using a minimum (if any) sodium or potassium metabisulfite. However, nowadays most winemakers carry out mild sulfiting and then add cultivated wine yeast, which for fino production includes flor yeast (Section 2.10.3). Thus by choice of initial fermentation temperature and yeast strains, the winemaker can direct production of fino and oloroso with reasonable confidence: a process that used to be more or less random. In December, the wines are tested by smell, taste and appearance and are classified as fino (light, floral, pale wines with flor formation) or oloroso (heavier wines with little or no flor formation) (Figure 2.10.3). The wine is then racked off its lees into fresh oak butts that are marked according to the destiny of the wine: fino or oloroso. The casks are filled to only 5/6 of their capacities, after fortifying with grape spirit (~95% ethanol), gradually to avoid shock, up to 15.5% alcohol (v:v) for fino and up to ~17% for oloroso. The vast majority of these casks enter the criadera/solera ageing and blending system, but some shippers produce single vintage (añadas) wines from a few casks that do not enter the solera system. A good example of this is Añadas of Gonzalez Byass. Thus nearly all Sherry is a nonvintage blend of wines of differing ages and also usually of different vineyards, although Valdespino Ynocente comes from a single Macharnudo vineyard (see Figure 2.10.1).

During the maturation of fino Sherry, a characteristic crust or film, known as flor (Section 2.10.3) forms on the entire surface of the wine, usually of 2 or 3 cm thickness. This flor velum protects the wine from oxidation and promotes certain reactions that add to the complexity and quality of the wine. It should be present during the whole of the fino's maturation in the criadera/solera system (four or five years usually). If the flor on a wine in a particular fino cask dies after more than three years maturation in the solera system, the wine becomes rather darker and more pungent due to extra oxidation, and is designated amontillado. This name is derived from Montilla, whose wine it resembles (Section 2.10.4). This cask is no longer included in the fino solera system, instead being blended with similar wines from other casks and usually further fortified with grape spirit. Alternatively, the winemaker can choose to divert a fino to amontillado by increasing the degree of fortification to ~17% ABV, which kills the flor. If the flor dies at an earlier stage in the maturation process, the wine will probably end up in a rather anonymous medium blend (which may actually be called amontillado!), as it is unlikely to be suitable as either a fino or genuine amontillado.

Oloroso wines do not develop a crust of flor because this yeast will not grow in a medium with more than 17 or 17.5% ethanol (v:v); 15.5% ABV is just about the limit. The wine in some oloroso casks may

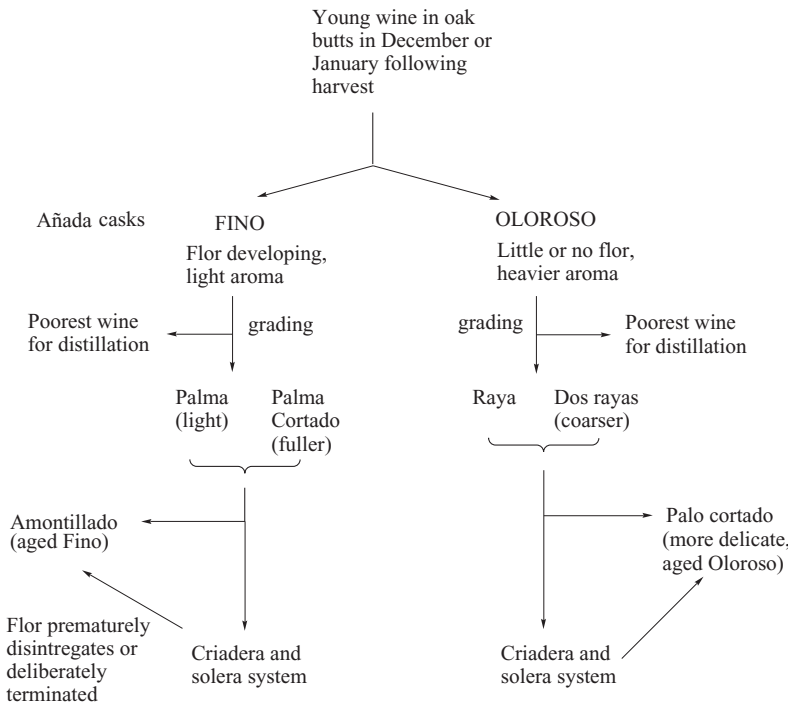


Figure 2.10.5 *Origin of Sherry types. The number of grades of fino and oloroso is higher than that shown. Only four are included here, for clarity*

well have developed a thin velum of flor at the first classification (in December following the harvest), but this is killed by fortification with spirit up to ~17.5% ABV. The lack of flor velum during maturation has two consequences for oloroso wines. Firstly, they are more oxidized than fino wines, having a more pungent and resinous character, as well as being darker. Secondly, through preferential evaporation of water, oloroso wines actually increase in alcoholic strength with age. It is not unknown for oloroso wines that have been in the solera system for more than seven years to have ethanol contents well in excess of 20% (v:v) (Lichine, 1982a). For the same reason old amontillado wines can also have high ethanol contents. In contrast, a film of flor prevents evaporation and, moreover, actually metabolizes a small amount ethanol during its lifetime. Consequently, many fino wines are further fortified (up to 16 or 17% ABV) before being bottled for export. At home in Spain, they are preferred at their natural strength (~15% ABV).

Before wines in the añada casks are entered into the criadera/solera system, they are further graded (Figures 2.10.3 and 2.10.5) according to their likely qualities, after inspection by skilled tasters. This means that the wine in a particular añada cask will enter a solera system (in a particular bodega, there will be many of these) judged to be of similar quality and characteristics. Thus, fino wines may be graded as palma (light and floral) through to tres palma (exceptionally delicate). Heavier finos are classed as palma cortado. Similarly, oloroso wines may be classified as raya (the best) and dos rayas (less good) (Lichine, 1982a). Some lighter, but high quality olorosos with a nutty character may be classed as palo cortado. Tasters play an important role in the production of Sherry: firstly, they grade and classify añada wines to match those already in the solera system and secondly, they are responsible for formulating the blends that eventually go into bottle, particularly for export.

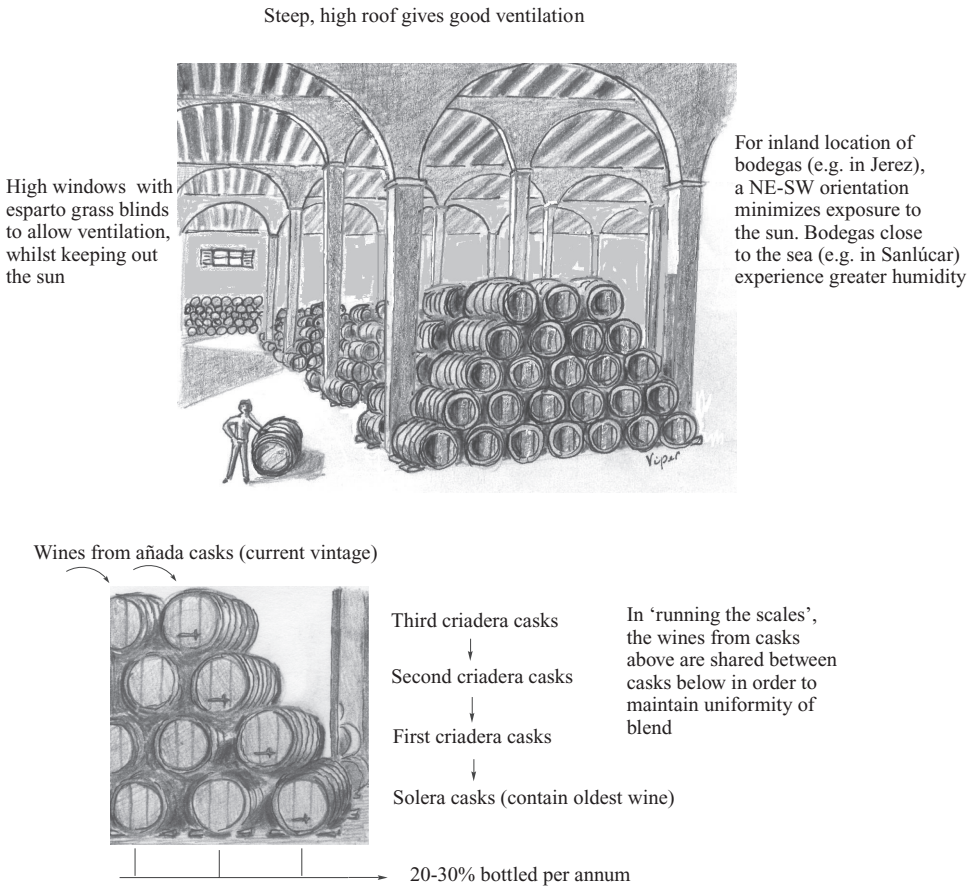


Figure 2.10.6 *Typical Sherry bodega and solera/criadera system*

The solera system is a dynamic ageing method that continuously blends wines of different vintages. Older wines are refreshed from time to time by quantities of younger wines. This ensures that the noble characteristics of the older wines are passed on to the younger wines, whilst at the same time enabling a consistent quality and character. The butts in a solera are arranged in rows (scales), one on top of the other, usually four or five casks high (Figure 2.10.6), but solera systems have more rows than this. The bottom row (on the floor) is known as the solera (the name solera is a corruption of suelo, meaning ground) and the casks here contain the oldest wine. The row of casks resting on the solera casks is called the primera (first) criadera; these butts contain the next oldest wine in the system. The top row of casks, therefore, contains the youngest wine in the system.

Wine for bottling is drawn from the solera butts in such a way that no more than about 20% (~100 l) is taken from each butt at any one time, usually once a year. The ullage in the solera butts, caused by removal of wine for bottling, is replaced by transfer of a similar volume of younger wine from the primera criadera casks immediately above the solera butts. This starts a cascade replenishment/refreshment process known as 'running the scales.' The youngest wines in the top casks are refreshed with wine from the añada casks (the wine from the latest vintage). For consistency of quality and character, a little wine from each cask in a

higher row is added to all the casks of the row below it. In a fino solera system, transfer of wines is carried out using a 'canoe' and 'sprinkler,' which ensure a gentle of trickle of wine, so that the all important layer of flor is undamaged. In oloroso solera systems, this precaution is unnecessary.

In this way, a solera system may be kept in operation for many years, even over a century. It is possible to determine the average age of the solera wine at a particular bottling by knowing the number of scales (rows of casks) in the system, the percentage of wine transferred at each bottling, the frequency of this operation and when the solera was laid down. At the discretion of the regulatory council of the Denomination of Origin, after strict analysis, a shipper may be allowed to state the average age on the labels of certain wines from especially old solera systems. The minimum average age of Sherry is three years, and a typical fino or manzanilla will have had four or five years in the solera system before bottling. Amontillado, oloroso and palo cortado wines are aged longer; upwards of seven years in a solera system is not unusual. The añada wines (fresh wines from the latest vintage) are subject to three possible destinies. Much is used to refresh the wine in the top rows of existing solera systems, some is used to lay down a new solera system and (depending on the shipper) a little is statically matured in cask for the production of a single vintage (añada) wine. The solera system originated in Jerez in the early nineteenth century, but it is not unique to that area. Solera systems of varying kinds are used in Madeira, Malága, Marsala, Montilla-Moriles and in countries that produce Sherry style wines, such as Australia, Cyprus, South Africa and the USA (California).

Sherry is a naturally dry wine and many are bottled as such. Fino and manzanilla are always dry as are many of the best amontillado, oloroso and palo cortado wines. A dry Sherry nowadays often undergoes clarification (often with egg whites) as well as filtration, cold stabilization and then in line sterile filtration as it is bottled under an inert gas to prevent extraneous oxidation. Sulfur dioxide (i.e. metabisulfite) is never used at this stage. Once they are drawn from the solera butts, fino and manzanilla wines have lost their protective velum of flor and hence it is imperative that these wines are not exposed to oxidation. The best way to prevent oxidation is to drink the wines 'young' (i.e. fresh from the cask); indeed to sample the best fino or manzanilla wines directly from the cask is one of the greatest wine experiences. Once in bottle, fino and manzanilla wines should not be kept for more than a few months, and once the bottle is opened, its contents should be consumed within a few days. That is why half bottles (375 ml) of these wines are popular in Spain.

In contrast, amontillado, oloroso and palo cortado wines will keep for many years in bottle and remain vital for a long time after the bottle is opened. Many wines of the oloroso type are sweetened and maybe colored before being bottled, as there is still a considerable demand for sweet Sherry in countries such as the UK and USA.

Apart from the Sherry shippers (bodegas de crianza y expedición) in the Sherry area (Jerez de la Frontera), El Puerto de Santa Maria and Sanlúcar de Barrameda, see Figure 2.10.1), there are smaller companies that make and mature wines in private cellars exclusively for sale to the shippers. These firms are known as bodegas de crianza y almacenado or simply as 'almacenistas.'

Sherry style wines are made all over the world, especially in regions with similar hot climates, such as Central Valley, California; Cyprus; New South Wales and South Africa. Often grapes of local varieties are used, rather than Palomino or Pedro Ximénez grapes. Examples include Gordo Blanco (New South Wales), Flame Tokay and Mission (California), Steen, also known as Chenin Blanc (South Africa) and Xynisteri (Cyprus). The emphasis is on medium to sweet oxidatively aged and moderately priced wines of moderate or even modest quality. A common practice of accelerating the ageing process for Sherry style wines in California is to heat the wine in bulk (up to 60 °C) and then leave it to mature in small casks in the cellar. Another way, practiced in Cyprus, is to expose casks of wine to the sun for two or three years. However, higher quality wines are being inceasingly made from Palomino grapes with flor formation, biological ageing and solera methods of maturation and blending. Notable amongst these is Llords and Elwoods (San José, California), Keo (Cyprus) and Cavendish (South Africa). In South Australia (Barossa), the Seppelt winery

produces flor Sherry style wines (a fino and amontillado type) and an oloroso style wine from Palomino grapes, using solera systems. These wines have a fragrance, nuttiness and rancio character (respectively) that are reminiscent of Jerez wines, despite differences in climate and soil types. Similarly, the Quady winery (San Joaquin, California) makes a solera aged amontillado style flor wine from Palomino grapes. Presumably this wine is produced by a period of biological ageing under a velum of flor, followed by oxidative ageing after removal of the flor, as in Jerez.

2.10.3 Flor and Oxidation in Sherry Wines

Flor is the name of the layer (velum) that can form on the surface of white wines, provided the alcohol content does not exceed about 15% (v:v). It is essential for the production of fino and manzanilla Sherries, Montilla Moriles wines, Vernaccio di Oristano and vin jaune. Wines like these that are aged under a layer of flor, protected from oxygen, are said to 'biologically aged.' In contrast, most other wines are aged in the presence of oxygen (albeit in a controlled manner) and are said to be oxidatively aged. In this section, the oxidatively aged wines include oloroso Sherry, Madeira wines, tawny Port wines, Marsala and vins doux naturels. The velum of flor not only protects the wine from extraneous oxidation and spoilage by microorganisms such as *Acetobacter*, but produces certain attractive aroma/flavor compounds, notable amongst which are Sherry lactones, like solorone. It also reduces the ethanol content somewhat, because ethanol forms a small part of the metabolism of the flor yeast. Under the same conditions, the same white wine without flor would undergo enzymic and nonenzymic browning, and infection by microorganisms may also occur. Flor is killed at ethanol concentrations greater than 15–15.5% (v:v), so oxidatively aged wines are usually fortified to 17–18% ABV. Under these conditions, *Acetobacter* and other spoilage organisms are inhibited, but oxidation results in browning and the formation of pleasant characteristic aroma and flavor compounds.

Fino and manzanilla Sherries can spend four years or so under a velum of flor. If the velum begins to grow thin or shows signs of dying before the end of the maturation period, the cellarmaster in many Bodegas may increase the alcoholic strength to 17–18% (v:v) by adding grape spirit. Ultimately, this produces an amontillado Sherry, a bit darker and more oxidized than a fino, because of a number of years of ageing without the protection of the flor velum. During the ageing of fino Sherries, the dead flor yest cells are continuously shed and fall to the bottom of the cask, being replaced by younger cells in the velum; the life cycle is similar to that described in Chapter 2.1. The layer of dead cells gradually release peptides and glycoproteins, providing nutrients for the living cells in the velum and inhibiting crystallization of tartrates. Many species of the genus *Saccharomyces* are able to form flor layers; most of these are races of *S. cerevisiae*, but *S. bayanus* can form a flor velum, albeit a rather thin one (Mauricio and Ortega, 1997). In the making of fino Sherry wines, the main races of *S. cerevisiae* are beticus, cheresiensis and montuliensis (Martinez *et al*., 1998). The first two mentioned produce flor velum rapidly and hence tend to predominate in young wines, whereas the last mentioned, which produces higher acetaldehyde concentrations, appears in older wines in the solera system (Martinez *et al*., 1998). These *S. cerevisiae* races usually make up the bulk of some 1126 different microorganisms that been identified in fermenting Sherry (Esteve-Zarzoso *et al.*, 2001), but *Candida stellata*, *Dekkera anomola*, *Hanseniaspora guilliermondii*, *H. uvarum* and *Issatchenkia terricola* have also been identified in frequencies higher than 2%. Most bodegas use inoculation of commercial flor yeast strains rather than rely on the wild yeasts for the formation of fino type Sherries, so that whatever strain is used dominates the microflora of the solera system (Esteve-Zarzoso *et al.*, 2001). In this way, the organoleptic qualities of the wine can be controlled up to a certain point (Martinez *et al.*, 1997).

Molecular characterization of flor strains by karyotype and mitochondrial DNA (mtDNA) restriction analysis usually indicates high polymorphism, but with certain patterns predominating (Mesa *et al.*, 2000),

usually corresponding to a small number of genotypes. Flor yeasts give different mtDNA restriction patterns to nonflor yeasts (Esteve-Zarzoso *et al.*, 2001). Further to this, a restriction analysis of the 5.8S ITS region from a large number of *Saccharomyces* flor strains (isolated from the velum of Sherry wines from different wineries) indicated that they all had the restriction patterns using endonuclease cfo I, thus suggesting the universal presence of the 24-bp deletion (Fernandez-Espinar *et al.*, 2000). The above results suggest the genetic origins for the observed adaptive mechanisms shown by flor yeast strains compared with nonflor strains. These are expressed as morphological, physiological and chemical characteristics, which are discussed next.

Flor yeast cells show differences in their cell shape, size and hydrophobicity compared with nonflor strains of *S. cerevisiae*. Hydrophobic interactions between cell surfaces allow the cells to cluster into colonies that are less dense than the wine, especially because of the buoyancy caused by trapped CO_2 bubbles released by metabolic processes (Martinez *et al.*, 1997). The population consequently floats and grows on the surface of the wine. Flor yeast strains are tolerant of ethanol concentrations of up to about 15.5 % (v:v) and are also tolerant (to a greater or lesser degree) of acetaldehyde and acetic acid (often known as 'volatile acid'). Different strains have different tolerances of these components: the cheriensis race is less tolerant of ethanol and acetaldehyde, but is more tolerant of acetic acid than the beticus strain (Mesa *et al.*, 2000). The race montuliensis is likewise more tolerant of acetaldehyde than the race cheriensis. These generally high tolerances of metabolic products have been shown to be the result of an especially efficient antioxidant defence system of flor yeasts, which is related to their generally high superoxide dismutase activity (see also Section 5.8.4) (Castrejon *et al.*, 2002). This confers a high resistance to oxidative stress in general and a tolerance of ethanol and acetaldehyde in particular.

The growing cells in a flor velum obtain most of their energy requirements from aerobic metabolism, during which specific nutrients from the wine are required. A modest amount of ethanol is used as a carbon source, but there is high uptake of acetic acid, ethyl acetate and glycerol (Berlanga *et al.*, 2006). Thus, the ethanol content decreases somewhat (by about 0.5–1.0% v:v) during biological maturation of a fino Sherry and there is a significant level of acetaldehyde in the mature wine. Dissolved oxygen in the wine is depleted during growth of the flor velum, but once this has gone, nitrogen compounds (mainly amino acids and ammonium salts) are taken up by the yeast cells and used as nitrogen sources or as electron acceptors. Amino acids are metabolized to more reduced amino acids (especially (*S*)-leucine), carbonyl compounds and alcohols, including acetoin (2,3-butanediol), 1-propanol, isoamyl alcohol and isobutanol (Martinez *et al.*, 1998; Mauricio and Ortega, 1997; Berlanga *et al.*, 2006). Some of these metabolic products make important contributions the aroma and flavor of fino Sherry. The amino acids in wine are of diverse origin: some come from the original must, others are synthesized by yeast during growth and yet others are released by yeast cell autolysis or by redox regulation (Berlanga *et al.*, 2006). The principal source of nitrogen for growing flor yeast cells is (*S*)-(or L)-proline (Mauricio and Ortega, 1997). The general metabolism of flor yeast cells may well be regulated by the most efficient balancing of redox potentials, coenzyme reoxidation pathways (e.g. $NADH \rightarrow NAD^+ + H^+ + 2e^-$) being used as alternatives to the electron transport chain (Section 5.8.3). It has been found that supplementation of amino acids led to an enhanced release of certain metabolic end products, depending on the supplement. Thus, all supplements led to the release of L-leucine, L-glutamate led to enhancement of ethyl acetate, L-leucine to isoamayl alcohol and L-valine to isobutanol. 1,1-Diethoxyethane was also produced (Berlanga *et al.*, 2006).

The concentrations of characteristic odor compounds in fino Sherries depend not only on the amount of nutrients present, but also on the status of biological ageing; indeed the concentrations of certain aroma/flavor compounds can be used as an indicator of the extent of biological ageing (Zea *et al.*, 2007). By using statistical analysis of concentrations of aroma compounds in fino wines of different biological ageing (1, 3 and 5 years), it was found that sotolon, 1,1-diethoxyethane and 2-whisky lactone were the best indicators of biological ageing (Moreno *et al.*, 2005).

Ageing of oloroso Sherry wines occurs in oak butts in the absence of flor velum; it is often known as oxidative ageing and leads to a darker product due to 'browning' and to a different aroma profile to corresponding biologically aged wines. Oxidative browning mechanisms have been reviewed recently (Li *et al.*, 2008). Browning is undesirable in most white wines, including fino Sherries, but forms an essential part of the ageing process for oloroso Sherries and certain other wines, such as Madeira (Section 2.10.5) and the 'rancio' styles of vins doux naturels (Section 2.10.9). The major cause of browning in wine is the presence of many phenolic substances, especially flavan-3-ols, which are able to undergo regenerative coupled oxidation reactions, as well as nonoxidative browning reactions (polymerizations) (Li *et al.*, 2008). Nonenzymic browning appears to be prevalent in wine, whereas enzymic browning tends to be more important in grape must. Both general mechanisms require the presence of Fe^{2+} and Cu^{2+} ions. Polyphenol oxidase is a copper containing enzyme and peroxidase contains iron (Section 5.8.4); these and tyrosinase (catecholase) are the major agents for enzymic browning. Nonenzymic browning mechanisms involve Fe catalyzed oxidation of flavan-3-ols to *o*-benzoquinones, ethanol to acetaldehyde, and (+)-tartaric acid to glyoxalic acid. The quinones can form polymer pigments by reaction with phenols and it is known that both acetaldehyde and glyoxylic acid can be involved in flavan-3-ol polymerization (Li *et al.*, 2008, Lopez-Toledano *et al.*, 2004).

Although browning is an essential feature of the ageing of oloroso wines, it is most undesirable in biologically aged wines, such as fino Sherries and fino Montilla-Moriles wines. In order to stabilize browning, by the removal of flavan-3-ols, these wines are fined and often filtered before bottling. Fining agents include activated charcoal, PVPP and a mixture known as Riduxhigh™: these all remove most of the flavan-3-ols after initial fining with casein or bentonite (Barón *et al.*, 2000). Dehydrated baker's yeast cells have been used for a similar purpose, the cells absorbing many brown compounds and flavan-3-ol derivatives (Razmkhab *et al.*, 2002). Likewise, yeast cells immobilized on carageenan gel were effective in reducing the concentration of browning compounds, without drastic modification of the wine's sensory properties (Lopez-Toledano *et al.*, 2004).

Oloroso wines have different aroma profiles to fino and similar wines, whereas amontillado wines possess profiles that are intermediate between those of fino and oloroso wines. The differences in profiles have been linked to the differences in the extents of biological versus oxidative ageing for wines of the Montilla-Moriles region, which are similar to Sherry (Zea *et al.*, 2001). Thus, fino wines were richer in acetaldehyde, acetoin, carboxylic acids and lactones, whereas oloroso wines were richer in ethyl acetate, other acetate esters, fusel alcohols, diethyl malate, diethyl succinate and ethyl lactate (Zea *et al.*, 2001). More specifically, the aroma compounds that best distinguished fino wines were butanoic acid, 3-methylbutanoic acid, γ-butyrolactone, pantolactone, γ-decalactone, farnesol and octanal. On the other hand, compounds that distinguished oloroso wines included 1-butanol, 2-butanol, methyl acetate, ethyl acetate, butyl acetate, hexyl hexanoate, hexyl lactate, isobutanoic acid, furfural and methionol. The contribution of flor yeasts and oak to the aroma profile of fino wines can be organized into eight ordorant series: balsamic, chemical, empyreumatic, floral, fruity, spicy and vegetable (Zea *et al.*, 2001). During biological ageing, the fruity series (related to flor yeast activity) and spicy series (related to release of oak compounds) most closely represented the changes in aroma.

Maintaining wines in the criadera/solera systems for at least four years, despite the advantages that have been described, is a costly business, making it impossible to reduce the price of Sherry or similar wines, such as those of Montilla-Moriles. For this reason, there has been some interest in methods of accelerated biological ageing and experiments have been conducted with fino style wines on laboratory and pilot plant scales. Recently, experiments involving periodic and controlled microaerations of fino wines (without disturbing the flor) produced, in a few months, wines that had similar aroma profiles to cellar aged wines (Muñoz *et al.*, 2007). Thus biological ageing time can be shortened by controlled periodic microaerations in stainless steel tanks, followed by a period in oak cask.

2.10.4 Other Flor Wines

The fino style wines of the Montilla-Moriles region, near Córdoba, are the other great flor wines of Spain. This area, being some 170 km northeast of Jerez and well away from the influence of sea breezes, experiences even hotter summers than the Jerez area. Pedro Ximinez is the major grape variety, rather than Palomino, and the grapes are usually harvested in early, to mid August. Although Montilla-Moriles wines have a distinctive character that sets them apart from Sherry wines, their methods of production are similar to those of Sherry and the family resemblance is clear to those who taste the wines. Similarities of production include biological ageing for fino wines, oxidative ageing for oloroso wines and the use of criadera/solera systems, so much that has been written in Section 2.10.3 applies to the wines of Montilla-Moriles. Indeed, production similarity is such that there is much discussion of these wines, alongside Sherry wines, in Section 2.10.3. The major differences include main grape variety, climate, terroir and the fermentative organisms, especially the flor yeasts. The Pedro Ximinez grapes are harvested some weeks earlier than Palomino grapes in Jerez. After drying in the sun for some time, they can make a wine containing up to 16% ethanol (v:v), thus removing the need for fortification with grape spirit for the fino style wines. The amontillado and oloroso wines, like their Jerez equivalents, are fortified up to about 18% ABV. Another interesting difference (although one which probably has little bearing on organoleptic differences between the wines of Montilla and Jerez) is the occurrence of fermentation in earthenware jars (tinajas), in the more traditional bodegas. However, many bodegas, like their Jerez counterparts, now use stainless steel fermentation vessels. Ageing is carried out in oak butts arranged in a criadera/solera system, as in Jerez. Also, like Sherry, the wines of Montilla-Moriles are naturally dry, but can be sweetened and given a deeper color by the addition of dolce and vino de color, respectively. Montilla-Moriles wines do not have such a strong export tradition as Sherry, probably because of the relative remoteness of the vineyards from the sea. They do, however, enjoy a great reputation in Spain. Principal bodegas include Alvear, Carbonell and Montuila.

Veranaccio di Oristano is a flor wine of about 15% ABV made on the island of Sardinia from Vernaccio grapes. Traditionally, the flor is formed after the first vigorous fermentation by wild yeasts in the bloom of the grapes. This was rather haphazard and led to variations in style and quality. In the past few decades, inoculation of the lightly sulfited must with flor forming yeast strains (mainly *Saccharomyces bayanus*, *S. cerevisiae* var. *prostoservodii* or *S. cerevisiae* var. *rouxii*) has become common for biologically aged fino style wines (Galletti and Carnacini, 1996). Inoculation accelerates the biological ageing process and makes it easier to control. *S. cerevisiae* var. *prostoservodii* was generally more successful at producing desirable ester components than *S. bayanus*, but inoculation with either led to a wine richer in desirable aroma components than a corresponding wine aged under sterile conditions (Carnacini *et al.*, 1997). Fortified liquoroso type Vernaccio di Oristano wines, with ethanol contents up to ∼18% (v:v) are also made. Wines aged for a minimum of four years in oak casks are called Riserva, provided that the ethanol content is at least 16% (v:v). Three year old wine (again oak cask aged) of at least 15.5% ABV is called Superiore. Major producers include Cantina Sociale della Vernaccia, Carta, Contini, Puddu, and Sella and Mosca.

Vin Jaune is a flor wine made from grapes of the vine variety Savagnin (called Gewürztraminer or traminer elsewhere) in Château-Chalon and L'Étoile, two small areas in the Jura region of France, between Burgundy and Switzerland. The grapes are harvested late, giving a must of high sugar content, which is fermented to dryness, producing a wine with up to 15% ethanol (v:v). The wine is stored in incompletely filled oak casks, whence a velum of flor develops and promotes the type of biological ageing described in Section 2.10.3. The wine remains in these casks undisturbed for up to six years, after which time it is bottled. Although it has its own unique character, vin jaune is rather like a cool climate version of fino or manzanilla, perhaps with the nutty aroma more reminiscent of an amontillado. The distant similarity with Sherry must result from biological ageing under a velum of flor, since most other factors are different: soil, climate, grape variety and static maturation, as opposed to to the dynamic criadera/solera system of maturation.

Table 2.10.1 *The main vine varieties of Madeira and the styles of wine they produce*

Variety	Main pseudonyms (probable or *possible* origins)	Type of wine produced	Approx. alcohol content in % v:v (and residual sugar in ° Brix) after final fortification
Sercial	White Riesling, Rheinriesling, Esganinho (Rhineland)	Sercial (dry)	17 (0.9–2.7)
Verdelho	Gouveio, Vidonia (Portugal)	Verdelho (medium dry) and 'Rainwater'	17 (2.7–4.5)
Bual	Boal (Portugal)	Bual (medium)	17 (4.5–5.5)
Malmsey	Malvasia (Turkey)	Malmsey	17–18 (5.5–12)
Tinto Negra Mole	(Iberian Peninsular)	Malmsey*	17–18 (5.5–12)

*Since 1986, must be minor component of Malmsey (<15%)

2.10.5 Madeira

Madeira wine is made from the grapes of five principal *Vitis vinifera* varieties growing mostly on terraces on the steep volcanic hillsides of the island of Madeira (57 km × 23 km), lying some 575 km off the coast of Morocco and some 850 km distant from Lisbon. Madeira and its neighboring islands, Porto-Santo and the Desertas, belong to Portugal and so production of its wines, like those of the mother country, is governed by European Union regulations. The volcanic soil of Madeira is very rich, indeed enriched by the ashes of thousands of trees that were burnt by the explorer João Gonçalves ('Zarco') in the early fifteenth century. Small clusters of trees from the ancient forest still grow around the island, amongst the vines and many other agricultural crops. The rich soil and moist warm climate influence the quality of the final product, but a greater influence is exerted by the grape varieties and the altitudes at which they are grown, together with the highly specific ways in which Madeira wines are made, especially the heating process known as estufagem.

The main varieties are all of the species *V. vinifera* and are described in Table 2.10.1. With the general exception of Tinta Negra Mole, Madeira wines are varietal and bear the name of the variety: Sercial, Verdelho, Bual (Boal) and Malmsey (Malvasia). Tinto Negra Mole (the 'soft black' grape) is now recognized as a good, but not classic variety for Madeira wine production. It used to be a substantial, even major part of the island's sweet Malmsey wines, along with Malvasia, which gives the wine its name. This has changed since Portugal entered the European Union in 1986, whose wine laws state that wines named after a specific grape variety must contain a minimum of 85% of that variety. Nowadays, most Tinta Negra Mole vines are grown at low altitudes, where they ripen early (from mid August onwards). Much of their fruit is made into the cheaper Madeira wines that are so popular for cooking, by an accelerated estufagem process (Section 2.10.6). This was once the role of Jacquet or Black Spanish, a hybrid (*V. vinifera* x *V. aestvalis*, probably) that produces black skinned and red juiced grapes. The vines on Madeira were especially badly affected by *Phylloxera vastatrix* in the early 1870s and so hybrids like Jacquet, Canica (Cunningham) and Isabellinha played important roles both as rootstocks for the *V. vinifera* varieties and as direct producers of wine. Today, they are all on the wane, since the European Union generally forbids the use of hybrids or non-*vinifera* species for the production of quality wine. Table wines for local consumption still originate from hybrid vines and also from minor *V. vinifera* varieties, such as Barrete de Pedre, Carão de Moca, Listrão, Terrantez and Triunfo. Newer, but still

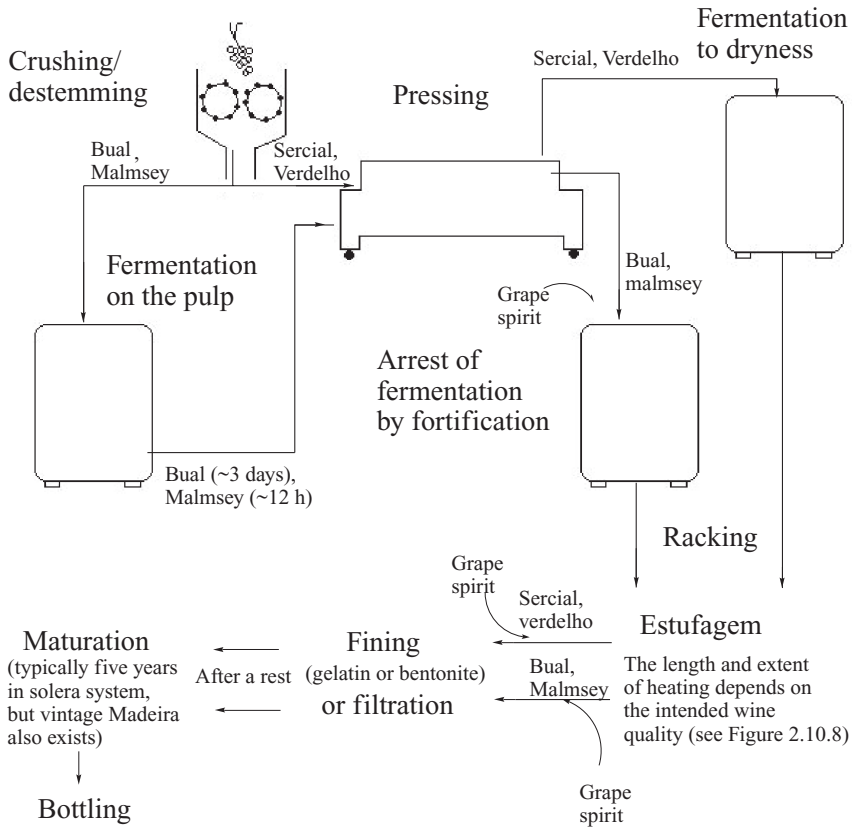


Figure 2.10.7 *Making Madeira wine. Not drawn to scale*

minor varieties used for Madeira wines include Complexa and Tinto de Madeira. The vineyards of Madeira stretch from sea level up to ~700 m or so above sea level. This, and the five varieties having different natural ripening times, necessitates many different harvest times on the island. The first grapes, Tinta Negra Mole grown at low altitude are picked in early August. The last grapes to be picked are usually those of Sercial vines growing around the 600 m or 700 m contours, often harvested in late October.

The traditional method of crushing grapes by foot in lagares (large wooden troughs) is uncommon now; most grapes are crushed and destemmed in an electrically powered crusher/destemmer (see Section 2.9.1). Sercial and Verdelho grapes are pressed (separately) immediately after crushing and destemming and are fermented to dryness (Figure 2.10.7). Bual and Malmsey grapes, on the other hand, are fermented on the pulp (again separately) for a period of time (Figure 2.10.7): until 50% of the sugar is fermented for Bual and for Malmsey until only 25% of the sugar has been used up. At these points, the fermentation is arrested by the addition of grape spirit, either before or after pressing, depending on the winery practices. After this, all the varietal wines are treated in similar ways, as indicated in Figure 2.10.7. They all go through a heating process known as estufagem and they are all fortified with grape spirit to bring their ethanol contents up to 17–18% (v:v). Some Verdelho wines are also colored and sweetened with vinho surdo, a mixture of concentrated unfermented must and grape spirit. Tinto Negra Mole grapes that are destined for the cheapest cooking Madeira wines, are treated somewhat differently from those shown in Figure 2.10.7. They are crushed and

pressed immediately, their musts are fermented to dryness and the wines are sweetened and fortified up to ~17% ABV by addition of vinho surdo, which is most likely made from Tinto Negra Mole must anyway. The wines are then given an accelerated estufagem (Section 2.10.6) in concrete vats, heated to 50 °C by a heating coil for three months.

After emerging from the estufa and having had 12–18 months rest (including fining and filtration), most young Madeira wines of medium to best quality are entered into a fractional or dynamic blending and ageing system, like the solera system of Jerez (Section 2.10.2). A few special wines are statically matured to produce a vintage Madeira wine, sometimes residing in the same cask for 30 or more years – but these wines are very rare. The really original part of Madeira wine production is the heating process known as estufagem. It is this process that gives the wines most of their special characteristics.

2.10.6 Estufagem and Cask Maturation of Madeira Wine

Like Sherry, the wines of Madeira were originally given fortification with grape spirit to preserve them on long sea voyages in cask to Great Britain or much further afield to the American continent and the Caribbean islands. It was found that these voyages, sometimes lasting six months or so, improved the wine quality remarkably. The high temperatures and wide fluctuations in temperature encountered on these long journeys, followed by a long period of rest (still in cask) at ordinary cellar temperatures, transformed the wines into something altogether better than those that started the journey. So, up until the latter half of the nineteenth century, Madeira wines were deliberately shipped around the world before landing at their destinations, often back home in Madeira, because more and more of the wine was being bottled on the island. However, nowadays, the wines in cask undergo a process known as estufagem in the wine shippers' lodges, mostly in Funchal, the island capital. Estufagem is intended to simulate the temperature conditions experienced by a wine that has been taken on a long sea voyage through tropical or subtropical zones. There are several versions of estufagem, in terms of heat intensity, fluctuation and duration, and which regime is chosen depends on the intended quality of the finished wine, as outlined in Figure 2.10.8. The majority of wines are subjected to the two regimes at the bottom of Figure 2.10.8, both of which involve the application of heat. The better quality wines are racked into 650 l casks (pipes), which are stacked in a building known as armazen de calor. The building is centrally heated by hot water ducts that maintain a temperature of around 40 °C for 6–12 months.

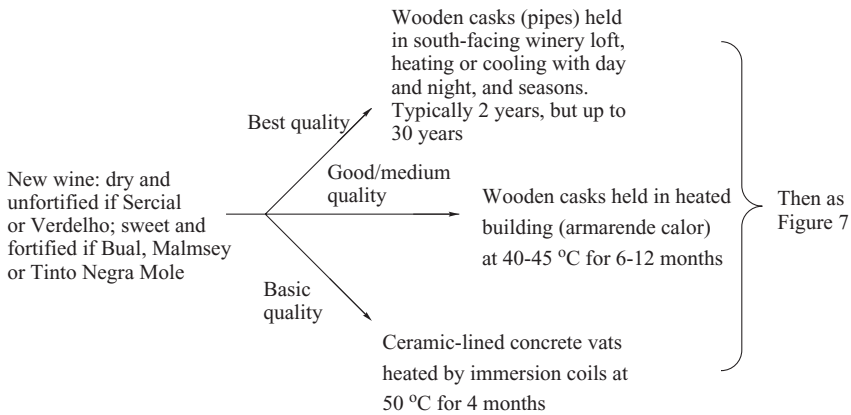
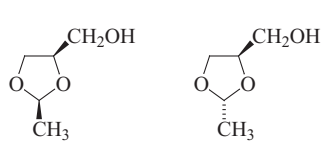
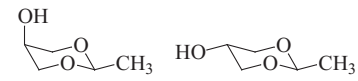


Figure 2.10.8 *Types of estufagem*



cis- and *trans*-4-Hydroxymethyl-2-methyl-1,3-dioxolane



cis- and *trans*-5-Hydroxy-2-methyl-1,3-dioxane

Figure 2.10.9 *Heterocyclic acetals in Madeira wines. Câmara* et al. *(2003)*

Wines destined to be the most basic are pumped into 500 hl ceramic lined concrete vats, where they are heated at 50 °C for about three months by electrical immersion coils. Wines that have been heated naturally, in south facing winery lofts or in large glass carboys kept outside in the sunshine (Figure 2.10.8) are called canteiro. Some of the best Madeira wines, including rare vintage wines, are produced by the canteiro method. Whatever the estufagem method, the wines are gradually allowed to cool to ambient temperatures over a period of a few weeks, whereupon they are fined and/or filtered, allowed a 12–18 month rest (estágio) and the best wines are entered into a solera system for four or five years, whereas the more basic wines may be blended and bottled after estágio (Figure 2.10.7).

During estufagem the relatively high temperatures and the presence of oxygen promote caramelization, Maillard reactions (Section 2.6.2) and oxidation, resulting in profound changes in aroma and flavor. Notable amongst these changes are enhanced concentrations of furans (Ho *et al.*, 1999). In particular, 5-hydroxymethyl-2-furaldehyde (5-HMF) was found in far greater concentration (361 mg/l) in 10 year old Madeira than in Banyuls, Port, Sherry and Tokay wines (average 61 mg/l) that had received long cask maturation times. However, the presence of many other volatile substances is more likely attributable to extensive cask ageing than to estufagem itself. Included here are heterocyclic acetals (1,3-dioxolanes and 1,3-dioxanes). In a study of Madeira wines of varying cask ages, Câmara *et al.* (2003) found a good linear correlation between the concentrations of the *cis*-1,3-dioxolane (I) and the *cis*-1,3-dioxane (II) (Figure 2.10.9) and cask age. These heterocyclic acetals are well known to be formed from acetaldehyde and glycerol, and similar ratios of *cis:trans* isomers have been found in Port wines. Concentrations of all four heterocyles were not significantly affected by the extent of estufagem. Similarly, an excellent correlation between the concentration of *cis*-oak lactone and the length of cask ageing of Madeira wines, was found by Alves *et al.* (2005).

Oxidized non-Madeira white wines have been found to possess enhanced levels of methional (3-thiomethylpropanal) and this compound has been demonstrated to be responsible for a cooked vegetable off flavor. Methional is thought to originate from the α-amino acid methionine by Strecker degradation, probably brought about by *o*-benzoquinones (oxidized polyphenols, probably flavan-3-ols) (Escudero *et al.*, 2000) (Figure 2.10.10). Recent studies of Madeira wine aroma profiles using the highly sensitive solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE), followed by GC/MS (see Sections 4.2.4 and 4.3.2) (Alves *et al.*, 2005) have revealed only low levels of methional. This may indicate that extended exposure to temperatures in excess of 40 °C and extended ageing in oak casks result in the loss of methional by evaporation and/or degradation. Phenylacetaldehyde is another Strecker aldehyde that has been found in oxidized white wines (da Silva Ferreira *et al.*, 2007), presumably produced by the Strecker degradation of phenylalanine. Moreover, da Silva Ferreira and coworkers (2007) were able to detect α-keto-γ-(methylthio)butyric acid and β-phenylpyruvic acid, as their quinoxaline derivatives, in Madeira wines by HPLC with UV/fluorescence detection (Section 4.3.3) in concentrations up to 5.7 mg/l and 9.6 mg/l, respectively. Thus, for the first time, these α-ketoacids were detected in wine and are presumed to be Maillard intermediates, and in particular intermediates of Strecker degradation (Figure 2.10.10). Other Maillard products have been found at trace levels in Madeira wines from all the major vine varieties. In particular,

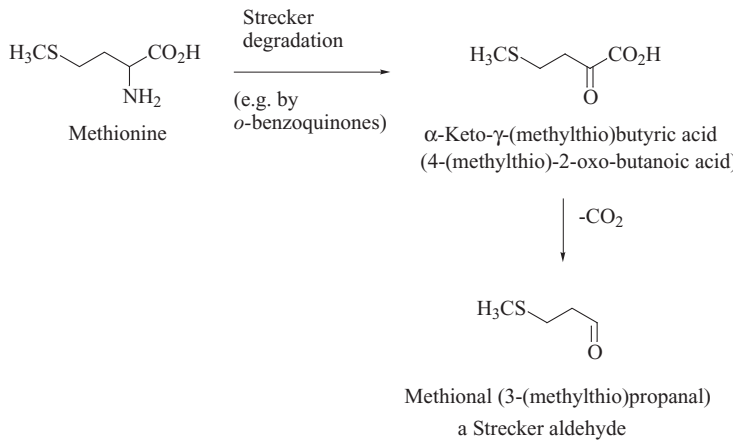


Figure 2.10.10 *Formation of methional during maturation of Madeira wine via Maillard reaction/Strecker degradation. da Silva Ferreira* et al. *(2007)*

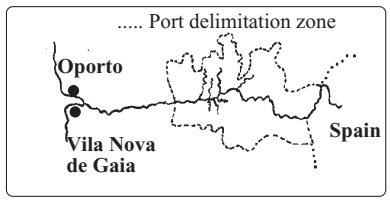
low levels of 5-hydroxyfurfural and 2,3-dihydro-6-methyl-4*H*-pyran-4-one (DDMP) have been found, using SBSE-GC/MS (Alves *et al.*, 2005). This highly sensitive technique was able to detect trace amounts of *cis*- and *trans*-oak lactones in some young wines and at quantifiable levels in older wines.

Apart from the Maillard products mentioned above, analysis of the volatile components of young Madeira wines has revealed the presence of many compounds that have been found in other wines (Alves *et al.*, 2005; Câmara *et al.*, 2007). Prominent amongst these were esters, with the most abundant being ethyl hexanoate, diethyl succinate, ethyl octanoate and ethyl decanoate (the last named not in Tinto Negra Mole wines). Tinto Negra Mole wine also had high levels of ethyl butyl 3-methylbutanedioate. The C_{13} norisoprenoids, β-damascenone and β-ionone, were also present. Interestingly, phenylacetaldehyde diethyl acetal was detected in all young varietal wines. Presumably this is formed from phenylacetaldeyde (a Strecker degradation/Maillard product) and ethanol.

2.10.7 Port

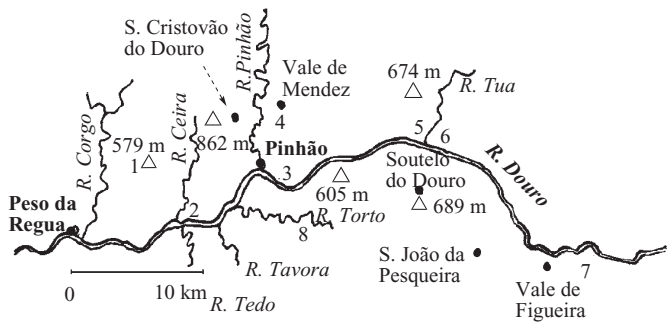
Port is the generic name for the fortified wines of the Upper River Douro, in northeastern Portugal, close to the border with Spain (Figure 2.10.11). The vineyards and wine estates ('quintas') are situated on the hills (often on terraces on steep slopes) that line the Upper Douro and its tributaries, such as the Pinhão, the Torto and the Tua. The best vineyards are to the east of the town of Peso da Regua, with some of the very best being around the town of Pinhão. Some of these vineyards are owned by the big Port lodges (Port wine companies), whose cellars are concentrated in the small port of Vila Nova de Gaia (Figure 2.10.11), across the river from Oporto. Others are owned or run by small companies or independent families who sell their grapes to the various Port lodges. In these vineyards, the granite and fragile schist soils, the hot, dry climate and the numerous grape varieties all contribute to the basic wine. Different vinification and maturation methods are then responsible for producing the range of Port wines, from white, through tawny to red, with the latter two being the most important.

The black grape varieties include Alvarehão, Bastardo, Sousão, Tinta Amarella, Tinta Barroca (the same as the Spanish Tempranillo), Tinta Cão, Tinta Fransisca, Tinta Miuda, Touriga Fransesca and Touriga Nacional.



Major Port lodges at Vila Nova de Gaia

Barros
Borges & Irma
Burmester
Butler & Nephew
Cálem
Churchill
Cockburn
Croft
Dalva
Delaforce
Dow
Feist
Ferreira
Fonseca-Guimaraens
Gould Campbell
Graham
Hunt
Hutcheson
Kopke
Krohn
Mackenzie
Martinez
Niepoort
Noval
Offley
Osborne
Pocas
Quarles Harris
Ramos-Pinto
Rebello Valente
Royal Oporto
Sandeman
da Silva
Smith Woodhouse
Souza
Taylor
Warre



Notable vineyards

Quinta	Shipper
de Boa Vista (1)	Offley
de Bonfim (3)	Dow
da Sra. da Ribeira	Dow
do Crasto (2)	
do Cruzeiro	Guimaraens
de Foz	Cálem
dos Malvedos (5)	Graham
de Noval (4)	
de S. Luiz	Kopke
de Tua (6)	Cockburn
de Vargellas (7)	Taylor
das Lages (8)	Graham

Figure 2.10.11 *Sketch maps of Douro region, central upper Douro region, list of major Port shippers and notable vineyards. Note that many of the companies above are amalgamated (e.g. Graham, Cockburn, Dow and Warre), others (e.g. Offley) are part of larger companies (e.g. Sogrape)*

All these varieties contribute different character to the wine, but the general opinion is that Tinta Cão, Touriga Fransesca and Touriga Nacional generally produce the best wine. The major white varieties are Esgana-Cão, Folgasão, Malvasia Fina, Rabigato, Verdelho and Viosinho. The grapes are mostly harvested by hand, mechanization being difficult on the steep and wild terrain of the vineyards. They are taken to the nearby quintas for vinification, where nowadays electrically driven crusher/destemmers and pneumatic presses do most of the work (Figure 2.10.12). Traditional crushing by foot in lagares (large, shallow troughs made of granite or hard slate) is relatively rare, but is still used in the best years for small quantities of the finest wines in some quintas (Halliday and Johnson, 1992). Once the grapes are crushed, fermentation of the pulp begins very soon and is allowed to occur with skin contact, as for standard red wines (Section 2.9.2). The difference with Port wine is that the pulp fermentation will be brief, soon to be halted by mixing with grape spirit, so to achieve maximum extract, continuous agitation of the must is required during fermentation. Traditionally, this is done in lagares by the relatively gentle motion of many treading feet, which continuously break up the pulp and submerge it. The large surface area of a lagar ensures fermentation at temperatures a little below 30 °C. Much more common these days are the open tank vats with pump over to keep the pulp cap moist. Also common are closed system autovinifiers, where the must is forced up and over the cap because of the build up of pressure caused by the CO_2 evolved during fermentation. Efficient as these pump over and autovinifier systems are, they lack the facility to extract that little bit of extra color and flavor that traditional manual treading was able to achieve from the skins and pulp. In recent years, the company of W. & J. Graham have

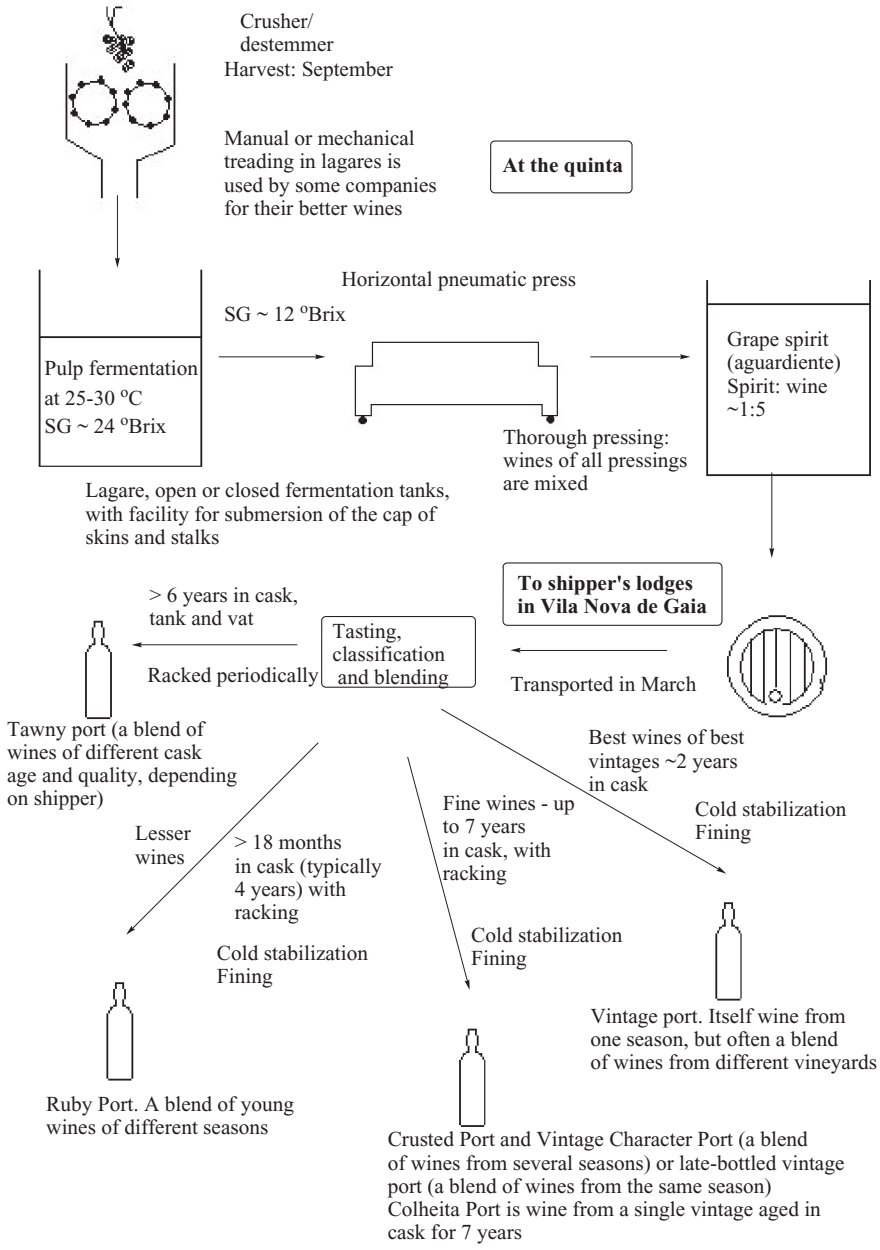


Figure 2.10.12 *Making Port wine from black grapes. All port wines are made from grapes of several varieties. Most are blends of wines from dif ferent sites and many are blends of wines of dif ferent vintages*

built and operated automated treading machines at Quinta dos Malvedos. These machines use pistons whose gentle up and down motion simulates the action of the human foot by pressing the grape skins and pulp gently against the floor of the lagar. The system has the added advantage of good temperature control.

At some point, whilst considerable unfermented sugar still remains in the must, fermentation is arrested by pumping the must into a tank one quarter filled with grape spirit (aguardiente) of about 76% ABV, so that the must to spirit ratio is about 5:1. The timing of this fortification step is important; it is based on calculations made from charts or tables that relate volume and ethanol content of the wine and spirit, initial sugar concentration, concentration required at the time of fortification (measured using a hydrometer) and the final ethanol concentration required. The winemaker's intuition and experience may also play a part in the fortification step. Generally, fortification occurs when approximately one half of the sugar content has been fermented, leaving about 12 °Brix of unfermented sugar. After fortification and subsequent processing, Port wines have typically ∼20% ethanol (v:v) and ∼8 °Brix of unfermented sugar. The pulp is then pressed thoroughly and the wine is transferred to 523 l casks ('pipes'), which are then transported by rail and/or road to Vila Nova de Gaia, where the Port lodges are situated. This journey used to be done down the River Douro on special sailing boats known as barcos rabelos; a romantic, but hazardous journey because of the rapids and strong river currents. This practice stopped after the Douro was damned for hydroelectric power, but the boats are still used for fishing.

Like Jerez and Madeira (and Marsala, initially), the modern day Port wine styles owe much to the enterprise and perseverance of British merchant companies, most of which are still prominent, although often amalgamated (e.g. Dow, Graham, Offley, Taylor, Warre and many others) (see Figure 2.10.11). The Dutch also have a footing (e.g. Burmester, Niepoort, for example) and, of course, there are many Portuguese lodges (Barros, Noval, Pocas, Sousa and others). Some companies, like Croft, Gonzalez Byass and Sandeman are also Sherry shippers, having bodegas in the Jerez area (see Figure 2.10.1 and Section 2.10.2). Originally (in the seventeenth and early eighteenth centuries), the wines were shipped unfortified, but were not popular in England as alternatives to red Bordeaux and other red wines (Lichine, 1982b). Fortification was found to improve the wine quality, by curing the travel sickness. However, the wines were still generally rather harsh and of course dry, so the merchants gradually moved to sweeter wines and the practice of arresting the fermentation was more or less in full favor by the late eighteenth century. As with Sherry and Madeira, high quality grape spirit (in the sense of fine old Cognac or Armagnac) is not necessary for the production of the best wines (Halliday and Johnson, 1992); the rough, high strength (∼77% ABV) grape spirit used even for the best Port (vintage Port) is made by distillation of basic wines from the Douro region or southern Portugal, without either the dilution or long cask ageing usually afforded to brandy (Section 3.6.1) and pomace spirits (Section 3.7.2). To emphasize this, shortage of grape spirit for the very good 1904 Port vintage meant that many lodges had to buy German potato spirit, but the resulting vintage Port wines were of high quality (Halliday and Johnson, 1992). Nowadays, the Port lodges choose their grape spirit according to aroma quality and price (Pissarra *et al.*, 2005), with most winemakers preferring a neutral spirit. The spirit aroma is due mainly to aldehydes, higher alcohols and esters, the major aldehydes being acetaldehyde, but with propanal, 2-methylbutanal, 4-methylpentanal, methylglyoxal and benzaldehyde having significant concentrations. Decreased levels of grape anthocyanins, coupled with increased hue angle (yellowing effect) and increased chromaticity (color saturation) of Port wine were positively correlated with the aldehyde content of spirit used for fortification (Pissarra *et al.*, 2005). Grape spirit with high aldehyde content gave Port wines with more intense colors and flavors; it is probable that aldehydes, especially acetaldehyde, participate in vitisin type anthocyanin formation, as well as in copolymerization between anthocyanins and flavan-3-ols (Figure 2.10.13). These aspects and the color of Port wine are discussed in the next paragraphs.

As with other red wines, the initial anthocyanin pigments of Port wine undergo progressive copigmentation reactions with other wine components, notably phenols, during maturation, so that after ∼2 years very little of the original anthocyanins remain (Romero and Bakker, 2000). The result of prolonged red wine maturation

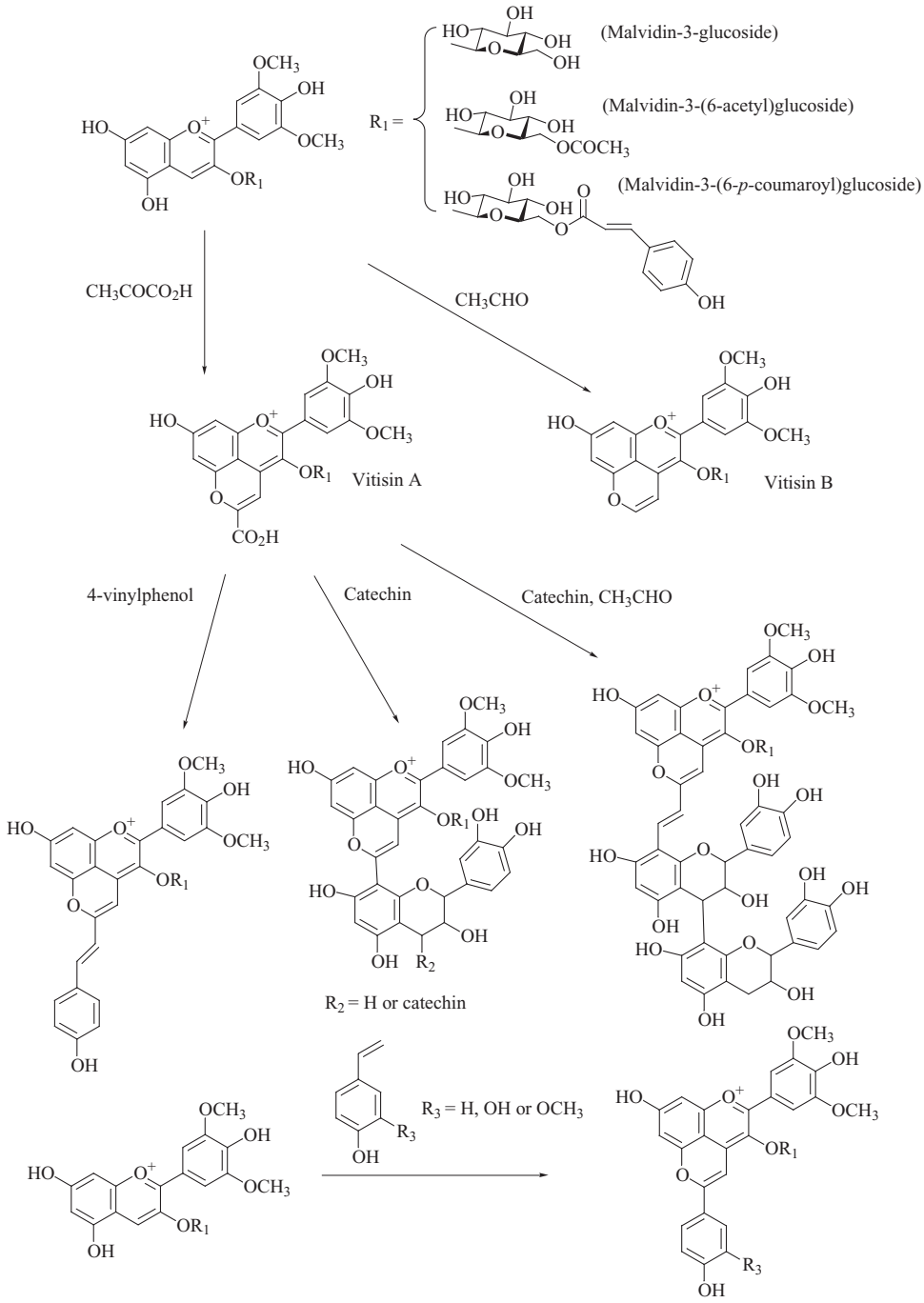


Figure 2.10.13 *Formation of pigments in maturing Port. Mateus* et al. *(2006) and Tempranillo wines, Morata* et al. *(2007)*

is a gradual shift of color from purple (~528 nm) through various hues of red to tawny, given enough time and the right conditions. This corresponds to the formation of new pigments with lower wavelengths of absorption (see Section 2.9.5) (actually lower λ_{max} values – see Section 4.4.3). Initially, the relatively labile anthocyanins, mainly malvidin-3-glucoside and its acylated derivatives, react with pyruvic acid and acetaldehyde to give pyranoanthocyanins, vitisin A and vitisin B, respectively (Figure 2.10.11) (Romero and Bakker, 2000). This occurs during the first two years of maturation, alongside other reactions, including some with flavan-3-ols to produce proanthocyanin polymers (Section 2.9.5). Acetaldehyde is a product of alcoholic fermentation and of ethanol oxidation, whereas most pyruvic acid is produced during malolactic fermentation. Acetaldehyde, along with other aldehydes, is also present in the grape spirit used in fortification. Vitisin concentrations have been increased by the addition of pyruvic acid (Romero and Bakker, 2000). Similarly, both vitisin A and vitisin B concentrations have been increased by addition of pyruvic acid and acetaldehyde, respectively, to young non-Port wines made with grapes of Tempranillo (itself one of the many Port varieties – known as Tinta Barroca) (Morata *et al.*, 2007). Vitisin A has also been produced in model wine solution by reaction between malvidin-3-glucoside and pyruvic acid, its formation being favored by low pH, high concentration of pyruvic acid, absence of acetaldehyde and low temperatures (10–15 °C) (Romero and Bakker, 1999). At wine pH (~3), the vitisins are characterized by both higher color intensity (large ε_{max} values – see Section 4.4.3) and somewhat lower λ_{max} values than those of the original anthocyanins; their colors are slightly to the red side of purple. The vitisins are less reactive and are relatively unaffected by SO_2, whereas the original anthocyanins are readily bleached by this widely used preservative (Ribereau Gayon *et al.*, 2000b; Bakker and Timberlake, 1997). However, on further maturation of the wine, the vitisins gradually undergo polymerization reactions with flavan-3-ols (with and without acetaldehyde bridges) and simple phenols, such as 4-vinylphenol (Figure 2.10.13) (Mateus *et al.*, 2006). The products of the latter reaction are known as vinylpyranoanthocyanins; that formed from vitisin A and 4-vinylphenol actually has a color on the bluer side of purple (λ_{max} ~538 nm) than malvidin-3-glucose (λ_{max} ~528 nm) at wine pH. This compound was characterized by mass spectrometry, NMR spectroscopy and by synthesis from authentic vitisin A and 4-vinylphenol at pH 2.6 (Mateus *et al.*, 2006). It also appears that malvidin-based anthocyanins react directly with vinylphenols to give pyranoanthocyanin-like adducts, as discovered in wines made from Tempranillo (Figure 2.10.13) (Morata *et al.*, 2007). These have colors rather on the red side of purple (λ_{max} <520 nm).

The polymerization reactions that lead to the gradual change of color from ruby to brown occur more rapidly in the presence of oxygen. Port wine matured in incompletely filled oak casks is transformed into tawny Port in less then 10 years, especially if racking with forced aeration has been carried out. Compared with this, vintage Port, with only 18 months cask maturation and stored in well sealed (waxed) bottles can take well over a century to reach a similar color to tawny Port. In a study of the vessel type on the changes in color and phenolic composition during the early stages of Port wine maturation, it was shown that wine maturing in oak casks developed more rapidly than those matured in stainless steel or glass vessels (Ho *et al.*, 2001). The cask wines had higher dissolved oxygen concentrations and higher redox potentials than tank or glass carboy wines. Furthermore, racking with forced aeration over a period of 15 months appeared to have an accelerating effect on the rate of maturation.

Flavonoid phenols and other colorless phenolic compounds are important in red wines, because of their copigmentation reactions with anthocyanins and vitisins (Figure 2.10.13), their contribution to the wine's bitterness and their contribution to the wine's antioxidant properties. Although there is information on these phenolic compounds in wine (Section 2.9.2), fruit wine (Section 2.11.2), cider (Section 2.8.2) and beer (Section 2.6.9), there appears to be limited data for Port wine grape varieties and Port wine (Andrade *et al.*, 2001). In a preliminary study of some major port varieties, Andrade *et al.* (2001) identified 12 phenolic compounds: five flavonols, (+)-catechin, (–)-epicatechin and the tartrate esters of caffeic acid, coumaric acid, *p*-coumaric acid, ferulic acid and syringic acid. The flavonol heterosides were predominant in most of the varieties, except Tinta Cão and Rufete, where (–)-epicatechin was predominant. The major flavonol

heteroside was found to be isorhamnetin 3-glucoside for most varieties, with myricetin 3-glucoside being the predominant flavonol in Tinta Roriz. Kaempferol 3-rutinoside was the least abundant flavonol.

Carotenoids in Douro grape varieties and port wines are of interest because of their antioxidant value and because they are precursors of C_9-C_{13} norisoprenoids, some of which are important flavor compounds. Certainly, carotenoids are biochemical precursors of aroma active norisoprenoids such as β-damascenone, α-ionone and β-ionone in grapes (da Silva Ferreira and Guedes de Pinho, 2004). It is possible that carotenoids are gradually degraded in maturing Port wine to the above norisoprenoids, plus 2,2,6-trimethylcyclohexanone (TCH) and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (Mendes-Pinto *et al.*, 2005). Some 13 carotenoids and three chlorophyll derived compounds were found in the Douro grape varieties Tinta Cão, Tinta Roriz, Touriga Francesca and Touriga Nacional (Mendes-Pinto *et al.*, 2005). Port wines were found to have 19 carotenoids or chlorophyll derived components, but chlorophyll b was not amongst them. The latter, present in grapes, appears to be degraded during the winemaking process to pheophytins and other products. The youngest wines generally had the highest levels of total carotenoids (up to 720 μg/l), with β-carotene and lutein being the most abundant. Of the varietal wines, Tinta Roriz showed the highest carotenoid content, while Touriga Francesca had the lowest. Mendes-Pinto and coworkers (2005) showed that aged wines had higher β-carotene:lutein concentration ratio than young wines, suggesting that lutein degrades more rapidly than β-carotene in maturing Port wine.

Of the many grape varieties used to make Port wine, Touriga Nacional is thought by many to be the finest, giving a wine that is characterized by herbal floral aromas, often described as citrus, bergamot and violet. Correspondingly, Touriga Nacional wines were found to have higher total terpenoid contents than the other major Port varieties (Guedes de Pinho *et al.*, 2007). The compounds largely responsible for bergamot notes were found by GC-O ('sniff GC'), GC-MS and aroma dilution analysis to be linalool and linalyl acetate (de Pinho *et al.*, 2007). This was supplemented by a study of bergamot essential oil, where it was found that linalool, linalyl acetate and α-pinene were the major aroma contributors. Although these studies were carried out on table wines, it is not unreasonable to suppose that the results should relate to Port wine, where Touriga Nacional usually forms a significant part of the blend.

Most recent studies on Port wine aroma compounds have focused on the influence of ageing, winemaking and technological factors on the aroma profile. As with many wines, norisoprenoids, such as β-damascenone, β-ionone, TCH, TDN and vitispirane make important contributions to Port wine aroma. The first two compounds contribute flowery and violet notes, respectively, TCH is responsible for 'rock rose' aroma, whilst TDN and vitispirane contribute 'kerosene' and 'tea leaf' notes, respectively. These compounds are of particular interest in Port wine because the short fermentation (stopped by fortification with grape spirit) results in the presence of carotenoids and other grape material in young wines. da Silva Ferreira and de Pinho (2004) have shown that young wines were richer in β-damascenone and β-ionone, whereas old cask aged tawny Ports had TDN levels some 15 times higher than in young Port wines. TCH and vitispirane were also present at higher levels in old oak aged wines. Furthermore, these authors demonstrated that pH, temperature, free SO_2 concentration and dissolved oxygen concentration all had influences on the norisoprenoid levels in young Port wines as they were stored over a period of 54 days. In particular, TCH and TDN (initially at very low levels in young wines) were formed during storage time, especially at low pH and high temperatures ($\geq$45 °C). Oxygenation increased the concentrations of all the norisoprenoids after a short storage time, but thereafter their levels fell drastically. The concentrations of β-damascenone and β-ionone were maintained by the presence of SO_2, although this compound had the effect of retarding the formation of β-damascenone. Thus, it is possible to rationalize the very different aromas of old vintage Port wines and old tawny Port wines on the basis of their different norisoprenoid compositions.

Many other changes in aroma and flavor occur in Port wines as they are aged for long periods in oak casks. The enhanced nutty, spicy notes that are characteristic of tawny Port wines have been attributed to the presence of sotolon (3-hydroxy-4,5-dimethyl-2(5*H*)-furanone) (da Silva Ferreira *et al.*, 2003a). This compound was

found in concentrations of up to 958 μg/l in old wines, and since its odor threshold value (OTV) in Port wine is only 19 μg/l, it was concluded that it made a significant contribution to the aroma of all the old tawny Port wines studied. Indeed, there was a linear correlation between sotolon concentration and cask age. Sotolon is a common wine compound, being found in Sherry wines (Section 2.10.3), vins doux naturels (Section 2.10.9), vins jaunes (Section 2.10.4) and various botrytized wines, such as Sauternes, as well as Asian rice 'wines' (Section 2.7.1).

It is probably produced in flor wines by aldol condensation between acetaldehyde and 2-oxobutanoic (2-ketobutyric) acid, which itself is derived from threonine by flor enzymic action. However, this mechanism may not be possible in Port wines, so a Maillard reaction origin for sotolon formation in these wines has been suggested by da Silva Ferreira and coworkers (2003a). It can be produced by the condensation of butane-2,3-dione (diacetyl) and hydroxyacetaldehyde. This suggestion is compatible with the observed high correlation between sotolon and 5-hydroxymethylfurfural (5-HMF, another Maillard product) concentrations and poor correlation between sotolon and 2-oxobutanoic acid levels in tawny Port wines.

Volatile sulfur compounds often exhibit unpleasant odors, even when present in very low concentrations, and hence they are often regarded as wine spoilage compounds. Compounds such as methional, methionol and 2-mercaptoethanol contribute aromas of cooked vegetables, cauliflower and burnt rubber, respectively, if present in wine at concentrations close to their OTVs. Other sulfur compounds, like dimethyl sulfide (DMS), provide pleasant odors (truffles or quince for DMS) if present at levels close to (but not exceeding) their OTVs. In general, sulfur compounds are much less in evidence in tawny Port wines than in young ruby Port types (da Silva Ferreira *et al.*, 2003b), so it has been suggested that the long oxidative ageing in oak casks experienced by many tawny Port wines leads to depletion of sulfur compounds. In controlled storage experiments with young Port wine at varying pH, temperature, SO_2 content and dissolved oxygen content, it was found that high O_2 content in particular led to depletion of methionol and 2-mercaptoethanol and build up of DMS and the odorless dimethylsulfone ($(CH_3)_2SO_2$). No methional (with cooked vegetable notes) was formed, nor could dimethyl sulfoxide ($(CH_3)_2SO$) be found (da Silva Ferreira *et al.*, 2003b). It also appears that 2-mercaptoethanol (burnt rubber notes) is converted under these oxidative ageing conditions to the odorless bis (2-hydroxydiethyl) disulfide. In wine, DMS and dimethylsulfone may have the same (as yet unknown) precursor, whereas in beer, the major precursor of DMS is known to be *S*-methylmethionine (SSM) (Section 2.6.2). These experiments explain generally why tawny Ports never suffer from sulfur compound off flavors and also why truffle or quince notes often feature in the aroma of such wines.

Young ruby Port wines often possess almond or hazelnut notes amongst the floral, fruity, herbal, vinous and other aromas. It has been found that 1,3-dimethoxybenzene (*m*-DMB) was responsible for sweet, medicinal, hazelnut notes in young ruby Ports (Rogerson *et al.*, 2002). This compound was present in concentrations up to ~20 μg/l, and although its OTV in Port wine is 47 μg/l, it was considered to contribute to the wine aroma. 1,3-Dimethoxybenzene is thought to be originate partially from the grapes and partially from the grape spirit (aguardente): it was found in Douro grape musts and Port wines, but not in unfortified Douro table wines, where the much longer fermentation process appears to result in degradation of this odorant (Rogerson *et al.*, 2002).

Many different styles of Port wine (except for white Port) are summarized in Figure 2.10.12. As with Sherry and many other fortified wines, blending is of prime importance for most Port wines to ensure consistency of quality and character from year to year. Blending is performed on the basis of the advice of experienced expert tasters who are familiar with the shipper's general wine styles and with the character of the base wines from year to year. Even most vintage Ports are blends of wines from different vineyards, although there are many single vineyard Ports, such as Calem's Quinta de Foz, Quinta de Noval, Offley's Boa Vista and Taylor's Vargellas (see Figure 2.10.11). Vintage Ports are generally reckoned to be the greatest products of the Douro. They are produced in the best seasons only, as decided by the numerous shippers (in theory) independently. Not all shippers 'declare' a vintage Port for a particular good season; a shipper's judgement is based on the

developing quality of the young wines of that season in cask, compared with the quality of previous recent declarations and the projected quality of newer harvests. Vintage Ports are all matured in cask at the shippers' wine lodges in Vila Nova de Gaia for 18 months to three years, with two years being typical. It is shipped in bottle for sale; not long ago, some vintage Port was shipped in cask to be bottled abroad, especially in England. Vintage Ports continue to mature slowly in bottle and may take many decades to develop their full potential. In doing so, they 'throw' a heavy dark, powdery deposit (crust), composed mostly of tannins and tartrate salts, and hence these wines need careful decanting before drinking.

High quality Port wine that was tentatively put down as a vintage wine, but for one reason or another was not declared, is aged in cask for another 2–3 years to become late bottled vintage (LBV) Port. Alternatively, it can be blended with wine of similar quality, but of different ages and bottled as crusted Port, sometimes after extra cask maturation. Although generally of lesser quality than vintage Port, crusted Ports are held in high esteem and will throw a sediment after some time in bottle. Vintage character Port is similar, but the average time in cask is less than that of crusted Ports. All the aforementioned styles (like the basic ruby Port style) are a rich ruby color at the time of bottling; careful racking and topping up of casks ensures a lack of oxidation. The red Port wines generally have 20% ABV and ∼6 °Brix (∼24 °Oe) of residual sugar. They generally have around 3–5 g/l of acidity, expressed as tartaric acid.

Tawny Port is held by many to be the other great product of the Douro, although it is totally different in style to vintage Port. As discussed earlier, oxidation is encouraged by a long maturation in cask and vat, and also by forced racking (Figure 2.10.12). The art is to strike a balance between oxidative ageing and fruit character, something that requires great skill and experience on the part of winemaker and blender. Tawny Ports are usually sweeter than ruby Ports, often having about 9 °Brix (∼36 °Oe) of residual sugar, but this is balanced by a higher acidity, sometimes almost 6 g/l, expressed as tartaric acid.

White Port is a relative newcomer. It is generally made from white grapes without skin contact, from varieties such as Códega, Malvasia Fina, Rabigato and Viosinho. White Port is usually a bit less alcoholic (18–19% ABV) than ruby or tawny Port wines (∼20% ABV) and also slightly less sweet (∼5 °Brix or ∼20 °Oe of unfermented sugar); it is enjoyed as an aperitif in many European countries.

Port style wines are made in many countries, but notably Australia (especially South Australia) and the USA (California, particularly). In South Australia, companies such as Burge, Château Reynella, Hardys and Penfolds make ruby, tawny and vintage style wines from Rhone varieties, particularly Grenache, Mourvedre and Syrah (Shiraz). Especially impressive are the tawny wines, many of which have average ages (most are blended) of over 20 years. Also in South Australia, Seppelt Winery makes a 100 year old 'Liqueur Tawny' wine with an alcohol content over 23% (v:v), almost certainly the result of a long rest in cask. The same company also uses Touriga varieties, along with Grenache and Shiraz, for vintage Port style wines. The tawny wines have the characteristic intense nutty flavors and rancio character associated with the real article, although they tend to be less sweet, typically having ∼3 g/l of residual sugar, as opposed to the more typical value of ∼9 g/l for tawny Port wines.

In California, Port style wine (and other fortified wine) production is concentrated in the hotter Central Valley regions, with some of the best coming from the San Joaquin Valley, especially from Madera County. Here, Ficklin Vineyards use Tinta Cão, Touriga Francesca, Touriga Nacional and other Douro varieties to make wine in the Portuguese style. Likewise, Quady (a winery that specializes in fortified wines – see Sections 2.10.2 and 2.10.9) makes its 'Starboard' brand from Douro varieties in the Douro style. As the producers suggest, differences in character between San Joaquin and Port wines arise from differences in climate (San Joaquin is hotter!), terroir, cask ageing (in 267 l casks, rather than in 623 l 'pipes') and in spirit used for fortification. The same argument could be used for the Australian Port style wines, with different varietals being added to the list.

Elsewhere in California, Port style wines are more likely to be made from the grapes of varieties such as Carignan, Grenache, Syrah, Zinfandel and others; indeed, the Quady winery made its first Port style wine

from Zinfandel grapes. These wines are all made in modern wineries (e.g. Fairbanks-Gallo) with emphasis on quick maturation. They tend to be considerably sweeter than the corresponding Port wines, in contrast to many Australian Port style wines.

2.10.8 Marsala and Málaga

Marsala is a DOC (Denominazione di Origine Controllata) wine of Italy and is that country's most famous fortified wine (see also Vernaccia di Oristano, Section 2.10.4). The vineyards are situated around the city of Marsala, on the west coast of Sicily. The grapes used include Catarratto, Damaschino, Grillo and Inzolia (white), as well as Calabrese, Nerello, Mascalese, Pingatello and Perricone (black). These varieties are also used to make unfortified white and red wines in Sicily and Southern Italy. The best grape for Marsala wine is considered by many to be Grillo. Like Madeira, Port and Sherry, British wine merchants played an important early part in developing the style of Marsala. In this case, it was a Liverpool merchant John Woodhouse who first fortified the wines for export to Great Britain and distant parts of the British Empire in cask, originally, then in bottle. The wine became so popular that he set up a winery in Marsala, where the general style of production of Marsala wine was established. Unlike Madeira, Sherry and Port, British influence in Sicily has gone, but modern methods of production owe much to Woodhouse. Like Sherry, all Marsala is fortified and there is wide variation in sweetness, color and quality, indeed some wines are maturated using a solera system. Unlike fino and amontillado Sherry, there is no formation of flor and Marsala wines therefore undergo oxidative maturation like oloroso Sherry, to which they have a distant resemblance. All Marsala is initially dry, containing some 14% ABV (minimum ethanol content is 12% (v:v)). Depending on quality level and style, the base (or young) wine is fortified with grape spirit and supplemented with musto cotto – grape must that has been concentrated to a syrup by heating. Alternatively, sifone, a blend of sweet wine made from semi-dried grapes and grape spirit, can be added. Just as likely a mixture of cotto and sifone (concia) may be used produce medium and sweet wines, depending on the blend of cotto and sifone.

The three basic styles of Marsala are seco (dry), made with added grape spirit only; semiseco (medium) and dolce (sweet), made with concia, cotto and spirit, or sifone. The styles and quality types of Marsala wine are summarized in Figure 2.10.14. The highest quality levels are vergine riserva and vergine; these are unsweetened seco wines which have undergone long cask maturation. Fine is the most basic quality level, with cask maturation for up to one year. This is followed by superiore and superiore riserva with longer time

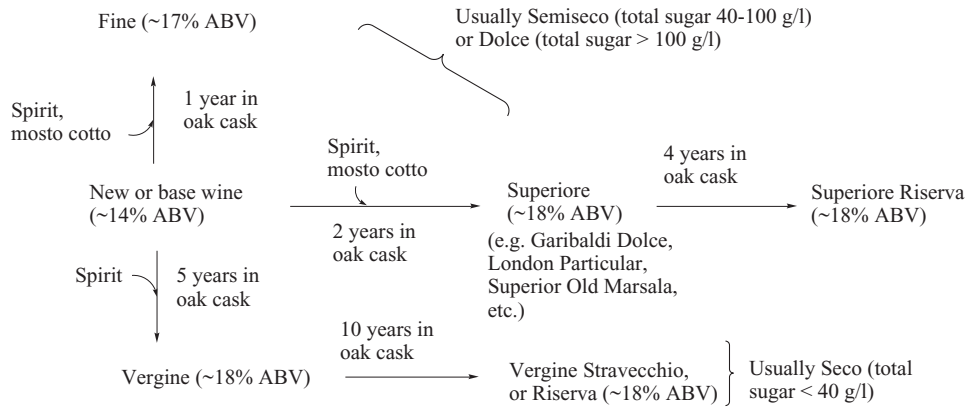


Figure 2.10.14 *Quality levels and styles of Marsala wine*

in cask. These wines are either semi-seco or dolce (i.e. sweetened), but seco superiore riserva wines exist too. The vergine wines are golden (oro) or amber (ambra), but the other quality wines are usually amber, the depth of color depending on the quality and depth of color of the concia used in the winemaking. The amber colors originate not only from the physical concentration of the grape must, but also from caramelization of sugars and Maillard reactions that occur during must heating. Less common are the ruby (rubino) Marsala wines, made from black grapes.

The grapes are harvested in August and September. Most are vinified to give the base wine, but some are semi-dried, fermented and converted to sifone by addition of grape spirit. Yet others, mostly Catarratto, are crushed, pressed and converted to musto cotto. Most Marsala wines are aged statically in oak casks (~450 l typically), which are stacked in rows to the winery ceiling. Blending is a highly skilled business – achieving consistent and high quality from a large number of wines of different ages, degrees of color, density, flavor and alcoholic strength. A few wineries are reputed to still use a solera system of dynamic aging/blending, like that of Jerez. Major producers are Vito Curatolo Arini, Florio, Di Guiseppe, Carlo Pellegrino, Diego Rallo and Scia-Scia.

It may well be difficult for amateurs to differentiate the various categories of Marsala by organoleptic analysis, but recently canonical discriminant analysis (CDA) of analytical results allowed classification according to phenolic compound and heavy metal compositions (La Torre *at al.*, 2008). Highest discriminant power was found with caffeic acid, catechin, kaempferol, lactose, procyanidin B1, quercetin, rhamnose and tyrosol, as well as copper, lead and zinc.

Málaga is a port on Spain's Mediteranean coast, situated east of Jerez and directly south of Montilla – Moriles. The strong wines of Málaga were once as famous as those of Jerez, but the area has never recovered from the ravages of *Phylloxera vastatrix* in the 1870s and now produces a small fraction of its former volume of wine. The best vineyards are found to the north of the city (Zona Norte), where Pedro Ximenez, Moscatel and Airen are the main vine varieties. The wines are often blends of wine from the above varieties, but varietal wines are also made from Pedro Ximenez or Moscatel grapes. Málaga wines are made in a range of styles from dry (seco) to sweet (dulce) and most are fortified. Like Marsala, the cheaper Madeira wines and sweet Sherry wines, Málaga wines are given a range of color and sweetness by the use of sweetening and coloring 'wines,' Arrope is must that has been reduced in volume by two thirds during heating. Vino de color (or pantomina) is an even more concentrated and viscous cooked must. Vino maestro and vino borracho are blends of grape must and grape spirit. To achieve the desired color, sweetness and alcoholic strength, a combination of the sweetening and coloring 'wines' is used to give blanco (white), dorado (golden), rojo-dorado (tawny), osuro (dark) and negro (black) Málaga wines. Very sweet wines were once made from the free run press juice (labelled Lagrima, which means tears). The best Málaga wines are matured in solera systems like those of Jerez. To qualify as Málaga DO (Denominación de Origen) wine, the wine must be made from grapes of specified varieties growing in four designated areas around the city and must be matured in bodegas in the city. Major shippers include Vinícole Andalucía, Hijos de A. Barceló, Luis Barceló, Felix Garcia Gómez, Flores Hermanos, López Henmanos, Scholtz Hermanos, Carlos J. Krauel, Larios, Campañia Mata, Morón, Juan Mory & Cía, Manuel Pacheco, Casa Romero, José Garijo Ruiz, Guillermo Rein Segura, Pérez Texeira and Hijos de José Suárez Villalbe.

2.10.9 Vins Doux Naturels, Liqueur Muscat and Similar Wines

Vins doux naturels (VDN) are French fortified wines made in 12 locations (appellations) in the south of the country, bordering or close to the Mediterranean Sea (Figure 2.10.15). Like all Appellation Contrôlée Wines, their production is strictly regulated from vineyard to bottle. For example, each appellation has its own defined territory, with certain grape varieties being grown, harvested in well defined yields, at well defined

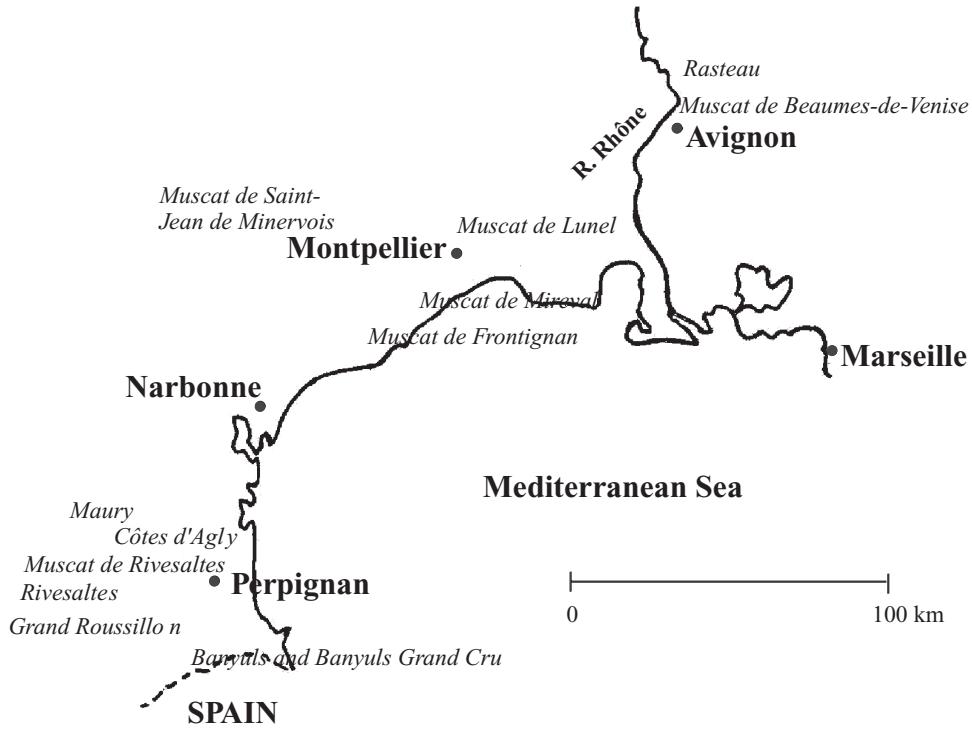


Figure 2.10.15 *Approximate locations of French vins doux naturels production*

maturity (Riberean-Gayon *et al*., 2000a). For example, yields must not exceed 30 hl/ha and the juice must contain in excess of 253 g/l of sugar (about 14.5% potential alcohol by volume). Permitted grape varieties include Grenache Blanc, Grenache Gris, Maccabeo, Malvoisie, Muscat d'Alexandrie and Muscat a Petits Grains (all white), along with Grenache Noir (black). The three major regions of VDN are Roussillon, Hérault and Vaucluse, all in the south of the country. The best areas within these regions have their own restricted appellations. In Roussillon, there is Banyuls and Banyuls Grand Cru on the rugged coastal hills very close to the border with Spain (Catalonia). Further inland are the Côtes d'Agly and Maury Appellations. All of these are well known for their VDN made from nonaromatic (non-Muscat) varieties. Also in Roussillon, just north of Perpignan, Muscat de Rivesaltes is made from Muscat d'Alexandrie and Muscat à Petits Grains. In Hérault, Muscat de Frontignan, Muscat de Lunel and Muscat de Mireval are made from the Muscat varieties near Montpellier and close to the mouth of the Rhône. A bit further inland, a smaller amount of VDN is made at St. Jean-de-Minervois. In Vaucluse, north of Avignon and close to Orange, Muscat VDN is made at Beaumes de Venise and non-Muscat VDN is made at nearby Rasteau, mostly from Grenache Noir.

Because of their different locations, grape varieties and details of winemaking, the VDN of southern France are of quite diverse character. However, they are all sweet and contain around 20% ABV, as required by the appellation laws. Some details of their production are summarized in Figure 2.10.16. Most of the Muscat wines are made by pressing the crushed grapes immediately, and fermenting the clear juice until the desired gravity has been reached, for fortification with grape spirit (mutage): usually 5–12 days.

A few Muscat wines are still made by either rough pressing or by allowing a short maceration period (fermentation on the pulp) before pressing and mutage. The greater extraction of aroma compounds, mainly

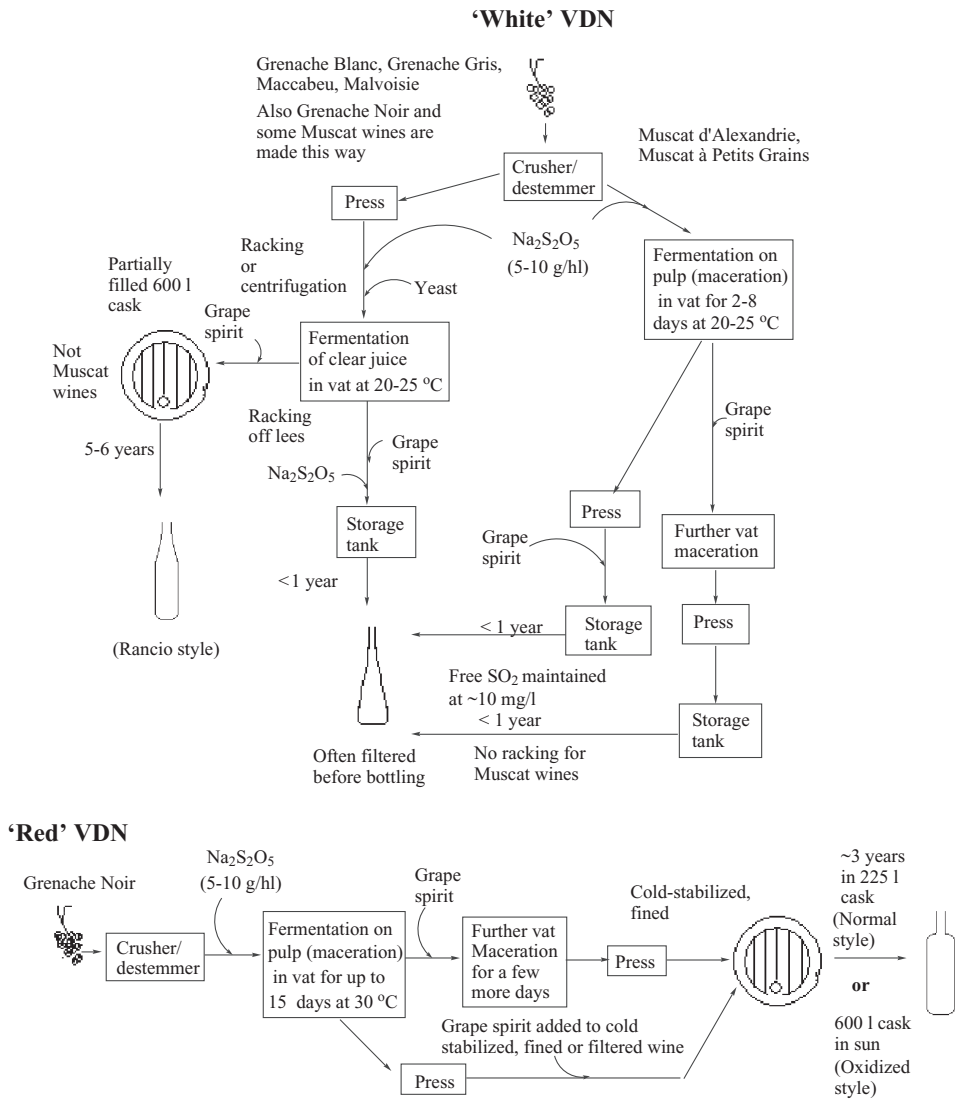


Figure 2.10.16 *Summary of methods for the production of vins doux naturels. White VDN is full gold to caramel, red VDN is brick red to dark amber*

alcohols, esters, norisoprenoids and terpenoids, give a wine with a more pronounced Muscat 'nose.' Muscat VDN are aged for up to a year in concrete or stainless steel tanks with minimal racking and with sufficient free SO_2 to prevent oxidation and preserve the bouquet. They tend to be bottled and sold in the spring or early summer following the harvest, whereas a few decades ago, two or three years maturation was not unusual for Muscat de Beaumes de Venise, for example. The Muscat VDN are the most floral and delicate wines of the type and should be consumed while still fresh – after three or four years in bottle. Muscat à Petits Grains is the more important variety in Beaumes de Venise, but this variety is subservient to Muscat d'Alexhndrie in most of the other Muscat VDN appellations (Frontignan being an exception). Although Muscat à Petits Grains is

classed as a white grape variety (its full name is Muscat Blanc à Petits Grains), there are clones that produce berries of colors ranging from greenish-yellow through pink to purple. The last type (grains noirs) are not dark enough to give a red wine but they do impart a copper color that can be found in some Muscat VDN.

Non-Muscat vins doux naturels are also made by a variety of methods (Figure 2.10.16) with immediate pressing/fermentation being used for most white wines and with the (less common) red wines being made by an extended pulp fermentation (maceration). Many non-Muscat VDN are matured in oak casks for 2–3 years, after being subjected to cold stabilization and fining, following mutage. Racking is performed as necessary and levels of free SO_2 are kept at about 10 mg/l by sulfiting. These VDN are typically golden-brown (nonmacerated) or red-brown (macerated). A few wines, at Rasteau and Maury in particular, are given an oxidative maturation in oak casks for 5–6 years, whence they take on the 'rancio' character of oloroso Sherry or tawny Port; Rasteau Rancio is an example of this. Maturation occurs in 600 l casks that are partially (~5/6) filled. Either a certain volume is removed from the casks each year, the volume being replaced with newer wine (a kind of simplified solera/blending system) or the wine from the rancio casks is bottled all at once (essentially a vintage wine). A few decades ago at Rasteau, it was not unusual for rancio wine to reside in casks for 10 years. Rancio wines tend to be brown in color and have a roundness of flavor and character that are reminiscent of oloroso Sherry (Section 2.10.2) or tawny Port (Section 2.10.7).

Some red VDN, after fining and cold stabilization, are placed in 600 l casks and exposed to the sun for a period of time, rather in the style of estufagem (Section 2.10.6). Oxidation and the heating cooling cycles ultimately give brown wines with characteristic rancio and toasted aromas rather like Madeira wines (Ribéreau-Gayon *et al.*, 2000a). The traditional making of oxidized style VDN, using loosely stoppered large glass carboys (bonbonnes) for maturation has almost disappeared, but some Banyuls is still made this way, mostly from Grenache Noir grapes. As with other fortified wines, the fortification process (mutage) used for VDN production is crucial. The moment of fortification must be chosen correctly to give a final unfermented sugar content in accordance with both the legislature pertaining to the appellation and the style of product required. Either grape spirit (~90% ABV) or mistelle, a mixture of grape spirit and unfermented must, is used. Fortification can be achieved in one go or at intervals over several hours, but the timing of fortification is based upon the fermenting wines' gravity; the minimum residual sugar content is between 95 and 125 g/l, depending on the appellation. The quantity of spirit added at the time of mutage is required to be between 5 and 10% of the fermenting wine volume and the final product must contain between 15 and 18% ABV and at least 21.5% total alcohol (v:v). Total alcohol content is the sum of actual alcohol content and the potential alcohol content remaining in the residual sugar (= residual sugar concentration in g/l divided by 17) (Ribéreau-Gayon *et al.*, 2000a). Fortification tables exist to help the winemaker perform the mutage correctly (as in other regions where fortified wines are made, like the Douro); these are based on volumes of wine and spirit, residual and original sugar content, and appellation requirements.

Non-Muscat VDN have characteristic odors that depend on the ageing process. Typical description include cocoa (or chocolate), caramel, coffee, dried fruits, prunes, jam and spice. It has been found that nonoxidatively aged red Maury VDN made from Grenache Noir have a standard range of acids, esters, lactones and volatile phenols, with perhaps somewhat elevated levels of carbonyl compounds and lower levels of fusel alcohols, possibly resulting from the mutage spirit (Schneider *et al.*, 1998). On the other hand, oxidative ageing in ullaged casks or in carboys exposed to the sun, led to increased levels of acetals, acids, esters, lactones and phenols. GC sniffing experiments indicated that ethyl 2-hydroxyglutarate (chocolate), 4-carboxy-γ-butyrolactone (coconut) and ethyl pyroglutamate (honey) made considerable contributions to wine aroma. These were found in nonoxidized wines, but were present in much higher levels in oxidized VDN (Schneider *et al.*, 1998). Certain other compounds, notably diethyl and monoethyl succinate, diethyl malate and ethyl lactate were also found at higher levels in oxidized wines. Interestingly, sotolon (3-hydroxy-4,5-dimethyl-2(5*H*)-furanone), with its curry or spicy notes, was found in only nonoxidized Maury VDN, and even then below its OTV. However, sotolon has been found at elevated concentrations in white non-Muscat Rivesaltes

Table 2.10.2 *Some major producers of Vins Doux Naturels*

Region	Producers
Roussillon	Les Vignerons de Maury, Mas Amiel (both Maury). Les Vignerons des Côtes d'Agly, SA Destavel (Banyuls and Rivesaltes), Cellier de Templiers (Banyuls and Banyuls Grand Cru), Société Coopérative l'Étoile (Banyuls), SCV Le Dominicain (Banyuls), Cave de l'Abbé Rous (Banyuls Grand Cru). Ch. de Canterrane, Ch. Las Collas, Ch. Pradel, Domaine Mounié, Domaine Rancy (all Rivesaltes, some Muscat de Rivesaltes).
Hérault	Ch. de la Peyrade, Ch. de Stony (both Frontignan). Les Vignerons du Muscat de Lunel, Ch. de la Devèze, Ch. Verargues, Ch. Tour de Farges (all Muscat de Lunel). Domaine du Mas Rouge, Domaine du Mas Neuf, Caves de Rabelais, Ch. Exindre, Domaine de Capelle (all Muscat de Mireval). Domaine Barroubio, Domaine Montaluc, Val d'Orbieu (all Muscat de Saint-Jean-Minervois)
Vaucluse	Coopérative Intercommunale des Vins et Muscats, Domaine Durban, Domaine des Bernadines, Domaine de Coyeux, Domaine St.-Saveur (all Muscat de Beaumes-de-Venise). Domaine de Char-à-vin, Domaine de la Grangeneuve, Francis Vache (all Rasteau).
Corsica	Domaine Gentile (Muscat du Cap Corse and Muscat de Patrimonio)

Source: Grand Roussillon Appellation is not shown. Roussillon information from www.vivexpo.org

VDN that had been aged in oak casks (especially if new) in a non-air-conditioned winery, where temperatures varied between 8 °C and 33 °C (Cutzach *et al.*, 2000). It was above its OTV, and hence made a significant contribution to odor, in all cask aged samples and also in a non-air-conditioned vat aged sample, after just 30 months maturation time. 5-Ethoxyfurfural and cis/trans-methyl-γ-octalactones were also found at higher levels (above their OTVs) in cask-matured wines from non-air-conditioned wineries. These results support the idea of a Maillard reaction origin for these compounds, as suggested by da Silva Ferreira *et al.* (2003a) for Port wines (see Section 2.10.7). It is not clear why sotolon was not detected in oxidized red VDN (Schneider *et al.*, 1998), a possible reason being that in this study, a four year old cask aged wine was kept at a constant cellar temperature of 17 °C and the sun exposed wine was matured in demijohns for only a year. Hence, it appears that the spicy character of red Maury VDN can be enhanced by cask ageing in a warm (or fluctuating) environment. Principal producers of VDN can be found in Table 2.10.2, including Muscat wines produced further east on the island of Corsica.

Both Muscat and non-Muscat are made around the world in the VDN style (i.e. via mutage). In the eastern Mediterranean there is Moscato di Pantelleria, the sweeter versions of which (liquoroso and passito) are fortified. Pantelleria is a small island, viticulturally and administratively linked to Sicily, but geographically closer to Tunisia. The sweet Muscat wines are reckoned by many to be amongst the best in Italy. The annual production of all Moscato wines on Pantelleria is around 1 000 000 l and the biggest producer is Agricoltori Associati di Pantelleria. The grape variety is Zibibbo, a clone of Muscat of Alexandria, and the wines usually have around 22% ABV after fortification. Further north, on Sardinia, some Malvasia di Bosa is fortified (called liquoroso), although not all of it by mutage. There are also fortified versions of Moscato di Sorso-Sennori and Cannonau in northern and eastern Sardinia, respectively. In Portugal, the well known Moscatel de Setúbal is made from Muscat of Alexandria grapes in the VDN style.

The Australian liqueur Muscat dessert wines are made like VDN, by stopping the fermentation with grape spirit. The best wines are made from Brown Muscat grapes, from vines that are strains of the darker skinned fruiting clones of Muscat à Petits Grains, responsible for the finest VDN. They are made in the hot, dry country of northeastern Victoria (especially Rutherglen), not far from the border with New South Wales. Here, there is usually no problem in achieving musts from raisined grapes with sugar concentrations of at

least 29 °Brix (~125 °Oe). Cultured yeasts are used and fermentation may be stopped early (which then requires a greater volume of spirit for balance) or when there is about 14 °Brix (~57 °Oe) of unfermented sugar still in the must. A kind of solera system is used, the wines being aged for four or more years in old oak casks that are stacked high in the winery or in the roof space. The fluctuating temperatures subject the wines to an oxidative heat treatment, rather like the estufagem used for the best Madeira wines (Section 2.10.6). The result is an intensely flavored deep colored dessert wine that has the nutty characteristics and balancing acidity associated with tawny Port and Malmsey Madeira wines. The best known liqueur Muscat wines are probably those of Brown Brothers and Campbell wineries. The lower quality Gordo Blanco (Muscat of Alexandria) grapes, along with Palomino, Pedro Ximénez and Sultana, are used in making Sherry style fortified wines, usually sweet or semi-sweet (Section 2.10.2). An interesting difference between Australian liqueur Muscat wines and European fortified wines is that the former is often made from relatively mature cask aged brandy, rather than more neutral and less refined grape spirit. This can give the Australian wines an extra dimension of spiciness, derived from the oak aged brandy.

Interesting fortified wines are made by the Quady winery in San Joaquin Valley of California. The Orange Muscat wine, made from grapes of that name, is relatively lightly fortified (~15% ABV). The Red Muscat dessert wine is unusual (for California, at any rate) in being made from Muscat Hamburgh grapes that have fully ripened (25 °Brix). At this ripeness, Muscat Hamburg grapes produce wine with strong Muscat notes, whereas this character is muted or even absent in the wine of less ripe grapes.

2.10.10 Vins de Liqueurs and Similar Beverages

Vins de liqueurs are defined as alcoholic beverages made by the addition of grape spirit to grape must. Thus, all the alcohol is derived from the added spirit. By French law, vin de liqueur must have 15–22% ABV, but in practice, the majority have around 17.5% ethanol (v:v). Another name for them is ratafia, as used for the vins de liqueurs of the Burgundy and Champagne regions. Vins de liqueurs are different from fruit liqueurs (or fruit liqueur wines), where crushed (or sometimes whole) fruits are soaked in spirit (not necessarily grape spirit) with added sugar and sometimes for extended periods of time (Section 3.9.2). Vins de liqueurs are made in several regions of France, each production being governed by the specific regional Appellation d'Origine Contrôlée. The main beverages are summarized in Table 2.10.3.

The best known vin de liqueur is probably Pineau de Charentes, reputedly discovered by accident in 1589 when a winemaker added grape juice to a cask that he thought was empty, but which in fact contained a considerable volume of Cognac. Being vintage time, the winemaker was very busy and simply put the cask aside to get on with other jobs. After two or three years in cask, the drink was found to be very palatable and was soon produced at each vintage, becoming known as Pineau de Charentes. Today, Pineau is made in the départements of Charente and Charente-Maritime from Ugni Blanc, Folle Blanche, Colombard grapes and sometimes Semillon and Sauvignon Blanc grapes (white) or Cabernet franc, Cabernet Sauvignon and Merlot Noir (rosé). The rosé version is less common. For the white version, the grapes are destemmed, crushed and then pressed heavily. The must is mixed immediately with Cognac (60% ABV) and aged in oak casks for at least a year, but usually longer; five years is not uncommon. The Cognac itself will have had a minimum of one year in cask, and the must and spirit are added in proportions to give about 17.5% ethanol (v:v). The rosé version is made in a similar manner except that it is given a short maceration time to extract some color. Floc de Gascogne is probably the next best known vin de liqueur. It is made in the Armagnac region of Gascony, in the département of Gers mainly, but also in a few cantons (parishes) in Lot-et-Garonne and Landes departments. The grapes are Colombard, Gros Manseng and Ugni Blanc (white version), Cabernet Franc, Cabernet Sauvignon and Merlot Noir (rosé version). The method of production is similar to that of

Table 2.10.3 *Vins de liqueur*

Type (AOC)	Allowed grape varieties	Type of grape spirit used	% Ethanol (v:v)	Ageing in cask/ years	Producers or brands
Pineau des Charentes	Colombard, Folle Blanche, Sauvignon Blanc, Semillon, Ugni Blanc; Cabernet Franc and C. Sauvignon, Merlot	Cognac	17–18	>2 years	H. Bergey, L. Bouron, Dom. Estève, Gautier, Otard, Remy Martin
Floc de Gascogne	Colombard, Gros Manseng, Ugni Blanc; Cabernet Franc and C. Sauvignon, Merlot	Armagnac	18	~2 years	Dom. de Bilé, Ch. La Grangerie, Dom. de Joÿ, Dom. de Pujo, Ch. de Sales
Ratafia de la Champagne	Chardonnay, Pinot Meunier and P. Noir	Fine or Marc de Champagne	18	>2 years	Boulard, Dumont, Geoffroy, Moutard, Vranken
Ratafia de Bourgogne	Aligoté, Chardonnay; Gamay Noir, Pinot Noir	Fine or Marc de Bourgogne	17–18	1.5–2 years	Cartron, Defaix, Lejeune, Trenel, Védrenne
Macvin de Jura	Chardonnay, Savagnin; Pinot Noir, Poulsard, Trousseau	Marc de Jura	17	>1 year	Ch. Gréa, Dom. de la Pinte, Dom. de la Tournelle
Cataroise de Béziers (Picardin Doux)	Bourboulenc, Clairette, Grenache Blanc, Maccabeu, Marsanne, Picpoul, Rolle, Roussane, Viognier; Cinsault, Grenache Noir, Mourvedre, Syrah	Local eau-de-vie de vin	18	>1 year	Comte de Fontserrane, Douce Nuit

Pineau de Charentes and is made under similar regulations: the grape must and Armagnac brandy must come from the same vineyard, and the Floc resides in cask for about a year before being tasted by a committee of experts and given Appellation d'Origine Floc de Gascogne status. Additionally, the grape must should have enough sugar to give 10% ABV – although this ethanol will never be produced as such in Floc de Gascogne, because of the mutage. This rule ensures that ripe grapes are used, so giving a well balanced product, with some sweetness. Like some Pineau de Charentes, Floc de Gascogne is fined, filtered and cold stabilized before bottling.

The other vins de liqueur are mostly consumed locally, but can also be found outside their region of production. Macvin de Jura may well be the first documented example of a vin de liqueur (fourteenth century). Pommeau is a drink made from apple must and Calvados and homemade (noncommercial) liqueur

wines are made in Deux-Sèvres département (Pineau) and in the Vendée département (Troussepinette), although the latter is flavored with pine.

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2.11
Fruit Wines and Other Nongrape Wines

I am always happy making wine and writing about it, and if people would only grow fruit to be eaten or drunk it would help them achieve and maintain good health.
—Peggy Hutchinson, 1959

2.11.1 Overview and Scope

The word 'wine' has more than one definition and means different things in different parts of the world. The most restrictive definition, and probably the original and most widely accepted one, is 'the fermented juice of freshly crushed and pressed grapes.' This demonstrates the domination of the grape over other sources (fruits particularly) in the production of alcoholic beverages. In many countries alcoholic beverages made from fruits other than grapes enjoy a significant economic standing and a good reputation, although they often have to contend with deep seated prejudices against them, in favor of grape wine. Alcoholic beverages can be made from any source of fermentable carbohydrates: fruits, vegetables, cereals, roots, honey, sap, leaves and flowers, with or without the use of adjuncts. In English speaking countries, these beverages are often known generally as country wines and those made from fruits are often simply called fruit wines. This is the terminology that will be used in this section and the emphasis here will be on fruit wines, although others will be mentioned and mead has a separate section (Section 2.11.5). Cereal based fermented drinks are dealt with under the headings Beer (Chapter 2.6) and Rice 'Wines' of Asia (Section 2.7.1). Similarly, cider and perry are treated together in Chapter 2.8. In many cases, the driving force behind the production of nongrape alcoholic beverages is the conversion to spirits by distillation. This includes the following conversions: 'grain or cereal wines' to Akvavit (Aquavit), gin, vodka, whiskey or whisky, 'rice wine' to soju or shochu, fruit wine to fruit spirit and Schnaps, potato and other 'wines' to aquavit or vodka, and sugar cane or molasses 'wine' to rum. These and others are discussed in Part 3. Many of the fruit based alcoholic beverages of Asia (such as those produced from green plums, yellow plums or blackberries in China, Japan and Korea) that have names that are loosely translated into English as 'wine' are technically not wines at all. Instead they are best classed as infused liqueurs, since they are usually made by extraction of the fruit with a sweetened spirit base that is derived from vegetables (such as sweet potatoes) or cereals (such as rice, sorghum or wheat). These beverages, which include maeshilju (Korea) and umeshu (Japan), are discussed in Section 3.9.2.

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

Table 2.11.1 *Sugar and acid content of crushed fruit used to make fruit wines. These are typical values in % w/w of fresh fruit, assuming fully but not overripe fruit*

Fruit	Apple	Apricot	Banana	Bilberry/ blueberry	Blackberry	Blackcurrant	Cherry
Sugar content	14	14	17	11/10	8	10	14
Acid content	1.0	1.5	0.3	0.8/0.3	1.2	3	1.0
Fruit	Crab apple	Damson	Gooseberry	Grape (wine)	Grape (table)	Loganberry	Mulberry
Sugar content	16	14	9	20	14	11	10
Acid content	2.0	2.0	1.5	0.5	0.3	2.0	1.0
Fruit	Orange	Pear	Plum	Raspberry	Strawberry		
Sugar content	11	16	15	11	8		
Acid content	1.0	0.6	1.2	1.5	1.2		

2.11.2 Comparison of Fruit for Winemaking

The most successful grape varieties are used as a yardstick for suitability of all other fruits for winemaking. This is because, when fully ripe, wine grapes give the optimum volume of juice (per mass of fruit) with the best balance of sugar and acidity. Additionally, grape must is usually low in protein and pectin content and hence it generally leads to wine that is easily clarified. In normal seasons, grape must requires little or no adjustments by way of adjuncts (sometimes called additives in country winemaking circles), including dilution with water. Table 2.11.1 shows some typical sugar and acid contents of fruits that are commonly used to make wine. Here it can be seen that ripe wine grapes are just about the only fruits that give a good yield of must, that can be fermented to produce an alcoholic beverage of acceptable alcoholic strength (say ~12% or more) and acidity (say pH ~3.2 or higher) without some kind of adjustment. Apples and pears give beverages of lower alcoholic content (5–9% ABV), but higher acidity and tannin content may partially compensate for this. All other fruits yield either too little juice or one that is deficient in sugar (which means a low alcohol content) and usually with excessive acidity. Some fruits, such as banana, although high in sugar content and of reasonable (if low) acidity, give musts that are both low in yield and are difficult to clarify. The majority of most other fruits would give musts that would result in a wine of low alcohol content (3–8% ABV) and high acidity.

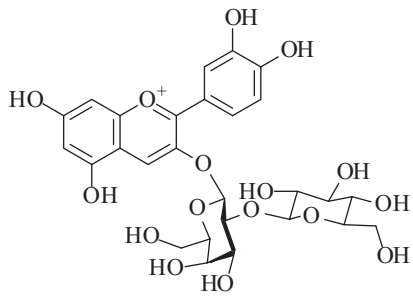
Despite these deficiencies, some fruit wines (as well as cider and perry) are produced commercially from their fruit musts without any or with only little adjustment. The majority, however, are made by methods that involve extraction of ingredients from the fruit into water, and involve adjustment of the extract by addition of sugar, acid and other adjuncts (Section 2.11.3). 'Other adjuncts' often includes a source of polyphenols (loosely called tannins), since despite the fact that many fruits and berries (e.g. damsons, elderberries, mulberries and others with dark skins or flesh) possess high total phenolic contents (TPC), these are considerably diluted by the extraction winemaking process.

Table 2.11.2 lists some polyphenols and anthocyanins (along with some typical concentrations) found in fruit that are commonly used to make fruit wines. Some of these, such as bilberry, elderberry, huckleberry and mulberry have TPC and/or anthocyanin contents that rival those of black grapes, but the polyphenol or anthocyanin profiles are quite different. The differences lie not only in the relative proportions of the aglycones (cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin), but also in the nature of

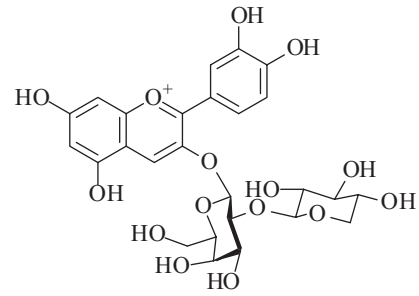
Table 2.11.2 *Total phenolic content and anthocyanin content of some fruits commonly used to make fruit wines. Unless stated otherwise, in mg gallic acid equivalents/100 g fruit weight and in mg cyanidin 3-glucoside equivalents/100 g fruit weight, respectively*

Fruit	Total phenolic content	Anthocyanin content	Principal polyphenols	Principal anthocyanins
Bilberry (*Vaccinium myrtillus*)	820*†‡	380†‡	Chlorogenic acid, syringic acid, caffeic acid, *p*-coumaric acid, flavonols†	Cyanidin 3-glucoside, delphinidin 3-glucoside, delphinidin 3-galactoside§
Blackberry (*Rubus* spp.)	962**	178**	Quercetin glycosides, ellagic acid derivatives**††	Cyanidin 3-arabinoside, cyanidin 3-malonoylglucoside, cyanidin 3-dioxaloylglucoside††
Blackcurrant (*Ribes nigrum*)	763‡‡	262‡‡		Cyanidin 3-rutinoside, delphinidin 3-rutinoside, delphinidin 3-glucoside§§
Blueberry (*Vaccinium* spp.)	359***	238***	Quercetin glycosides††	Malvidin 3-galactoside, delphinidin 3-galactoside, cyanidin 3-galactoside, delphinidin 3-arabinoside††
Cherry (*Malus*)	72†††	11†††	Neochlorogenic acid, *p*-coumaroylquinic acid, chlorogenic acid, (-)-epicatechin, rutin†††	Cyanidin 3-rutinoside, cyanidin 3-glucoside, pelargonidin 3-rutinoside†††
Huckleberry (*Vaccinium ovatum*)	1169‡‡‡	563‡‡‡	Chlorogenic acid, flavonol glycosides‡‡‡	Cyanidin 3-galactoside Cyanidin 3-arabinoside Dephinidin 3-arabinoside Peonidin 3-galactoside Malvidin 3-galactoside‡‡‡
Raspberry (*Rubus idaeus*)	1769§§§	43§§§	Ellagic acid derivatives, flavonol glycosides††	Cyanidin 3-sophoroside, cyanidin 3-glucoside††
Strawberry (*Fragaria ananassa*,			Ellagic acid derivatives, coumaroyl glycosides, flavonols††****	Pelargonidin 3-glucoside, pelargonidin 3-rutinoside, cyanidin 3-glucoside††****

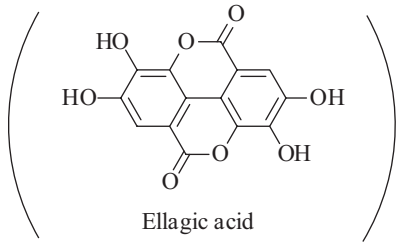
*Anthocyanins, flavonols and hydoxycinnamic acid derivatives only. Individual phenolic compounds determined by HPLC; †Hukkanen *et al.* (2006); ‡Castrejón *et al.* (2008); from high bush (*V. corymbosum* L.) varieties, determined by HPLC; §Faria *et al.* (2005); **Siriwoharn and Wrolstad (2004); ††Seeram *et al.* (2006a); HPLC/MS/MS; ‡‡Plessi *et al.* (2007); mean values for six varieties; §§Slimestad and Solheim (2002); ***Ehlenfeldt and Prior (2001). There are data for 87 cultivars here: these values are for *V*. corymbosum L, var. 'Little Giant'; †††Usenik *et al.* (2008); mean values for 13 varieties, individual components determined by HPLC; ‡‡‡Lee *et al.* (2004); §§§Pantelidis *et al.* (2007); mean values for three red cultivars (summer/autumn fruiting); ****Seeram *et al.* (2006b).

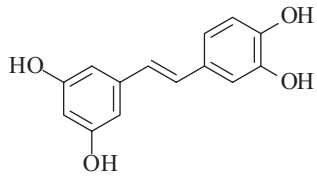


Cyanidin 3-sophoroside
(Raspberry, *Rubusidaeus*)

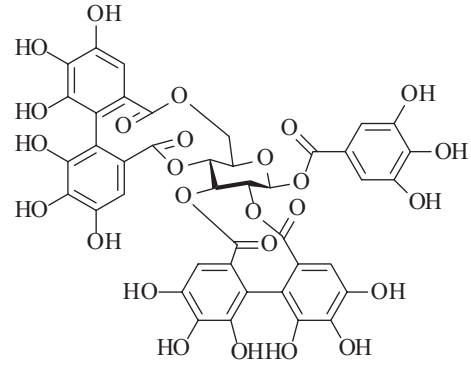


Cyanidin 3-sambubioside
(European elderberry, *Sambuca nigra*)
Jordheim *et al.* (2007)





Piceatannol (a stilbene)
(Blueberry, *Vaccinium* spp.)
Rimando and Cody (2005)



Ellagitannin (galloyl-bisHHDP-glucoside)
(Strawberry, *Fragaria* ssp.)
Seeram *et al.* (2006a)

Figure 2.11.1 *Some characteristic phenolic substances of fruits used to make fruit wine*

the carbohydrate moieties (e.g. galactose, glucose, rafinose, xylose, etc.). In black grape must and young red wine, the dominant anthocyanin is malvidin 3-glucoside, whereas in most other musts of dark skinned or dark fleshed fruits, the major anthocyanins are cyanidin-based (Table 2.11.2). Thus adulteration (such as the use of elderberry pigments to deepen the color of red wine) is often readily detected by chromatography (Bridle and Garata-Viguera, 1996). Figure 2.11.1 shows some prominent anthocyanins and polyphenols of dark fruits. Polyphenol profiles of fruits used in winemaking also vary widely, although this variation has been less frequently used to detect adulteration or fraud.

Ellagic acid derivatives (Figure 2.11.1) are found mostly in seeds and pips (embryos), as well as in more woody parts of plants. Thus they tend to be more prominent in the must of berry fruits such as blackberry, raspberry and strawberry, where normal winemaking practices of crushing/infusion/pressing causes some leaching. As with grape must, flavonols (especially quercetin and kaempferol derivatives) are present in fruit juices. Likewise, flavan-3-ols (–)-epicatechin and (+)-catechin are found in many fruit musts,

although often in low concentrations (Siriwoharn and Wrolstad, 2004; Seeram *et al.*, 2006a). Advances in technology, especially LC/MS/MS (Sections 4.3.3 and 4.4.5), have significantly widened the scope of natural phenolic compound analysis, so that not only are minor components being newly reported, but more reliable structural information is obtained for more complex components (including condensed molecules), and in some cases correcting earlier structural assignments (Seeram *et al.*, 2006a; 2006b). Similarly, GC/MS in SIM (selected ion monitoring) mode (Section 4.3.2) has been able to detect and quantify the derivatized stilbenes, resveratrol, pterostilbene and piceatannol (Figure 2.11.1) in blueberries (*Vaccinium* spp.) (Rimando and Cody, 2005).

Simple phenolic acids are present in fruit juices (often as glycosides); these include chlorogenic acid, cinnamic acid derivatives and neochlorogenic acid in huckleberries (*Vaccinium* spp.) (Lee *at al.*, 2004), neochlorogenic acid and *p*-coumarylquinic acid in sweet cherries (Usenik *et al.*, 2008), and chlorogenic acid and neochlorogenic acid in sweet rowanberries (Hukkanen *et al.*, 2006). Mattila *et al.* (2006) have determined the phenolic acid composition of a very wide range of berry fruits, where caffeic acid was found to be particularly concentrated in bilberry, blueberry and sweet rowanberry, protocatechuic acid in bilberry and blackcurrant, *p*-coumaric acid in bilberry and sweet rowanberry, *p*-hydroxybenzoic acid in strawberry, cinnamic acid in cloudberry (*Rubus chamaemorus*), and gallic acid in raspberry and strawberry. This study has provided much information on berry fruits that are not readily available outside specific areas of the world, especially northern Europe.

The major acids (other than phenolic acids) in fruit are malic and citric acids, as opposed to tartaric and malic acids in grapes. In cherries (as in apples and many varieties of pear – see Section 2.8.2) malic acid in predominant, but in many berry fruits, such as blackberry, raspberry and strawberry, citric acid (or isocitric acid lactone) is significant or predominant. As a result of this, malolactic fermentation may not be required (or may even be undesirable) in the production of certain wines made from berry fruits such as strawberry.

Like grapes, the major sugars present in fruit are the reducing sugars fructose and glucose, but unlike grapes, low concentrations of sucrose are also present. For example, bluberry, raspberry and strawberry sucrose contents are typically 10%, 20% and 10% (respectively) of the total sugar content. Additionally, sugar alcohols (especially sorbitol) are minor components of many fruit musts, as they are for grape, apple and pear juices.

Each kind of fruit has its own characteristic aroma compounds; in many cases over 100 such compounds have been detected, and often quantified, mainly by gas chromatography (Section 4.3.2). For a particular fruit, many of these compounds are present in concentrations that are well below their odor threshold values (OTV) (Section 4.7.2) yet they may be essential for the full (true) aroma of that fruit. Likewise, many of these compounds individually possess odors that are not reminiscent of the fruit aroma. However, there are often individual compounds or a combination of small numbers of compounds that obviously contribute most to the actual fruit aroma; their odors are a simplified essence of the whole fruit odor. Examples of such compounds are given in Table 2.11.3, where it can be seen that the majority are alcohols, carbonyl compounds, esters, terpenoids and norisoprenoids. Although the aroma profile of a particular fruit is altered during fermentation and other processing events, many of the characteristic aroma compounds persist, so that the resulting wine may often be strongly reminiscent of the original fruit. This is especially the case if no heat treatments (e.g. hot water infusion or boiling) are used in the winemaking processes.

2.11.3 Making Fruit Wines

There are two basic styles of fruit wines. The first, which is the more common, strives to maintain the fruit character of the base ingredient, giving it emphasis over all other aromas and flavors in the finished product. The second style aims to create a fruit wine that has a general resemblance to grape wines or even

Table 2.11.3 *Characteristic aroma compounds of some fruits*

Fruit	Characteristic aroma compounds
Apricot	Benzaldehyde, linalool, 2-phenylethanol, 4-terpineol and α-terpineol
Blueberry	Ethyl 3-hydroxybutanoate, hexanol, (*E*)-2-hexenol, linalool
Raspberry*	1-(*p*-Hydroxyphenyl)-3-butanone ('raspberry ketone'), (*Z*)-3-hexen-1-ol, β-ionone
Lychee†‡‡	(*Z*)-rose oxide, β-damascenone (rose-floral), dimethyl disulfide, dimethyl trisulfide, 2-methylthiazole and other sulfur compounds
Elderberry‡	Dihydroedulan, β-damascenone, 1-pentanal (fruity), 1-nonanal (flowery), 1-hexanal (grassy), 1-octen-3-ol (mushroom)
Banana§	Pentan-2-one, isobutyl and isoamyl acetates and butanoates
Peach**	Hexalactone, γ-decalactone
Strawberry††	2,5-dimethyl-4-hydroxy-2H-furan-3-one (DMHF)
Pineapple	Allyl carboxylate esters, ethyl and methyl 2-hydroxybutanoates and 2-hydroxyhexanoates
Cherry	Acetophenone, benzaldehyde, benzyl alcohol

*Fuganti *et al.* (1996); †Ong and Acree (1999); ‡Jensen *et al.* (2001) – many other compounds contribute to fruity, flowery, grassy and mushroom notes; §Salmon *et al.* (1996); **Tamogami *et al.* (2001) – determined as enantiomeric composition; ††Mura and Zabetakis (2002); ‡‡Mahattanatawee *et al.* (2007).

a resemblance to a specific wine type, such as Port or Champagne. The making of fruit wines is generally very similar to winemaking (Sections 2.9.1, 2.9.2 and 2.9.3), the major differences being at the early stages of the process, as outlined in Figure 2.11.2. Some fruits, such as apples, pears (see Chapter 2.8), peaches and some berry fruits give sufficient juice when crushed and pressed, with a reasonable balance between acids and sugars. After perhaps addition of sugar syrup to improve the acid:sugar ratio, or perhaps the addition of a little water, these juices can be fermented without any further additions or adjustments. This process seems to be best for wines of the first of the two aforementioned styles; the wines produced this way are often intensely fruity and are immediately recognizable as strawberry or peach or whatever. In order to accentuate the fruit

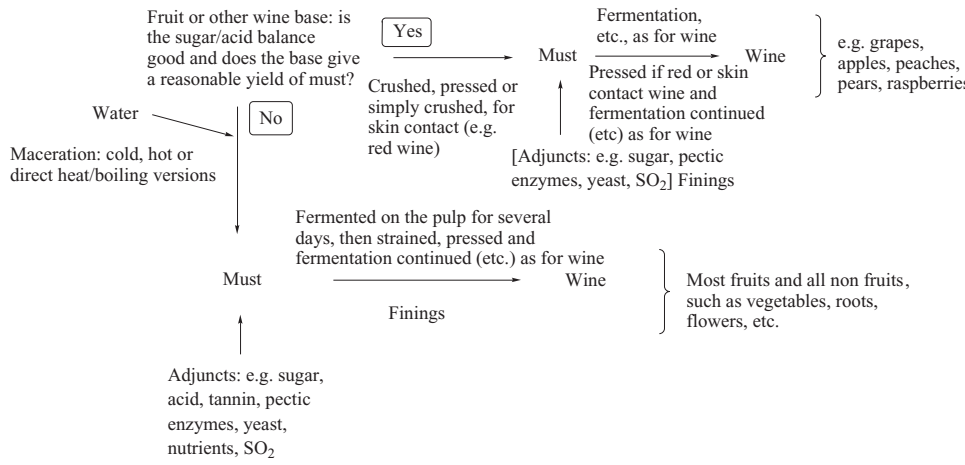


Figure 2.11.2 *Summary of processes used to make fruit wines and country wines. Optional items are indicated in square brackets*

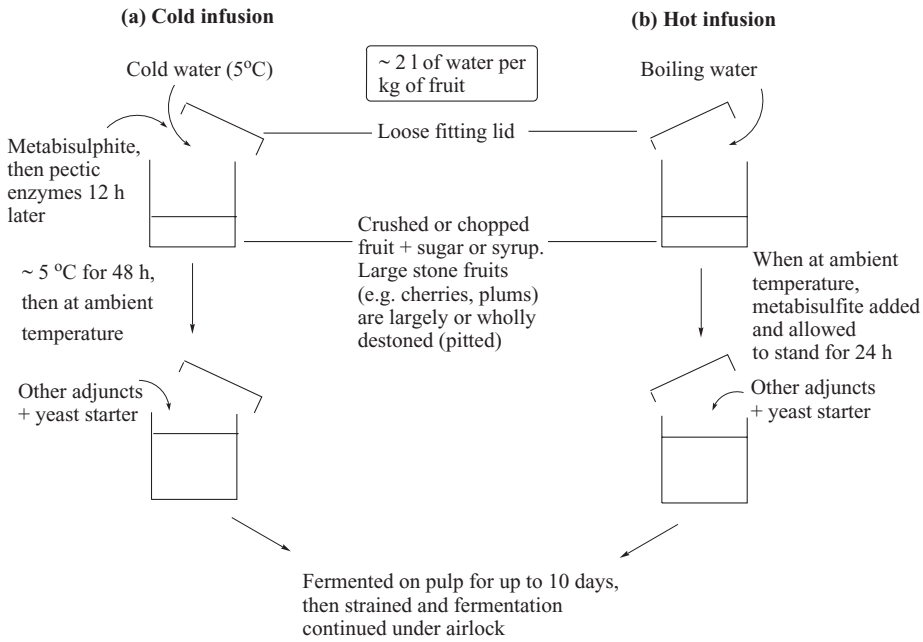


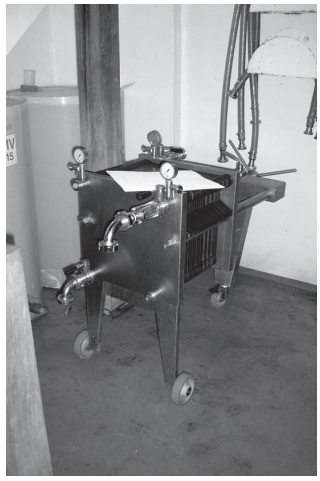
Figure 2.11.3 *Infusion techniques for making fruit wines*

character and also to balance the usually high acidity, they are often made semi-sweet or sweet. This process, shown at the top of Figure 2.11.2, is similar to the making of wine, cider or perry. The alternative process – basically an infusion technique with water, followed by incorporation or readjustment of components by use of adjuncts – is essential for fruits that produce too little juice or juice with poor balance of acids and sugars. The cold and hot infusion versions are generally preferred over the direct heat or boiling version, as the last named technique leads to the loss of volatile components and the formation of 'cooked' flavors (via Maillard reactions, presumably). The hot infusion technique (Figure 2.11.3) involves mixing the crushed or whole fruit with boiling water and allowing the mixture to cool at ambient temperature.

Some adjuncts are usually added straight after mixing, but enzymes such as pectolases are added when cool. Adjuncts include sugar or invert sugar syrup, acids, yeast nutrient, pectic enzymes, grape concentrate, grape tannins, sodium or potassium metabisulfite and oak chips. Many wineries may only use a selection of these adjuncts, depending on the desired nature of the final product. The cold infusion technique is somewhat gentler than the hot method, but probably gives similar results. The hot water helps start the leaching process that is an essential part of the initial fermentation period, by breaking cell walls. It also aids sterilization before fermentation starts, although metabisulfite plays the bigger role in this respect. If not already crushed, the fruit is macerated with a mechanical or electrical device, such as the one shown in Figure 2.11.4. After adding and mixing of sodium or potassium metabisulfite, the must is allowed to stand for 24 h. Most wineries then add an activated pure yeast starter culture at this stage and fermentation on the pulp commences after a few hours. Fermentation on the pulp is an important part of the process – it allows and indeed facilitates continuation of the leaching process via breakdown of cellular wall tissues. The rapidly increasing ethanol concentration during this period helps extract flavor compounds and pigments from the fruit tissues. The presence of pectolytic enzymes also helps in this respect. In this way, the hot or cold infusion methods can produce wines that are almost as overtly fruity and (for rosé and red wines) as deeply colored as those made by the direct crushing and pressing method.

Impeller type fruit macerator at Lurgashall Winery, UK.

Destoning and pulping machine for plums and other stone fruit. *Photograph by courtesy of Vigo Ltd., Cullompton, UK.*



Sheet filtration equipment at Lurgashall Winery.

Figure 2.11.4 *Some equipment used in the production of fruit and country wines. Photo courtesy of Vigo Ltd, Devon, UK*

The overall character of the wine can be influenced markedly by the nature of the adjuncts used in the infusion techniques. Apart from sugars and metabisulfite (and in some cases pectic enzymes), the most essential adjuncts are acids and tannins. In former times, amateur country winemakers in Britain used (maybe some still do!) lemon juice and cold tea as a source of acid and tannin, respectively. Nowadays food grade organic acid and grape tannin have largely taken their place. As the dominant organic acids of fruits other than grapes are either malic or citric acids, tartaric acid may be the best acidity regulator for use in the making of most fruit wines. The acid strengths of the common fruit acids are malic acid ($pK_a(1) = 3.40$) < citric acid ($pK_a(1) = 3.13$) < tartaric acid ($pK_a(1) = 2.98$). Whatever acid (or mixture) is used, care in taken to keep the

pH of the final must (i.e. before addition of the yeast starter) between 3.0 and 3.4, in most cases. The weight of sugar used to make a particular volume of fruit wine is determined by experience, according to the type of wine desired (i.e. its % ABV and degree of residual sugar); the winemaker will measure the original gravity of the must before adding the yeast starter. If incorrect, the original gravity can be altered by the addition of more sugar syrup or more (sterile) water. Grape concentrate can be used in place of some (or all) of the sugar. Its main advantage lies in the better balance it often imparts to fruit wines, along with a certain increased vinosity that underlies, but does not dominate, the finished wine. The major disadvantages are the extra cost and the fact that, according to the particular country's food labeling regulations, its presence will need to be indicated somewhere on the label. The presence of all other adjuncts will also need stating on the label in some countries (e.g. the USA), a situation that may deter certain kinds of consumers. Wines made by the hot or cold infusion technique with a grape concentrate adjunct tend to have more vinous character, at the expense of overt base fruit character. Indeed, with careful choice of base fruit, adjuncts and yeast strain, fruit wines can be made to resemble certain commercial grape wines. Commercially, this style of fruit wine is in a minority, although it is very popular with amateur winemakers in the UK. Probably the most commercially emulated grape wine style is Champagne or sparkling wine – witness the sparkling (and even bottle-conditioned) cider and perry (Sections 2.8.5 and 2.8.6) and sparkling wine made from nongrape bases such as green gooseberry, rhubarb, peach or elderflower (Section 2.11.4).

Oak chips can be added just before pitching the yeast starter. As with wine (Section 2.9.5 – see also Section 2.8.7), this is an inexpensive way give the wine oak character and it makes possible the emulation of well known commercial grape wines, such as Rioja. Experience has shown that highly toasted oak chips tend to be much more successful than low toasted types, but care must be taken to avoid overoaking, especially with white fruit bases. The generally recommended amount is 100 g of oak chips to 100 l of must; the 7–10 day pulp fermentation for red wines (less for white wines) should then give about the right level of oak flavor, as the oak chips are filtered off with the pulp and debris during the straining and pressing processes. Of course, oak chips can be added at the maturation stage and there are also oak extract materials (both solid and liquid versions) that can be used in the place of oak chips. In the experience of many fruit winemakers, however, the best oak flavors are obtained after the fruit wine has been subjected to a maturation period in an oak cask. As with wine, the larger the cask, the longer the maturation period that is possible. The very slow and controlled ingress of oxygen through the wood pores into the wine results in a modest amount of oxidation, something that is not possible with wines stored over oak chips in non-porous containers. Furthermore, malolactic bacteria are present in the wood pores and if the conditions are favorable, MLF will occur during the maturation period in wood, provided that there is sufficient malic acid in the wine. These factors probably make up the biggest differences between oak matured and oaked red fruit wines. Indeed, using a base like elderberries or blackberries (or a mixture), it is possible to make a red fruit wine that bears a striking resemblance to Rioja or other high quality oaky red wines.

Pectolytic enzymes are useful not only for hydrolysis of natural pectins, but also for the degradation of fruit cell walls, thereby improving the efficiency of extraction of flavor compounds and pigments. The natural pectolytic enzymes of fruit will be largely denatured by the hot infusion technique and so enzymic adjuncts are added when the must is at ambient temperature and some 12 h following the addition of metabisulfite, since this acts as an inhibitor. Commercial pectolytic enzymes are of fungal origin and can be purchased as liquid formulations or powders.

Choice of yeast can influence the style of a fruit wine, and the winemaker has a wide range from which to choose. Many companies (Gervin, Lallemand, Red Star, Wyeyeast, Young – to name but a few) produce several cultured yeasts, some of which are recommended for specific results (e.g. delicate white, robust red, sparkling white, sweet white) or for particular types of fruit bases. Examples are shown in Table 2.11.4, but it should be kept in mind that there is quite a lot of tolerance and it is possible to use a particular yeast strain to satisfactorily ferment quite different fruit (or even nonfruit) musts. It is also possible to purchase dried

Table 2.11.4 *Possible cultured wine yeasts for production of fruit wines*

Fruit base	Wine style	Possible wine yeast (with comments)
Black stone fruit (plums, cherries, damsons and sloes) and black/red berry fruit (e.g. blackberry, blackcurrant, blueberry, elderberry, mulberry)	Full-bodied red (long maceration time) Medium-bodied red	Lalvin RC212 (varietal aroma enhancer) Lalvin B45 (Brunello di Montalcino strain) Gervin No. 2 or Red Star Montrachet strain (UC Davis 522 strain) Gervin No. 1 (Fermivin; Bordeaux 7013 strain) Red Star 904 (Institute Pasteur strain) (varietal aroma enhancer)
White or pale fruit (e.g. apple, citrus fruit, gooseberry, pear, white currant)	Full-medium (dry-sweet) wines, with enhanced aroma	Lalvin ICV D-47 Lalvin 71B-1222 (reduces acidity due to ability to metabolize ~30% of must malic acid content) Lalvin EC-1118 (not for sweet wines), Red Star 904 (Institute Pasteur strain)
	Delicate, light, fruity wines	Gervin No. 5 (GVN) (good for low temperature fermentation) Gervin No. 9 (CC)
Acidic white fruit (e.g. some apples, gooseberries, plums)	Sparkling wine	Red Star 750 (Geisenheim strain), Red Star 796 (*S. bayanus*; good for restarting stuck fermentations), Gervin No. 6 (*S. bayanus* 8906 strain)
General	General	Lalvin 71B-1122, Lalvin K1-V1116 (highly tolerant of conditions), Red Star 595 (Institute Pasteur strain)

cultured yeast, nutrient and bentonite (finings) mixtures, so that a certain amount of clarification of the wine can take place during the early stages of the winemaking process.

Straining and processing the wort is carried out after 3–10 days of pulp fermentation, depending on the desired final result (especially with regard to depth of color for rosé and red wines). Rack and cloth presses (as used for cider and perry – Section 2.8.3), basket wine presses and pneumatic wine presses are all used by various commercial procedures. Thereafter, the fruit winemaking process is similar to that for cider and perry (Sections 2.8.4 and 2.8.5) and wine (Sections 2.9.1–2.9.5).

The chemical reactions (both enzymic and nonenzymic) that occur during fermentation and maturation are similar to those that have been described for wine (Chapters 2.2, 2.3 and Section 2.9.5). A large number of volatile aroma compounds are produced as end products of various yeast metabolic pathways and the aroma of these is superimposed on those of the surviving characteristic fruit aroma compounds (i.e. those that have not been degraded or otherwise lost during fermentation). As with most alcoholic beverages, the major aroma compounds are higher alcohols, esters, terpenoids and norisoprenoids.

Strawberry wine is reported to have several esters (amyl acetate, ethyl hexanoate, ethyl octanoate, ethyl 9-decenoate and ethyl cinnamate) as the major flavor compounds (Kafkas, *et al.*, 2006). As may be expected, limonene is a flavor component of clementine (*Citrus reticula* Blanco) wine, as are a number of esters, higher alcohols and an assortment of furfurals (Selli *et al.*, 2004).

In the making of red fruit wines, the anthocyanin pigments undergo a number of changes that are likely to be similar to those reported for red grape wines (Section 2.10.7). Thus, after fermentation and maturation, a substantial proportion of the fruit anthocyanins exist as derivative pigments: pyranoanthocyanins, acetaldehyde (and other aldehyde) mediated anthocyanin-flavan-3-ol condensation products and direct

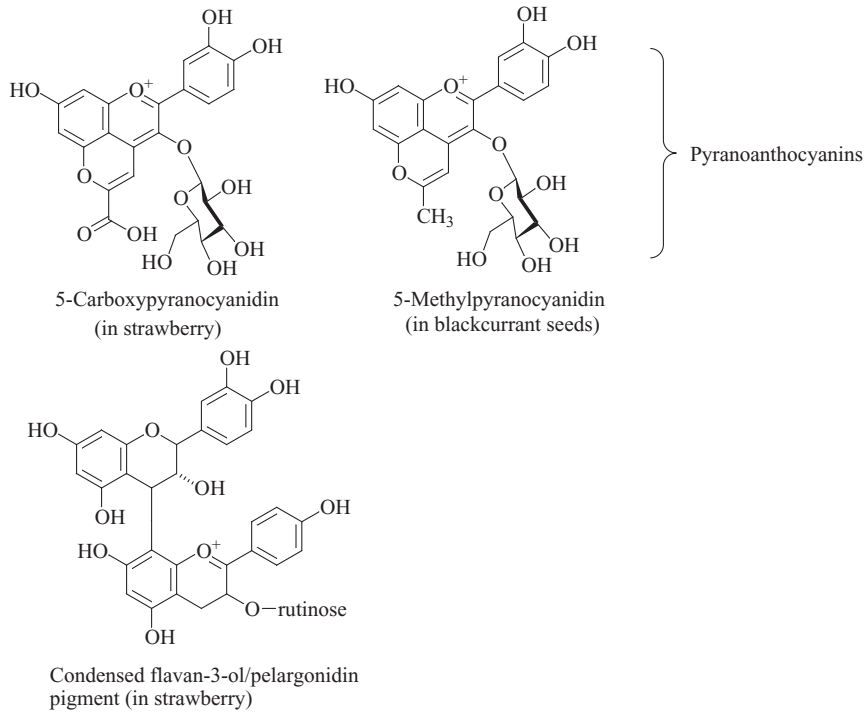


Figure 2.11.5 *Some anthocyanin-derived pigments in soft fruit. Gonzalez-Paramas* et al. *(2006) and references therein*

anthocyanin–flavan-3-ol condensation pigments (Figure 2.11.5), although it is claimed that some of these pigments are present in the original fruit (Gonzalez-Paramas *et al.*, 2006).

Apart from the changes outlined above, any winemaking process that involves a significant amount of heating (i.e. the hot infusion and especially the direct heat method) is potentially responsible for a certain amount of degradation of anthocyanins. Sadilova *et al.* (2006) have shown that exposure of pigment isolates from elderberry and strawberry to heat at 95 °C for 1 h leads to loss of ∼ 19% and 17% of anthocyanins, respectively. Furthermore, they showed that cyanidin sambubioside (glucosylxyloside), a major anthocyanin of elderberry, degrades via deglycosylation to the aglycone cyanidin, which then decomposes to protocatechuic acid and phloroglucinaldehyde. Similary, pelargonidin3-glucoside of strawberry is degraded to phloroglucinaldehyde and 4-hydroxybenzoic acid (Figure 2.11.6). These results were obtained using isolated purified pigments from commercial concentrates after dilution with water, but it is reasonable to suppose that similar reactions would occur in elderberry or strawberry must during winemaking procedures that use heat.

2.11.4 Country Wine Styles

Country wines, and fruit wines in particular, are made throughout the world. The largest producers appear to be Canada, the USA, the UK and France, although in the last named country most of the fruit wine in distilled to give the corresponding spirit (Chapter 3.8). The emphasis is generally on intense fruit character, with winemakers tending to use a high fruit load per volume of finished wine. Sugar syrup or water are added

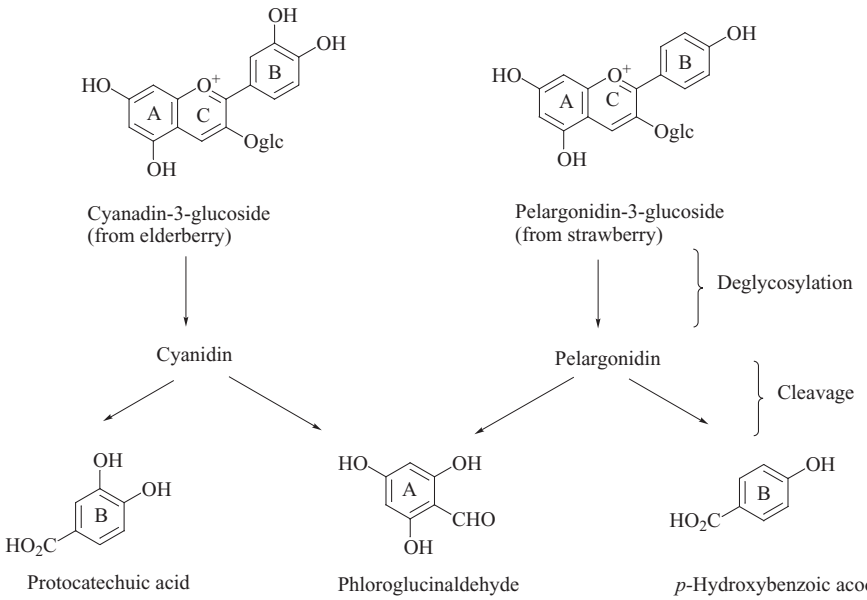


Figure 2.11.6 *Thermal degradation of anthocyanins from elderberry and strawberry. From Sadilova* et al. *(2006)*

in cases where sugar content of the juice is too low or the acid content too high, respectively. There is also a tendency to ferment to 9–11% ABV, in order to preserve maximum fruit character, a common opinion being that 12–14% ABV (or higher) detracts from the fruit character. These wines rely on a delicate balance between sweetness and acidity and because of their strongly fruity character, they are used as social wines, dessert wines or mixers. The lighter styles can be used as aperitifs and the drier styles as dinner wines, but these appear to be in a minority. Some wineries use first grade (i.e. eating quality) fruit, whereas others use 'culls' or 'seconds,' as long as no rotten fruit is included. Other wineries use at least a proportion of concentrate (e.g. cherry or raspberry) and some use frozen fruit, so that wine can be made at different times of the year, rather than just at harvest time (see Section 2.8.6). Freezing also helps to break down fruit cellular tissues, thereby making the extraction of a clearer juice easier. Nevertheless, pectic enzymes are extensively used throughout the fruit wine industry, as is fining (bentonite and gelatin being most popular) and filtration. Many fruit wines are given a polish pad (sterile) filtration immediately before bottling. The majority of these fruit wines are made from a single fruit type, although some mixed fruit or 'formula' types are made. Fermentation and maturation vessels are typically of stainless steel or fiberglass, and residence times are often less than six months, so that bottling is achieved with maximum retention of fruit character. Oak characters and lack of malolactic fermentation are of little consequence for most of these wines (but see later for exceptions).

In the USA, fruit wine production is concentrated in the Midwest and in the northern states of both the Atlantic and Pacific seaboards. The states of Illinois, Michigan, Minesota, Missouri and Ohio possess many fruit wineries, such as Diamond Ridge (Minesota), Lynfred Winery, Illinois Cellars (both Illinois), Chateau Grand Traverse (Michigan), St. James Winery (Missouri) and Wyandotte (Ohio). Some of these wineries specialize in fruit wines, whilst others also make wine from grapes (e,g. Diamond Ridge) or cider (Illinois Cellars). Further west, the Prairie Berry Winery of South Dakota makes an oak barrel aged wine from chokeberries (*Aronia arbutifolia* and *A. Melanocapa* – giving red and black fruit, respectively), as well as grape and other fruit wines. On the Pacific coast, famous (grape) wineries, such as Oak Knoll Winery (Williamette Valley, Oregon) and Bonny Doon Vineyard (California) make intensely flavored raspberry wines,

called Frambrosia and Framboise, respectively. The latter is made to resemble Crème de Framboise from Alsace, Burgundy and other European locations, and so is a fortified fruit wine. There are also fruit wineries in the eastern states, although many of these also produce grape wines, such as Chateau Renaissance (Finger Lakes, New York) and Sand Hill Berries (Pennsylvania).

At present (2009) only grape wines are allowed by the Alcohol and Tobacco Tax Bureau to carry a vintage indication on the bottle, provided at least 95% of the wine in the bottle is derived from grapes harvested in the stated year. Since the presence of a vintage year on the bottle is a common perception of superiority and given the fact that fruit and other country wines, like grape wines, have a wide range of ageing capabilities, it would be a very useful step to change the regulations regarding statement of vintage to nongrape wines.

In Canada, the National Fruit Wine Committee was set up to oversee fruit wine production quality and standards in the regions where fruit wines are in commercial production (Ontaria, Quebec, British Columbia and Atlantic Region). Regulations were drafted to help producers achieve high and consistent standards of quality for export markets. The biggest concentration of fruit wineries is in Ontario, but there are many wineries in other Canadian provinces, with the exception of the most northerly ones. Sunnybrook Farm Estate (Ontario) has a very big range of fruit wines including ice wine made by gently pressing frozen fruit, and unusual blueberry and chocolate wine. In British Columbia, Forbidden Fruit and Kermode Wild Berry Wines are well known for their produce, including fortified wines. East of B.C., Field Stone Fruit Wines (Alberta), Rigby Orchards Ltd (Killarney, Manitoba) and Cypress Hill Winery (Saskatchewan) make a good range of fruit wines, the first – named using wild Choke Cherry (*Prunus virginiana* – 'bird cherry') for some of its products. Fruit wineries also exist in Quebec, Nova Scotia and Newfoundland, Rodrigues Markland Cottage Winery (Newfoundland) being an example. In Australia and New Zealand, there are fruit wineries that concentrate on wines made from indigenous or tropical/subtropical fruit, making wines that are probably made nowhere else in the world. Barossa Fruit Wines (South Australia) produce wine from Quandong (Australian national peach or *Santalum acuminatum*), as well as from apricot, blueberry and mulberry. Even more exotic is Paradise Estate (Queensland); here tropical fruit wines are made from mangosteen, banana, coconut and pineapple (white), and red mango, dragon fruit and banana (red). Fruit wine production is quite extensive in New Zealand (especially North Island). Examples of wineries include Lothlorien (Ahuroa), Manaku Blue (Rotorua), Riverhead Estate Winery (Kumeu) and Sentry Hill (Taranaki). Lothlorien and Sentry Hill make wine using feijoa (*Feijoa Sellowiana*: pineapple guava or guavasteen) and the latter produces several styles of red wine from boysenberry (a loganberry-dewberry-*Rubus ursinus* cross), quite rare in the fruit wine world.

In the UK, the two largest fruit wineries are probably Broadland Wineries (Norfolk) and Lurgashall Winery (West Sussex). The former produces a range of fruit wines and fortified wines from concentrate (British Wines), as well as mead. The latter makes around 500 000 bottles of fruit wines, liqueurs and mead. The fruit wines are made from fresh fruit by the cold infusion method (Section 2.11.3), but both wineries produce elderflower wine and Lurgashall also makes a wine from rose petals and one from silver birch sap.

Dedicated fruit wineries also exist in Scotland, the best known are probably Cain O'Mohr (between Perth and Dundee) and the Orkney Wine Company. Both companies concentrate on fruit wines (from both wild and cultivated fruit), including blaeberry (bilberry) and cranberry, rare for UK fruit wines. They also produce wines from leaves (oak) and flowers (hops, gorse), which may well be unique to the UK. Apart from these few dedicated country wineries, some English vineyards produce fruit wines, notably Avalon (Somerset), Carr Taylor (East Sussex), Kemp's Wine (Suffolk), Rosemary Vineyard (Isle of Wight), Sascombe (Suffolk) and Sedlescombe (East Sussex). Additionally, Old Walls (Devon) makes Elderberry and Regent, a mixed fresh fruit-grape wine, possibly unique in the UK at the time of writing (2009). Several of the above-mentioned vineyards, along with other English vineyards, make cider (Section 2.8.8), mead and liqueurs (Section 3.9.2). Most UK fruit wines are probably made by cold infusion methods; alcoholic strengths vary from ~10% (v:v) to nearly 20% (v:v) for some fortified country wines.

2.11.5 Mead

Mead is basically honey wine; in its simplest form it is an alcoholic beverage made by the fermentation of a solution of honey in water. Since honey, along with grapes, fruit and malted cereals, was a source of sugar for early man, mead probably has as venerable a history as beer, cider and wine. The first mention of mead is probably in the books of Rigveda, of the ancient Vedic religion of India (from the seventeenth century BC). Since then, mead is known to have been produced as a popular beverage in all three continents of the Old World, especially in Ancient Greece, and the Celtic and Germanic kingdoms of northern and western Europe, in the early centuries after the birth of Christ. For a variety of reasons, the popularity of mead waned in many parts of the world, in favor of beer or wine. It maintained some popularity in the Baltic States and some Slavic countries (especially Russia) and recently has undergone a revival of popularity in western Europe, like cider and ale. Likewise, its production in Australia, New Zealand and the USA has increased along with the number of craft breweries and wineries during the last two decades.

Like other alcoholic beverages, mead can be produced by natural yeasts (spontaneous fermentation) or by the use of cultured wine yeasts, the latter method predominating nowadays. However, some meads produced by craft brewers and specific types, such as Tej are made by spontaneous fermentation. Tej is home processed, but commercially available honey wine in Ethiopa. In a study of 200 samples of this beverage, it was found that over 25% of the yeast isolates belonged to the *Saccharomyces cerevisiae* genus along with *Kluyvermyces bulgaricus* (16%), *Debaromyces phaffi* (14%) and *K. veronae* (10%) (Bahiru *et al.*, 2006). The lactic flora consisted of *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptomyces* species, with the first named being the most abundant.

The aroma and flavor of simple mead originates from original honey flavor compounds, along with yeast fermentation and products. Also, many varieties of mead are made with adjuncts (herbs, spices, fruit juices, etc.), which will contribute to aroma and flavor – these are discussed later. The honey aroma compounds will be a mixture of those derived directly from blossoms and those synthesized by the bees. Since simple meads are immediately recognizable as being derived from honey, it is reasonable to assume that the aroma characteristics of mead are similar to those of the honey from which they were derived. There is a lack of information in the literature on mead aroma compounds, but there is much on honey aroma (see Alissandrakis *et al.*, 2007 and references therein), so this will be discussed next.

Honey aroma varies according to the source of nectar, although aroma differences are often subtle, suggesting that, despite the aroma profiles being different, honeys from different blossom sources may have many common aroma compounds (Moreira and De Maria, 2005; Alissandrakis *et al.*, 2007; Tanaki *et al.*, 2007 and references therein). Common compounds include *p*-anisaldehyde, benzaldehyde, benzoic acid, (*E*)-β-damascenone, decane, heptanal, linalool, linalool oxides, nonanal, phenylacetaldehyde, 2-phenylethanol, octane and octanal. Nevertheless, it is possible both to select marker compounds (compounds that are unique to or are in high abundance) and major contributors to aroma of particular honeys, according to their source of nectar. This is particularly the case with blossom honeys, but is not so easy with pine honeys (it requires statistical Kohonen self-organizing map – KSOM analysis), where aroma compounds are dominated by saturated, aromatic and terpene hydrocarbons. For example, lilac aldehyde isomers have been suggested as markers for citrus honey (Allisandrakis *et al.*, 2007) and 3-carene as a marker for Turkish pine honey (Tanaki *et al.*, 2007). Similarly, benzaldehyde, benzonitrile, methylbutanoic acids and 2-phenylethanol have been identified as major aroma contributors of cambará (*Gochnatia velutina*) honey (Moreira and De Maria, 2005), phenylacetaldehyde and coumarin of lavender (*Lavandula augustifolia*) honeys, and *p*-anisaldehyde, linalool, phenylacetaldehyde and 2- phenylethanol of haze (*Rhus Succedanea*) honeys (Moreira and De Maria, 2005 and references therein). Honey displays a wide range of radical scavenging activity according to its floral origin (Baltrušaitytė *et al.*, 2007), with phenolic compounds apigenin, chrysin, *p*-coumaric acid and kaempferol playing a major role. Additionally, honey is known to be rich in enzymic and nonenzymic

antioxidants, including ascorbic acid, amino acids, carotenoids, catalase, flavonoids, glucose oxidase, Maillard reaction products, phenolic acids, proteins and various organic acids. Common flavonoids appear to be chrysin, galangin, kaempferol, luteolin, pinobanksin, pinocenbrin and quercetin (Baltrušaitytė *et al*., 2007). Although antioxidant properties of honeys and honey extracts have been determined by many different methods, such as the $ABTS^{+\bullet}$ and $DPPH^{\bullet}$ methods (Section 4.4.3), there is once more a lack of data for mead. However, it is likely that a considerable number of honey antioxidants will survive fermentation and other winemaking processes, although some of the proteins and phenolic substances may well be removed by fining agents or filtration. Also, mead recipes that call for prolonged boiling or excessive heat treatment will not only alter the antioxidant activity of the honey, but will also cause changes in the aroma profiles, by loss of natural aroma compounds and the production of extra Maillard products. In particular, enzymes are denatured (reversibly or irreversibly, depending on the temperature and duration of heating), so that diastase activity is impaired and hydroxymethylfurfural (HMF or 5- hydroxyl methyl) furan-2-carbaldehyde concentration increases (Tosi, *et al.*, 2008).

Strictly, mead is the product of fermentation of a honey based medium, although different names are often used, depending on the adjuncts used. Wine flavored with unfermented honey is not mead at all, but commercial examples are available under the name of 'meade'; an older name is malsum. Unfermented honey can also be used to flavor liqueurs, such as Drambuie (Chapter 3.8). Plain mead – produced from honey and water – is nowadays given the name traditional mead. Meads in Latin countries are based on the name hydromel, e.g. hidromiel (Spanish), hidromel (Portuguese) and idromele (Italian).

The Slavic countries (except Poland and Russia) have medovina and medica. In Poland, meads are named according to the honey/water ratio: Czworniak, Trojniak, Dwojniak and Poltorak, in order of increasing honey content. Mead is often made with adjuncts, especially fruit juices. The general name for these is melomel, but specific names can be used to indicate particular fruit juices used – e.g. cider (apple), morat (mulberry) and pyment (grape). Metheglin is traditional mead with added herbs and spices, such as cinnamon, cloves, coriander, ginger, mace, nutueg, orange (or other citrus) peal, rosemary, sweet briar, tea and vanilla. In eastern Slavic countries, such as Croatia and Slovenia, these are given the name gverc, from the German Gewürz (spice). Mead brewed with malt wort adjunct is known as braggot; not to be confused with honey beer (Section 2.6.13), in which the honey is an adjunct. Like other fermented alcoholic beverages mead can be distilled or freeze distilled, but such drinks are uncommon.

Mead (mostly traditional, metheglin and melomel types) are made all over the world, often by companies or wineries that also produce fruit wines or grape wines, although there are many companies that produce mead (and honey products) only. In Europe, mead is frequently made by companies that produce other foodstuffs, such as juices, syrups and wines. Some companies use pasteurized honey, some filter the honey/water liquor prior to fermentation, some use sulfites and sorbates and many filter the product prior to bottling. The label or company information (e.g. at the website) will tell much about the product, and the presence of sulfite or sorbates needs to be stated on the label in many countries, and there will certainly be strictly monitored legal limits. Mead is a popular drink in many African countries, notably Ethiopia (already mentioned) and South Africa. A major producer in the latter country, Makana Iqhilika Meadery, makes mead based on an ancient recipe from honey, water and the roots of a local herb, called imoela. Perhaps unique to the world of mead, Makana uses a continuous fermentation process (Chapter 2.6). In Australasia, well known mead producers include Bemrose Estate, Celtic Winery (both New Zealand) and Maxwell Wines of McLaren Vale (South Australia). In the USA, there are meaderies from Alaska (Ring of Fire) to Hawaii (Volcano Winery), but the highest concentrations are in the Pacific States and Colorado – indeed in the last named state, there is an annual mead festival. The emphasis of many of these companies is on melomel styles of mead, using fruit juices from blackcurrant, raspberry and others.

Bargetto Winery (California) matures some mead in redwood vats. Notable mead produces in the USA, apart from the ones mentioned above, include Mountain Meadows (California), Chateau Lorane (Oregon),

Sky River (Washington), Redstone Meadery (Colerado), Trapper Creek Winery (Montana), Pirtle Winery (Missouri), New Day Meadery (Indiana) and Montezuma Winery (New York).

The mead producers in Canada include Intermiel (Quebec), Munro Honey and Meadery (Ontario), and Middle Mountain mead (British Columbia). Mead producers are scattered all over Europe, with particularly strong representations in Slavic and Baltic countries, including Krkonosska Mead (Czech Republic), Pasicka Maceij Jaros (Poland), Tenczynek (Poland) and UAB Lietuviskas (Lithuania). The last named company makes some interesting flavored meads, using acorn, cloves, hops, juniper and linden blossom, amongst others. Other well known mainland European mead producers include Zaubertrank (Hamburg, Germany) and Chouchen (Brittany, France). The former company also makes a range of fruit wines and ciders, whilst the latter makes some oak cask matured traditional mead. In the UK, the two largest mead producers are Lurgashall Winery and Broadlands Winery, both of whom also make fruit wines (Section 2.11.4). Mead is also made by the Orkney Wine Company and by a few English vineyards, such as Avalon.

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2.12
Aromatized Wines

There is something about a Martini,
A tingle remarkably pleasant,
A yellow, a mellow Martini;
I wish I had one at present. . .
—Cyril Ray, 1984

2.12.1 Introduction and Brief History of Aromatization of Wine

Aromatized wines are wines that have been flavored by the addition of aromatic substances of plant origin. This is performed by extraction of flavoring substances from bark, berries, flower heads, leaves, seeds or skins (peel) of plants, sometimes with the aid of heat (even distillation) and usually with the help of grape spirit for efficient extraction. Sugar syrup or mistelle (a mixture of grape must, sometimes concentrated by heating, and grape spirit) is added to many of these wines, such as vermouth (Section 2.12.2) and hence many are slightly fortified. The original purpose of aromatization, like fortification, was to preserve the wine from microbiological or oxidative spoilage. Nowadays, with vastly improved winemaking techniques, materials and equipment, the original purpose has been lost, but the taste for aromatized wines remains strong in many parts of the world. This is especially true of retsina, wine flavored with pine resin, which comprises close to 50% of wine production in Greece (Lichine, 1982a). The ancient civilizations of Egypt, Greece and Rome are all known to have used pine resin, usually smeared on the internal surfaces of earthenware wine jars. Some early wine jars may have been unglazed, so the film of pine resin performed the duel role of infusing preservative and antioxidant substances into the wine and keeping extraneous oxygen out by inhibiting the slow ingress of air into the wine through the porous earthenware.

The flavoring of alcoholic drinks with herbs and spices (known as botanicals) also has a long history and many of these beverages have survived to the present day, although in most cases, probably not in their original forms. These beverages include beer (Chapter 2.6), mead (Section 2.11.5), gin (Section 3.4.2), vodka (Section 3.4.3), absinthe and related drinks (Section 3.5.6), many liqueurs (Chapter 3.8), some Asian liqueur wines and spirits (Sections 2.7.1 and 3.4.4), vermouth and similar wines flavored with herbs and spices.

Vermouth is wine flavored with herbs, the combination and proportions of which are closely guarded secrets of the various manufacturers around the world. The term ‘vermouth’ was first used by a Piedmontese

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

winemaker and tavern owner named Antonio Carpano in the latter half of the eighteenth century. One of the main herbs was a species of wormwood, other species of which were already being used to flavor wine in Germany (Wermuth) and the spirit absinthe in France. Originally, vermouth was an alcoholic concentrate of herbs intended for addition to wine in measures called punti (points), according to the wishes of the customer. A popular measure in Carpano's tavern was one and a half points (punt e mes in the Piedmontese dialect of the eighteenth century). The first preblended vermouth was produced in 1786 by Carpano, in Turin; it was based on the same measure and was named 'Punt e Mes.' Vermouth rapidly gained popularity; many manufacturers sprang up in Piedmont and surrounding areas and many established wine and spirits companies tried their hands at vermouth. Such companies include Cinzano, Gancia, Martini and Rossi, and Stock. The proximity of several large ports, particularly Genoa, Livorno and Venice ensured that vermouth was soon being exported to many countries.

In the early nineteenth century, winemakers in the Midi began production of their own vermouth from local wine and herbs, in response to the influx of wine from Italy. Thus, Noilly Prat was established in 1813 in Marseilles, again convenient for export. A few years later (1821), a distinctive vermouth was being made by Dolin at Chambéry, Savoie. The Chambéry vermouth, originally a dry white version, was made from local high altitude wines and herbs, and was lighter and more delicate in flavor than the heavier vermouths of the Midi and Piedmont. Nowadays, vermouth is made in many wine producing countries: white, red and rosé, dry and sweet, and with a variety of herbal mixtures. Several techniques are used in winemaking, according to the manufacturer. These points are all discussed next.

2.12.2 Vermouth

As has been pointed out, the production of vermouth originally became established in Piedmont (southeast of Turin in northern Italy), followed by the Midi and Savoie (both in France) a few years later. In each of these regions there was a ready supply of white wine and aromatic or bitter herbs. The original purpose of infusion of herbal flavors into wine was to mask faults in the latter, particularly overoxidation, a common characteristic of white wines made in warm climates. The early attempts gained rapid popularity, to such an extent that deliberately oxidized white wine bases are still used by some vermouth manufacturers (e.g. Noilly Prat), although many other features have changed over the years, such as the botanical recipes and the methods of production. Oxidation occurs when the wines are stored in large casks in the open air, subject to sunlight and fluctuating temperatures. The main areas of original vermouth production are shown in Figure 2.12.1. It can be seen that, except for Chambéry, these areas are close to busy ports, making export relatively easy.

Dry, neutral white wine is the usual base for vermouth, with an ethanol content of 10% (v:v) upwards. A bland background is considered best for bringing out the herbal flavors, and besides, a well flavored, subtle white wine would be wasted on vermouth. Some of the original Piedmontese vermouths were made with Moscato wine, but this is no longer the case. Red wine can be used to make vermouth, but is not favored by commercial enterprises because the bitter herbal compounds, combined with red wine polyphenols ('tannins') can produce a wine that is altogether too coarse. Amateur red vermouths are sometimes made with red wine, and red wines are used as bases for other aromatized apéritif wines (Section 2.12.3). Red and rosé vermouths are generally made by including herbs in the botanical mixture that provide red pigments. Also, orange-brown colors can be imparted to the wine by the inclusion of caramel.

Originally, the white wine base was the product of local vineyards, but nowadays the source of wine is usually more prolific vineyards that produce cheap wine, which is transported (often over considerable distances) to the vermouth winery. Thus, most of the wine for Italian vermouth comes form Apulia or Sicily (Anderson, 1980) and the wines for Chambéry vermouth are mostly from the Midi and southwest France.

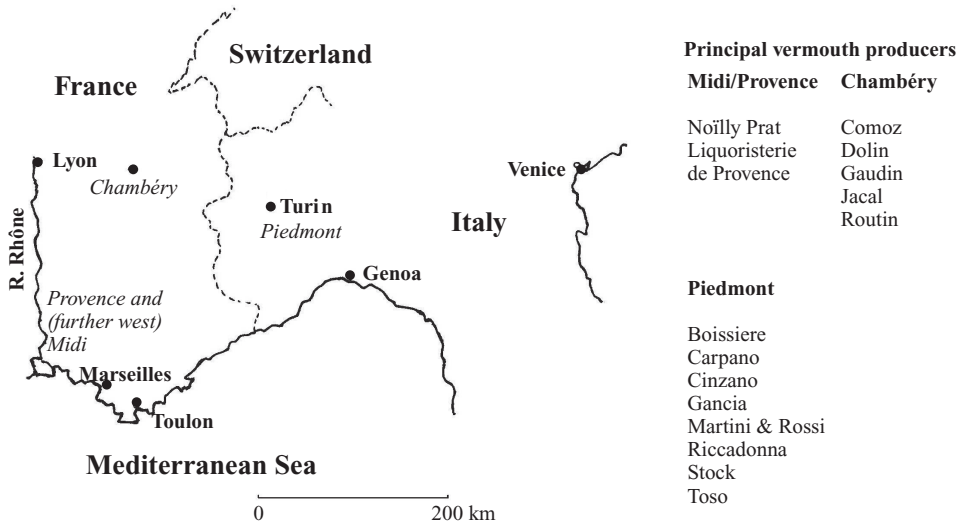

Figure 2.12.1 *Sketch map of earliest vermouth production and list of notable manufacturers*

Thanks to the success of the original vermouth producers in Piedmont, the Midi and Savoie, virtually all wine producing countries or regions make some vermouth nowadays, notable amongst which is California (e.g. Gallo and Tribuno).

Although there are many methods for the making of vermouth, most of these will have basic similarities to the two methods shown in Figure 2.12.2. Basically, vermouth is a fortified and sweetened wine that has been infused with herbal and spicy flavors. The various methods differ in details of the production process and each manufacturer has its own botanical recipe (the selection and quantities of herbs and spices used). Probably the most common methods are based on that labeled 1 in Figure 2.12.2; this is the basic method that has long been favored by northern Italian vermouth producers. Maceration in grape spirit (as opposed to wine or water) gives a more efficient extraction of flavor compounds, some of which are of low polarity (many are hydrocarbons – see Figure 2.12.4). Constant agitation of (e.g. by mechanical stirring, shaking or by ultrasound) is desirable for a week or so, and extraction is more efficient from milled herbs, which have a bigger surface area. Some companies and some amateur vermouth makers extract flavor compounds by simmering the herbal mixture in a sample of wine base. Others use distilled essences or essential oils of certain botanicals, along with the standard herbal infusion method. In the extreme, there are several commercially available clear ethanolic vermouth essences that can be added to bulk fortified and sweetened white wines. The clarified herbal extract is added, with stirring, to the wine in large vats, along with grape spirit and sugar, or mistelle. Caramel can also be added for red vermouth, giving an orange-brown mature tint, but the red pigments are from herbs (such as rose petals) or from permitted colorants (such as cochineal, E20). After a period of time, allowing the flavors to 'marry,' the wine is processed and bottled according to standard methods.

In the Midi and Savoie, rather more time consuming processes are used, similar to method 2 in Figure 2.12.2. Typically, herbs are macerated in a sample of the matured wine base (to which mistelle has been added) for many months to give a highly alcoholic, strongly flavored liquor. This is then racked off the debris and blended with the rest of the base wine and fortified with grape spirit.

The quantity of sugar syrup or mistelle determines the sweetness of the vermouth, which varies from extra dry, through dry to sweet. Dry vermouths (mostly white) normally have less than 30 g/l of sugar,

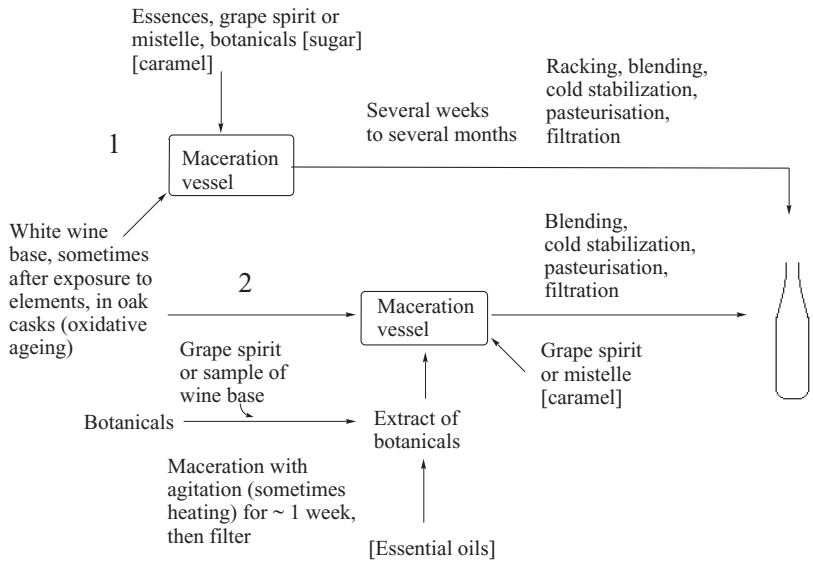

Figure 2.12.2 *Two methods for the manufacture of vermouth. In practice, there are many variations on the above, mostly involving short cuts. Caramel is used for red vermouth. Optional ingredients are in square brackets*

whereas sweet versions (red, rosé or white) have around 130 g/l or more of sugar. Similarly, the ethanol content of vermouth depends on the ethanol contents of the base wine and the mistelle or grape spirit, as well as on the ratio of mixing. Usually, dry vermouths possess ∼18% ABV, whilst sweet vermouths have ∼16% ABV.

The botanical ingredients in vermouth vary from company to company, and the recipes are closely guarded secrets. The Martini museum neat Turin lists over 200 herbs that have been used to produce vermouth throughout the years. Most modern vermouths are made with fewer than 50 botanical ingredients and sometimes as few as 30 (Lichine, 1982b). There is no doubt that, according to organoleptic evidence, botanical recipes have changed over the years, probably in response to changes in fashion and taste, and maybe also in response to concerns over the safety of certain ingredients. It is also evident (again from organoleptic evaluation) that some vermouth manufacturers use different botanical recipes for different products. A good example of this is the firm of Carpano (now part of Fernet Branca): it produces Antico Formula, Classico and Punt e Mes. The first named claims to be made by using the original botanical recipe; it is certainly very different from the other products.

The basis of the herbal contribution to the flavor of vermouth was originally the flower head of the wormwood plant (Wermouth in German), giving vermouth its name. Wormwood is a name used for many species of the genus *Artemisia* (the daisy family), but unfortunately many of these species are botanically quite similar and are not easily identified by amateurs. Furthermore, they have nonbotanical pseudonyms, and the same name from different literature sources may not necessarily refer to the same species. The most important wormwood species in vermouth is *Artemisia pontica*, also known as Roman wormwood, a native of southeastern Europe. Other species include *A. absinthium* (central Europe), *A. annua* (eastern Europe), *A. cinia*, *A. maritime* and *A. vulgaris* (mugwort). The different species of *Artemisia* probably have many flavor compounds in common, although the relative quantities probably differ considerably, and the flavor profile will also depend on which part of the plant is used. Examples of components commonly found in *Artemisia* species are artemisinin (active against malaria), 1,8-cineole, santonin and thujone (see Table 2.12.1

Table 2.12.1 *A short list of herbs used to make Vermouth and their typical aroma compounds and bitter agents (*)*

Common name (part of plant used)	Latin name	Major or typical aroma ompounds	Common name (part of plant used)	Latin name	Major or typical aroma compounds
Aniseed pericarp)	*Pimpinella anisum*	Anethole	Juniper (berry)	*Juniperus communis*	α, β-pinene, limonene, sabinene
Basil (flower, leaf)	*Ocimum basilicum*	Estragole, eugenol	Hyssop (flower)	*Hyssopus officinalis*	1,8-Cineole, linalool, pinocamphone
Camomile (flower)	*Chamomilla recutita*	α-Bisabolol, chamazulen	Mugwort (flower)	*Artemisia vulgaris*	1,8-Cineole, thujone
Cinnamon (bark)	*Cinnamomum verum*	Estragole, ethyl cinnamate, eugenol	Oregano (flower, leaf)	*Oreganum vulgare*	*p*-Cymene, γ-terpinene, *p*-thymol
Citrus (peel)		Citronellol, limonene, α,β-pinenes	Quinine* (bark)	*Cinchona spp.*	Quinine
Clove (pericarp)	*Syzygium aromaticum*	Acetyl eugenol, β-caryophilline, ugenol	Rose (flower)	*Rosa* spp.	Citrinellol, geraniol, nerol, 2-phenylethanol
Coriander (seed)	*Coriandrum sativum*	Camphene, camphor, linalool, sabinene	Sage (flower, leaf)	*Salvia officinalis*	Camphor, β-pinene, thujone
Gentian* (root or rhizome)	*Gentiana* spp.	Amarogentin, gentiopicroside, sweroside	Star anise (pericarp)	*Illicium verum*	Anethole
Ginger (rhizome)	*Zingiber* spp. *tesp. officinale)*	Zingiberene	Thyme (flower, leaf)	*Thymus* spp.	*p*-Thymol
Hop (flower)	*Humulus lupulus*	Humulene, myrcene	Wormwood (flower)	*Artemisia pontica*	Thujone

Source: Information from Williamson and Evans (1989); Santos-Gomes and Fernandes-Ferreira (2003) and Faleiro *et al.* (2005).

and Figure 2.12.3). *Artemisia absinthium* is famous as a major flavor ingredient (via its leaves) of the spirit known as absinthe (Section 3.5.6).

Details of some commonly used botanicals are given in Table 2.12.1 and the structures of selected components are shown in Figure 2.12.3, with indications of likely sources. It should be noted that each botanical ingredient has the potential to contribute hundreds of flavor and taste (especially bitter taste) components to the vermouth. Many components are common to several botanical ingredients. It should also be noted that botanical recipes vary from company to company and some of the ingredients in Table 2.12.1 may only be present in very minor amounts in a particular company's botanical recipe. So, some of the flavor compounds illustrated in Figure 2.12.3 may be present in concentrations below their odor threshold values (OTVs). Additionally, several different species of a botanical genus (e.g. *Artemisia*, gentian, ginger, sage

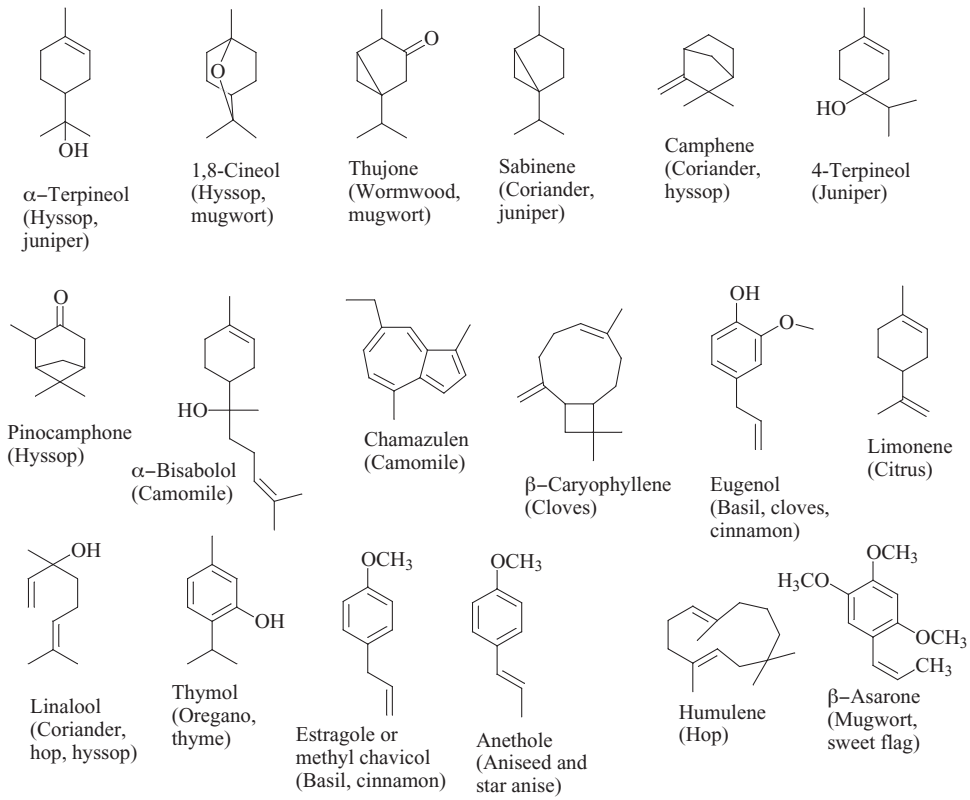

Figure 2.12.3 *Some flavour compounds in vermouth. Major botanical sources in parentheses. Many of these compounds are common to several herbs or spices*

and thyme) may be used in vermouths made in different parts of the world, and it is quite possible that a particular company may include more than one species of a particular botanical genus in its botanical recipe for a particular vermouth. Many (maybe most) of the flavor compounds can be found in most of the botanical ingredients, but it is usual for a handful of components to be present in much greater concentrations than other components, though this does not necessarily mean that these compounds contribute most to the herb flavor; this depends on their OTVs. For example, γ-terpinene and *p*-thymol are important components of the essential oils of oregano (Faleiro *et al.*, 2005) and thyme, estragole (methyl chavicol) is prominent in basil and cinnamon, anethole in aniseed and star anise, and thujone in *Artemisia* species.

The most important flavor compounds are monoterpenoids, as hydrocarbons (e.g. α- and β-pinenes in juniper and many others, camphene in coriander, sabinene in juniper and coriander), alcohols (e.g. α-bisabolol in chamomile, linalool in hyssop and many others), cyclic ethers (e.g. 1,8-cineol in hyssop and mugwort) and carbonyl compounds (e.g. camphor in sage; pinocamphene in hyssop, thujone in sage and wormwood). Aromatic terpene derivatives are also important vermouth flavor compounds. These include estragole (in basil, cinnamon and others), eugenol (in basil, cinnamon and cloves), acetyleugenol (in cloves), *p*-thymol (in oregano and thyme) and β-asarone (in mugwort and sweet flag). Anethole, another aromatic terpene derivative, is a major flavor component of aniseed and star anis. These two spices are the basis for the flavoring of many spirits, such as anis, ouzo, pastis and pernod (Section 3.5.6).

Some of these natural products are known to be toxic if ingested in large quantities – in far greater quantities, however, than is possible even for the most dedicated vermouth drinker. Of particular interest here are β-asarone, estragole, safrole and thujone. The first named has been shown to cause intestinal tumors in laboratory mice if present in their diet in concentrations of up to 250 ppm (Wojtowicz, 1976). The main sources of β-asarone are mugwort and (especially) oil of calamus, distilled from the dried rhizome of *Acarus calamus* (sweet flag). There are many varieties of this aquatic plant, with the European diploid varieties having as little as 5% β-asarone in their essential oils. This compares with Asian tetraploid varieties, whose essential oils can contain more than ten times that quantity of β-asarone (Amerine and Ough, 1980). Nowadays, addition to wine of flavorings that contain this compound is banned in many countries (e.g. the USA), but one of the European varieties of sweet flag may still be used as a minor flavoring agent in vermouth, whence concentrations of β-asarone are likely to be much less than 0.1 ppm (Wojtowicz, 1976). See Section 5.11.2 for a more detailed discussion on toxicity and regulations.

Thujone is a major component of *Artemisia absinthium* leaves and a relatively minor component of *A. pontica*, *A. vulgaris* and other herbs. If ingested in large quantities it can cause hallucinations. It was thought that this compound played a major part in the deterioration of psychological health shown by many heavy drinkers of absinthe, a spirit flavored with *A. absinthium*, which was popular in the latter half of the nineteenth century. Recent work has cast considerable doubt on this hypothesis (Lachenmeier *et al.*, 2008), but for a more detailed discussion, see Section 5.11.2. The most widely used species of wormwood in vermouth, *A. pontica* (as flower heads) has a much lower content of thujone than *A. absinthium* leaves. In all probability, all of the flavor compounds in Table 2.12.1 may prove to be detrimental to health in some way or other if ingested in large enough amounts. Some of the flavor components in vermouth derived from botanicals will be present in concentrations of a few ppm and many others will be present at lower levels, some at ppb or ppt levels. Thus, the health risk to the normal vermouth drinker due to the presence of herb flavor compounds can be discounted, and indeed, ingestion of small quantities could lead to health benefits (Section 5.11.2). The possible health risk due to the presence of ethanol is far more significant (Section 5.6.2).

Apart from the flavor components mentioned above, the botanicals in vermouth are also the source of bittering agents – (mostly nonvolatile) compounds that give a bitter taste. Principal amongst these is cinchona bark (from *Cinchona officinalis* L. or *C. pubescens* Vahl), the source of quinoline alkaloids, including quinine, quinidine and their dihydro derivatives, as well as cinchonine and cinchonidine. Cinchona bark is also an important ingredient in a number of vermouth-like wine based aperitifs, such as Campari (Italy) and Lillet (France) (Section 2.12.3), as well as various spirits (Section 3.4.3). Collectively, these beverages are sometimes called quinquina.

Interestingly, a medicine for malaria given to Louis XIV of France in the late seventeenth century was made from wine, cinchona bark, rose petals and lemon juice – predating bitter aperitifs by 100 years or so. Other bittering agents from herbs in vermouth are flavonoid phenols, such as rhamnetin and isorhamnetin, proanthocyanidins, gallic acid and catechol tannins and iridoids (Figure 2.12.4). Bitter constituents are commonly found in bark, rhizomes and roots, but are also present in flower heads, leaves and stalks. Apart from quinine and related alkaloids, some of the most bitter substances are the iridoids, such as those from gentian roots (usually wild gentian, *Gentiana lutea* L.). These include amarogentin, gentiopicroside (Figure 2.12.4), loganic acid, sweroside and swertiamarin (Carnat *et al.*, 2005).

Many of the botanicals used in the making of vermouth possess a host of other, mainly nonvolatile compounds. These include the sesquiterpenoid santonin, the chromones (1,4-benzopyrones) eugenin and eugenitin, lignans such as deoxyphyllotoxin, and tetracyclic triterpenoids, such as oleanolic acid derivatives, stigmasterol and ursolic acid derivatives. Some of these compounds are illustrated in Figure 2.12.4. Although many of these compounds are insoluble in water, they are soluble in ethanol or ethanol–water mixtures to a certain extent, so it is not unreasonable to suppose that low concentrations of these compounds can be found

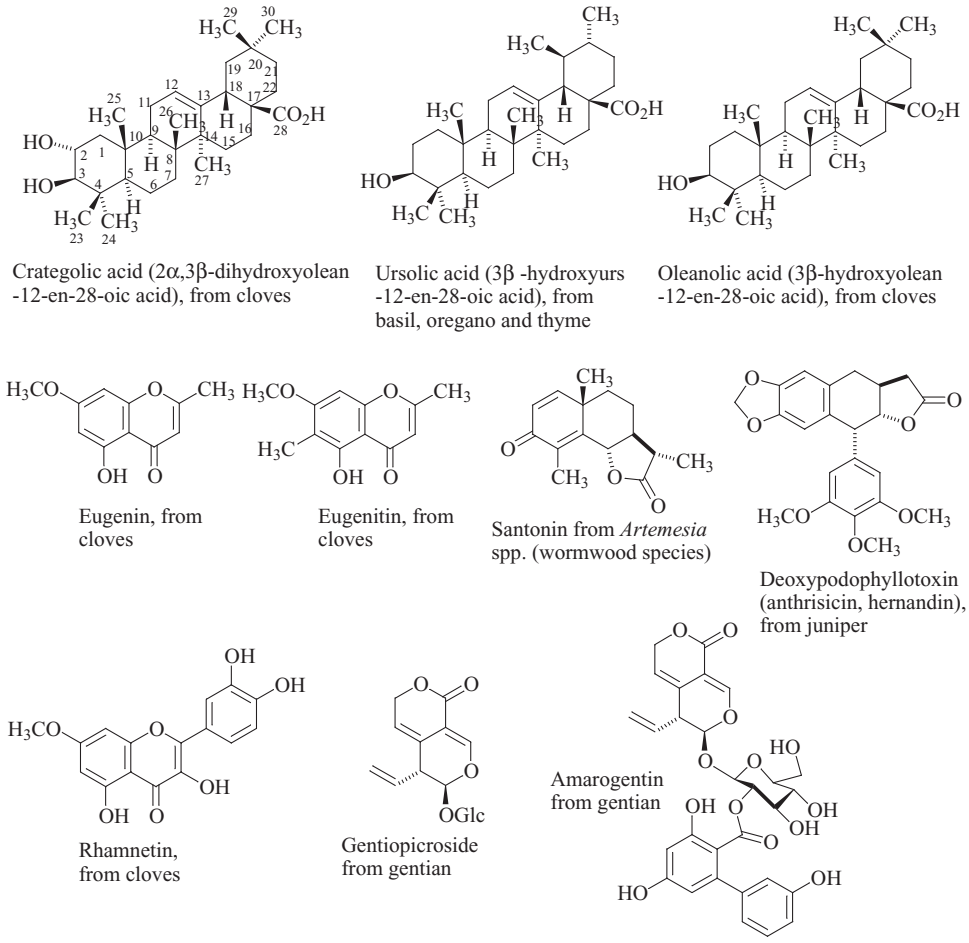

Figure 2.12.4 *Lignans, pentacyclic triterpenoids and phenolic compounds from botanicals used in the manufacture of vermouth*

in vermouth. Some, such as santonin and the triterpenoids are known to have bitter tastes. The latter may also have pronounced health benefits – see Section 5.11.2.

Apart from flavor and other components that arise from the added botanical ingredients, the rather limited literature on the composition of vermouths not surprisingly suggests that they are similar to standard white wines; sometimes more similar to oxidized white wines. Ethyl 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylate (MTCA-EE) is a β-carboline found in a range of alcoholic beverages (Herraiz, 1999). It is of interest because it is a structural analog of ethyl β-carboline-3-carboxylate, a potent benzodiazepine receptor inverse agonist and a compound that may play a role in alcohol addiction. Vermouths were found to have lower levels of MTCA-EE than almost any other kind of alcoholic beverage. On the other hand, the biogenic amines histamine and tyramine (Section 5.11.3) were found to be present in higher concentrations in vermouths than in sparkling wines and ciders (Vidal-Caron *et al.*, 1989). In a study to determine the levels of γ-hydroxybutyric acid (GHB) and γ-butyrolactone (GBL) in alcoholic beverages, vermouths were found to have concentrations

similar to ordinary white wines (Elliot and Burgess, 2005). GHB and GBL are drugs that are sometimes added to alcoholic drinks ('spiked drinks') (often surreptitiously) and so for forensic analysis, it is important to know the natural background levels of these substances in alcoholic beverages. Analysis of diethyl ether extracts of red vermouth demonstrated the presence of 5-hydroxymethyl-2-furaldehyde (5-HMF), a Maillard product (see Section 2.6.2) (Cartoni *et al.*, 1997). Its probable origin is caramel, which is a frequently used colorant of red vermouths.

2.12.3 Other Aromatized Wines and Apéritifs

Other aromatized wines fall into two major groups: the quinquinas or bitters (popular aperitif wines) and Retsina, pine resin flavored white wine, popular in Greece. The bitter group, like vermouth, are used in cocktails: Americano, Negroni (both with Campari) and Vesper (with Lillet) being prime examples. Like vermouth, the bitter aperitifs are fortified wines (white, sometimes red) infused with herbs (flowers and leaves), spices (dried bark, pericarp and seeds), bark (for bittering agents) and peel, or their essences. The botanical mixture has a different emphasis to those of vermouths, with more of an emphasis on bitterness, and with cinchona playing a more prominent role. There are other differences which will be highlighted in the next paragraphs. Probably the first of the bitter aperitifs (and probably the one that is most vermouth-like) is Dubonnet (founded in 1846) named after Joseph Dubonnet, the Parisian chemist and winemaker who developed it. This beverage was originally intended as a palatable source of quinine (from Cinchona bark) for troops and civilians living in French colonial Africa and other tropical or subtropical colonies, where malaria was rife.

Campari, a well known bitter aperitif was started by Gaspare Campari in 1860, as a rival of vermouth. The botanicals in Campari are quite different to those in vermouth, with cinchona and cascarilla bark being the main ingredients. Also included in the botanical recipe are ginseng (*Panax* spp.) roots, bergamot (*Monarda* spp.) essential oil and rhubarb (*Rheum rhabarbarum*) stalks. Close to 60 other herbs and spices are used in addition to these. Campari is made from a fortified white wine base, the bright red color coming from natural carmine cochineal (E20).

St. Raphael, like most bitter aperitif wines, has its origins in the latter half the nineteenth century, when such drinks were highly popular. It was dedicated to the Archangel Raphael by its originator, Dr Juppet, who is said to have regained his former eyesight upon formulation and testing of the recipe. Unlike the other aperitifs in this section, St. Raphael is really an aromatized vin de liqueur, since its alcohol base is not a fortified wine, but mistelle, a blend of grape must and grape spirit. The botanicals, which include cinchona bark, bitter orange peel, vanilla beans and cocoa beans, are macerated in grape spirit over a period of several weeks. The extract is then blended with the mistelle, cold stabilized, filtered and bottled. The aperitif is now (2008) marketed by Boisset, after a period of time under the Bacardi Martini group. It has 20.5% ethanol (v:v).

Lillet was first formulated in 1882 by the Lillet brothers (wine and spirits merchants in Bordeaux), using a fortified wine base and flavored with cinchona bark. Nowadays, since 1986, the bitter component has been decreased in favor of a citrus and vanilla flavor. The former flavor comes from an orange liqueur made by cold maceration of Haitian, Moroccan and Seville orange peel in brandy for several months, whereas the latter flavor comes mostly from cask ageing. The aperitif is made by blending wine made from Sauvignon Blanc and Semillon (white) or Carbernet Sauvignon and Merlot (red) with orange liqueur in the approximate ratio 85:15, giving a final ethanol content of 17% (v:v). The wines are from around the Podensac area of the Graves region, southeast of Bordeaux. The blend is matured in oak casks for up to 12 months, whence it is filtered and bottled. The original Lillet aperitif was white, the red version originating as recently as 1962. Both drinks are unlike other aperitifs, being lighter, more vinous, and without dominating herb flavors and bitter taste. Instead, they have a delicate citrus-spicy edge, but are still useful in cocktails (such as Vesper).

Retsina is the most famous and longest established member of a family of drinks known as resinated wines. It is the major survivor of the widespread practice of preserving and flavoring wine with pine resin, carried out by the great early civilizations in the countries bordering the eastern Mediterranean Sea. Today, the practice is more or less confined to Greece and the Greek speaking southern areas of Cyprus. The name 'Retsina' is an EU protected designation of origin for Greece and Cyprus: similar wines from elsewhere must be called resinated wine and not Retsina.

Although Retsina is produced in many parts of Greece, the major areas are in center of the country, at Attica (near Athens), Boeotia and Euboea. Local grape varieties are used for Retsina, with the most prominent being Assyrtiko, Athiri (on Rhodes), Rhoditis and Savatiano. The base wine is either white or (less commonly) rosé, but not red, and the winemaking techniques are similar to those used for making standard white or rosé wines (Sections 2.9.1 and 2.9.2). The pine flavor comes from small pieces of pine resin (usually from Aleppo pine) that are added to the fermenting must. After a settling period that follows the termination of fermentation, the wine is racked off the resin pieces and is treated as for ordinary white or rosé wine. Most Retsina wines possess alcohol contents in the region 12–12.5% (v:v).

Relatively few analytical studies have been carried out recently on Retsina. As expected, Retsina has higher levels of phenolic substances compared with nonresinated white wines, because of the additional contribution of the resin pitch (Proteos *et al.*, 2005). At especially elevated levels were (+)-catechin, *p*-coumaric acid, (–)-epicatechin, gallic acid and tyrosol. Biogenic amines, such as histamine and tyramine, although beneficial to humans at low concentrations in the diet, can be damaging to health if ingested at higher concentrations, especially in the presence of ethanol (Section 5.11.3). Their presence in wines at higher concentrations is thought to be largely the result of microbiological contamination arising from poor sanitation in the winemaking process. In a study of biogenic amines in Greek wines and beers, using HPLC of dansyl derivatives with fluorometric detection and atmospheric pressure chemical ionization mass spectrometry (APCI-MS; see Section 4.3.3), Loukou and Zotou (2003) showed that Retsina wines had generally less than average levels of biogenic amines, especially regarding histamine.

The most obvious feature of Retsina is its pine based aroma and flavor. The most usual pine resin used for making Retsina is that of the Aleppo pine (*Pinus halepensis* Miller), which is widespread in Greece. This resin contains and α- and β-pinenes, caryophyllene, cembrene, myrcene, limonene and terpinolene, as well as other terpene hydrocarbons, terpenols, terpene esters and nonterpene esters, similar compounds to the pine needles, but in different relative concentrations (Roussis *et al.*, 1995).

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2.13

Low Alcohol and Non-Alcoholic Beers, Ciders and Wines

2.13.1 Overview

Anyone who has tasted grape juice, apple juice and hopped malt wort against their fermented equivalents (wine, cider and beer, respectively) can hardly fail to notice the greater complexity of pleasing flavors of the fermented drinks. So, why has considerable effort been expended on producing a range of low alcohol beverages? Part of the answer is health related: some people need to avoid ethanol intake, either because of some metabolic malfunction or because of some medical condition that requires minimal ethanol content in the diet, either directly or indirectly, due to the prescription of ethanol incompatible drugs. Additionally, health conscious people who like to exercise, or athletes, are able to drink low alcohol beers of the high carbohydrate type for a boost of energy. Other reasons are more social or religious in character: stricter drink-driving legislation and greater awareness of the social implications and greater risks associated with driving after excessive alcohol intake, and religious avoidance of alcoholic drinks, for example. Hence, there is a considerable market for low alcohol or nonalcoholic beer, cider and wine that look and taste like their alcoholic equivalents. Prior to the last two decades, low alcohol beers existed at various times in different countries. During prohibition (*ca.* 1919–1933), breweries were allowed to produce 'near beer' of ∼0.5% ABV. In the Scandinavian countries of Finland, Norway and Sweden, commercial brewing is more or less closely regulated by government and has been influenced by temperance movements at various times. Low alcohol beers (∼2% ABV) form a significant part of beer production in these countries and are generally known as class I beers.

The definition of the terms 'low alcohol' and 'nonalcoholic' vary throughout the world. Nonalcoholic or alcohol free may be used to describe only drinks with no detectable ethanol content in the USA, and in the UK to denote drinks with no more than 0.05% detectable ethanol content. Thus, in these two countries, virtually all of these beverages are described as low alcohol drinks. In Germany and other European countries, drinks can be described as nonalcoholic if they have a maximum of 0.5% ABV. Interestingly, fruit juices and various other foodstuffs, especially bakery products, possess natural ethanol contents from 0.1% to over 0.5% (v:v). Also, in the USA, malt based drinks containing less than 0.5% ethanol (v:v) cannot be called beer; instead they are known as malt or cereal beverages.

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

2.13.2 Production of Low Alcohol Beverages by Limited or Checked Alcohol Methods

There are two basic ways of producing low alcohol beverages:

- Limited or checked alcohol methods
- Dealcoholization methods.

For low alcohol ciders and wines, only the second of the above techniques may be used, because the first technique would give too sweet a product, with too great a resemblance to apple juice or grape juice. The production of low alcohol beers presents one of the biggest challenges to the brewer because of the inherent balanced nature of the product. Use of limited or checked fermentation methods can lead to beverages that are unacceptably sweet and malty to many people. Also, use of dealcoholized methods can lead to loss of flavor compounds that will need to be replenished before the beer is packaged. On top of this, the production of low alcohol beers requires rigorous filtration and pasteurization regimes, because of the inherent biological instability caused by lack of ethanol and, in some cases, by the presence of fermentable sugars. A great deal of research has gone into improving the methods of low alcohol beer production, so that beers with better color, flavor/aroma balance and head retention can now be brewed (Hornsey, 1999). The first low alcohol beers were brewed using limited or checked fermentation methods (Figure 2.13.1) and some of these methods use conventional worts (with normal balance of maltose and α-glucans and normal hop bitterness). These methods will be discussed first, even though they are no longer widely used.

The checked fermentation method (Figure 2.13.1) involves arresting a normal fermentation (usually of a lager style beer fermenting at ~10 °C) by rapid cooling to 0 °C. Such beer has fermentable maltose and some fermentable α-glucans, and so rather than risk incomplete removal of yeast (by centrifugation and filtration regimes) it is usually heavily pasteurized before chilling, carbonation and packaging. Beers brewed by this method can have a rather harsh, cooked flavor.

The blended beer or Barrell process was also an early method. It uses two fermented worts of the same color, pH and hop bitterness. One batch of wort is of normal OG (typically 1040°), whilst the other is a low OG wort (usually around 1010°). During fermentation, the evolved CO_2 and flavor compounds from the normal wort are transferred to the weaker wort. The final ethanol content can be varied according to the ratio of blending, but lower ethanol products (say 1% ABV) tend to be rather thin and lacking in body.

The cold contact method (Murray and van der Meer, 1994) uses conventional boiled wort of 12–20 °Plato (48-80 °OG), whose acidity has been adjusted to pH 4.0–4.6 by the addition of food grade acid. To this is added a slurry of yeast, freshly harvested from the barm beer (Section 2.6.4) of a regular brew, so that the cell concentration in the wort/slurry is 40–80 × 10^6 yeast cells/ml. This is held at 0–7 °C for up to 30 h. The resulting beer is then separated from all but 0.5–2.0 × 10^6 yeast cells/ml. It may be supplemented with beer esters. After dilution with water, it is cold stabilized, filtered and carbonated.

The other methods of producing low alcohol beers by limiting production of ethanol involve either modified worts, high gravity mashing or by the use of mutant strains of *S. cerevisiae* that are deficient in certain enzymes of the tricarboxylic acid cycle, or by the use of other fermentative organisms. The modified wort method involves mashing at higher than normal temperatures (75–80 °C, as opposed to ~65 °C), so that α-glucans are formed, but are not hydrolyzed to maltose because, at that temperature, β-amylase is disabled (Section 2.6.2). Depending on the mash temperature, this method can produce a wort that yields around 0.5% ethanol (v:v) when fermented with a normal brewery yeast. The unfermented α-glucans are not as sweet as unfermented mono- or disaccharides, so the result is a sweetish, but full tasting beer. This is the original way by which the malt beers of England and Germany (Malzbier) were brewed (Section 2.6.13 and Chapter 5.8) and some beers are still produced by this method today. Methods that involve high gravity mashing are sometimes known as spent grain methods. The high gravity wort is filtered off the grains and then hot water is added to the spent

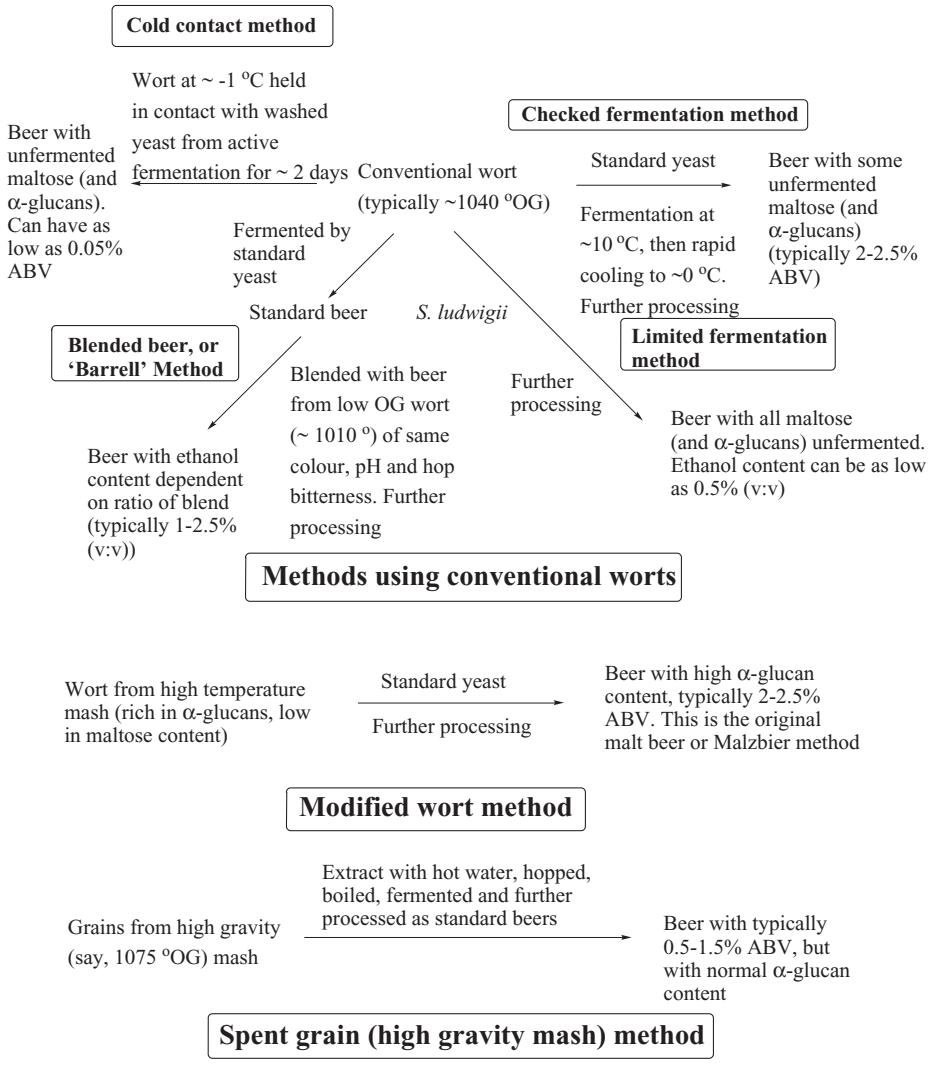

Figure 2.13.1 *Low alcohol beers from checked or limited fermentation methods. Standard yeast is* S. cerevisiae or S. uvarum. *Further processing is described in the text*

grains to extract residual sugars. This second, weak wort is then processed using standard techniques to give beers with ethanol contents of 0.5–1.5% by volume. This is an extreme case of the once common brewery practice of obtaining two beers from a single mash.

Strains of *S. cerevisiae* deficient in one or more enzymes of the tricarboxylic acid cycle have been used recently for the small-scale production of nonalcoholic beers (Selecký *et al.*, 2008). In particular, strains lacking in fumarase (fumarate hydratase) and α-ketoglutarate dehydrogenase produced beers with less than 0.5% ABV. These yeasts had disrupted *FUMI*, *KGD1*and *KDG2* genes and were notated by the symbols Δ *FUMI*, Δ *KGD* and *KGD1*, respectively. The beers had increased levels of carboxylic acids compared with that brewed by a standard brewing yeast strain (W96); for example, the beer brewed with Δ *KDG21* or

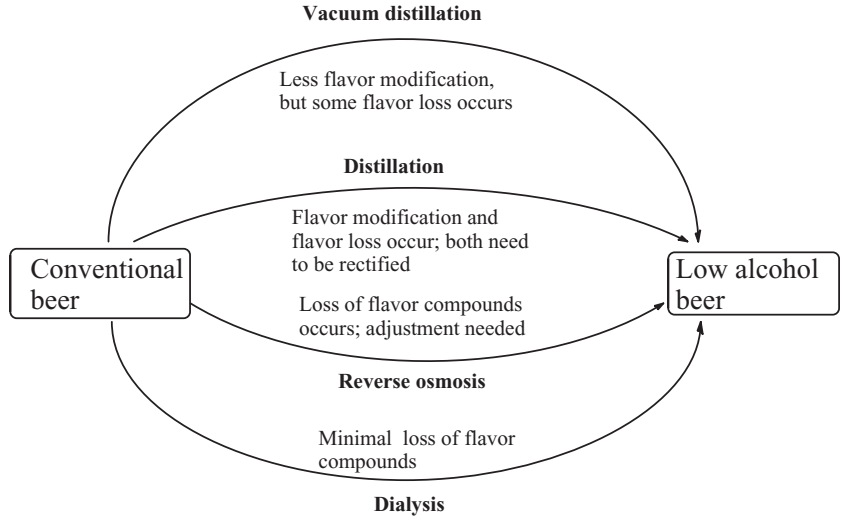

Figure 2.13.2 *Summary of methods for the dealcoholization of beer*

Δ *KDG2* had concentrations of lactic acid contents of ∼550 mg/l, as opposed to ∼50 mg/l for the beer brewed with W96. Far from being an organoleptic problem, the presence of lactic acid was considered to help protect the sugar rich beer from microbiological attack and to mask the unacceptable worty flavor often associated with nonalcoholic beers. Additionally, the nonalcoholic beers produced by the mutants had slightly increased color, hop bitterness, total nitrogen contents and total phenolic contents. However, high levels of diacetyl and low levels of esters negatively affected the beers brewed using the mutant yeast strains, so it was suggested it would be beneficial to produce a hybrid between brewing yeast and a laboratory strain (mutant), carrying the genetic properties needed for well flavored beer, yet lacking TCA enzyme genes (Selecký *et al.*, 2008).

Limited fermentation can be achieved by use of *Saccharomyces ludwigii* on conventional worts, in place of *S. cerevisiae* or *S. uvarum*. This organism will only ferment the monosaccharides fructose and glucose and the disaccharide sucrose; the major wort disaccharide, maltose, is not metabolized. Thus, beers brewed by this method have sweet, rich palates that are unacceptable to some people.

2.13.3 Dealcoholization Methods

Nowadays, most low alcohol beers are produced by dealcoholization methods. In most cases, flavor compounds are removed with the ethanol and this necessitates the addition of a flavor mixture to the dealcoholized product in order to regain the balance of flavor. Figure 2.13.2 outlines the major dealcoholization methods used to make low alcohol beer. Distillation methods are not much used these days, probably because of the effect of elevated temperatures on the flavor profile, as well as loss of volatile flavor components from the beer. The atmospheric pressure distillation method, in particular, causes isomerizations and degradations of flavor compounds, even though it can produce beers with 0.5% ABV (Hornsey, 1999). The vacuum distillation and film evaporation methods operate at lower temperatures because of the reduced pressure; the latter is often operated at 30 °C and 30 mm Hg (0.04 atm) pressure. Straight vacuum distillation is often used to remove flavor compounds that are more volatile than ethanol. These are kept aside and added back to the beer once the ethanol has been removed. The beer is then cold stabilized, carbonated and processed as for standard beers.

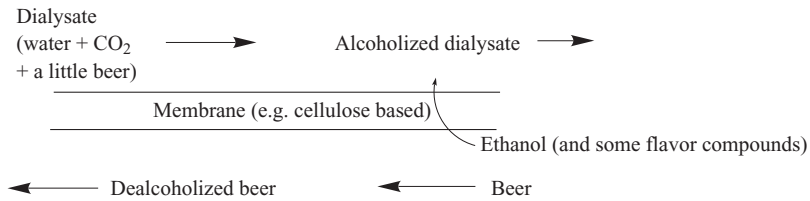

Figure 2.13.3 *Simplified scheme for dealcoholization of beer by dialysis*

The film evaporation method operates rather differently, leading to loss of CO_2, some water and some flavor compounds, as well as ethanol, because of the large surface area of evaporation. The resulting dealcoholized beer is then diluted with carbonated water and a mixture of flavor compounds is added to restore the original flavor characteristics of the beer. Beers with ethanol contents as low as 0.01% (v:v) can be made by this method.

The reverse osmosis method is basically a high pressure filtration of a normal, filtered (or clear) beer through a semi-permeable membrane. Ethanol, other small molecules (including flavor components) and some water is lost from the bulk beer as a result of reverse osmosis. The beer is diluted with deoxygenated water before reverse osmosis to make up for that lost during reverse osmosis and to avoid clogging of the membrane filter. This method can produce beers with ethanol contents of 0.5% by volume (but not usually much lower than this), with little modification of the flavor profile. On the negative side, it can be costly to operate (especially for beers with less than 0.5% ABV) and loss of flavor compounds to the permeate needs to be compensated.

Currently, some of the most commonly used methods for producing low alcohol beers are based on dialysis (Figures 2.13.3 and 2.13.4), sometimes known as isothermal membrane perstration. The beer flows through a module whose walls are constructed of a cellulose or poly(tetrafluoroethylene) membrane (Bowser and Dennison, 1995). At the same time, the outer walls are exposed to a flow of the dialysate or strip solution, pure water containing a small amount of the beer that is undergoing dialysis and CO_2 in order to prevent excessive loss of this gas from the beer during the process. Like reverse osmosis, there is no thermal stress on

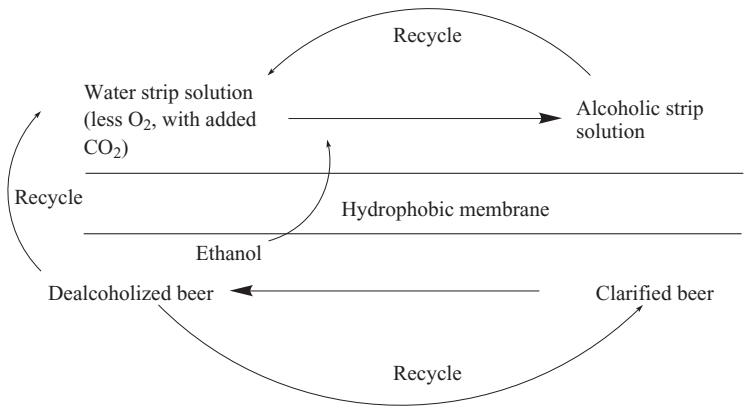

Figure 2.13.4 *Recycling in dialysis dealcoholization of beer to minimize loss of flavor compounds. Based on Michaels* et al. *(1998)*

the beer because the operation runs at ambient temperatures (typically ~12 °C) and near ambient pressure, the pressure difference across the membrane being only 0.1 bar. During dialysis, overall diffusion of ethanol (along with low molecular weight flavor compounds) is from beer to dialysate. The flavor compounds can be recovered from the dialysate and by vacuum distillation and added back to the dealcoholized beer before further processing.

A modified version of the dialysis method, which can be applied to cider and wine as well as beer, uses a polypropylene (or other synthetic polymer), as a hollow fiber membrane, and an initial strip solution consisting of deoxygenated and carbonated water (Michaels *et al.*, 1998). The beer to be dialyzed has been clarified by either cross flow membrane filtration or vortex flow membrane microfiltration. Dialysis is carried out as above, but application of chosen recycling regimes for some of the dealcoholized beer and/or the alcoholized strip solution (Figure 2.13.3) are used to determine the final ethanol content of the beer and to minimize loss of flavor components. Biomass removed from the beer by filtration can be returned to the dealcoholized beer, which is then aged and processed by standard methods. This process can be operated in either batch or continuous modes; it can be used to dealcoholize beers of 3–4% ABV to <1% ABV or to reduce the alcohol content of a 'hot' wine from say 15% to 12% by volume.

In addition to these methods, there is at least one continuous method (Section 2.6.8) for brewing low alcohol beers, using immobilized modified yeast. The Dutch brewery Bavaria (Lieshout) passes modified filtered wort through bioreactors containing a Cultor Spezyme GDC support medium with immobilized yeast colonies. Beers of 0.05–1% ABV can be produced by this method, according to the mode of operation. After fermentation, the beers are diluted, cold stabilized, carbonated, bottled and then pasteurized.

Low alcohol wines can be made by the reverse osmosis and dialysis methods described for beer in the previous paragraphs. The greater reduction of alcohol needed for wine (say 8–12% → 1% ABV) compared with beer (say 3–5% → 0.5% ABV) necessitates a more vigorous process, which means that more flavor compounds will be lost from the wine to the strip solution, if steps are not taken to prevent or minimize this. To this end, the strip solution used in the dialysis process is deoxygenated and sulfited to reduce loss of free (unbound) SO_2 from the wine. Either recycling of the strip solution and/or the dealcoholized beverage can be carried out as previously described (Figure 2.13.4), or a separate reverse osmosis or dialysis step can be used to remove flavor compounds from the wine, which are then added back to the dealcoholized wine (Michaels *et al.*, 1998).

On the other hand, reverse osmosis methods can be used to reduce the ethanol content of wine by, say, one third, without significant loss of many key flavor substances. Reverse osmosis and nanofiltration were used to reduce the ethanol content of a model white wine (containing flavor compounds diethyl succinate, 2-phenylethanol, *cis*-3-hexenol and isovaleric acid (the last being regarded as undesirable) from 12% to 8% ABV (Labanda *et al.*, 2009). Permeation was performed in batch retentate recycling mode with one nanofiltration and two reverse osmosis flat sheet polymeric membranes at 15 °C. Interestingly, of the four flavor compounds chosen for the model wine, isovaleric acid (with undesirable cheesy/rancid notes) was the one that suffered highest loss, an average of ~14%, compared with ~3% for the loss of 2-phenylethanol.

A more recent technique for the dealcoholization of wine is the spinning cone column (SCC) method; it can also be used on beer and cider. The apparatus is essentially a multistage mass transfer device that uses gas–liquid contact. Wine is entered at the top of the column, the interior of which contains a series of alternating stationary (fixed to the interior wall of the column) and rotating truncated cones (attached to the central spindle) (Figure 2.13.5). Wine flows down the surface of the stationary cones, but spreads itself into a thin film on the rotating cones because of the applied centrifugal forces. The stripping gas (e.g. deoxygenated N_2) enters the column from the bottom and flows counter current to the flow of wine, thereby stripping ethanol and the more volatile flavor compounds from the large surface area of wine on the rotating cones. In practice, the wine is run through the SCC to strip the more volatile flavor components first, which are then condensed, collected and are added back to the dealcoholized wine produced by subsequent runs through

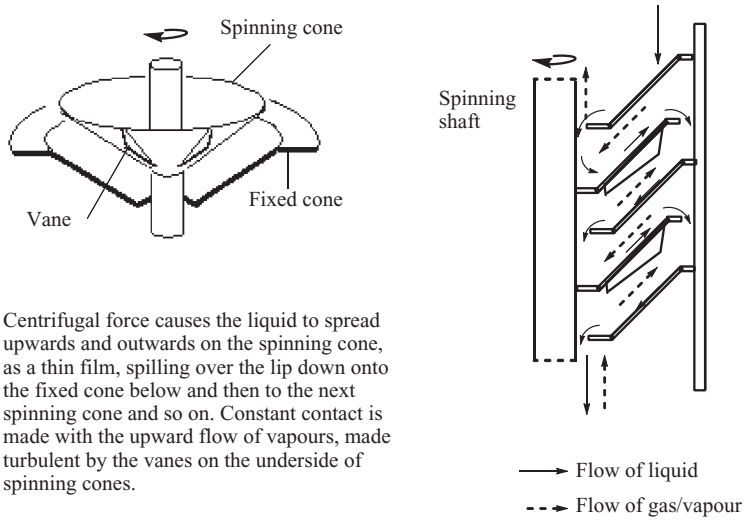

Figure 2.13.5 *Perspective section and horizontal half-section through part of a spinning cone column device*

the SCC. The method can be used to remove off flavors, such as acetic acid, ethyl acetate and hydrogen sulfide. It can also be used for obtaining industrial ethanol from crude yeast fermented mixtures (Wright and Pyle, 1996) and it can be used for essence and essential oil extraction from botanicals, as in the preparation of flavored wines (Section 2.12.2), flavored spirits (Section 3.4.2) and liqueurs (Section 3.9.6). Spinning cone columns are obtainable in sizes that process from1000 l to 10 000 l of wine or other liquid per hour. The smallest is ~2 m high and ~30 cm in diameter, whereas the largest is ~5 m in height and ~1 m in diameter.

In the production of a low alcohol wine, it is important that the product has an aroma profile that is as similar as possible to the original wine, or that at least it is well balanced and acceptable to consumers. As already discussed, present methods for the production of low alcohol wines lead to the loss of some volatile aroma components, thus altering the aroma profile. The lost components can be added back to the dealcoholized product, sometimes having been removed in a separate process prior to ethanol removal. An alternative is to add a particular cocktail of aroma compounds to dealcoholized wine, to give the product a particular well recognized and acceptable character (e.g. a Muscat character). Rather than use a mixture of synthetic food quality chemicals (which then must appear on the label as 'synthetic'), it is preferable to use a mixture of natural aroma compounds, separated from a fermenting wine as described by Schäfer *et al.* (1999). Here, aroma compounds were removed from fermenting Muscatel wine by organophilic pervaporation, using a polyoctylmethylsiloxane on polyetherimide membrane. The best and most faithful Muscatel wine aroma (as judged by a sensory panel) was recovered from fermenting musts of densities 1075–1055 g/l. Higher must densities gave too few aroma compounds, whereas lower density musts (with higher ethanol contents) gave unbalanced aroma profiles, with 'solvent-like' notes.

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Part 3
Distilled Spirits

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Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

3.1
Introduction: Distillation Methods and Stills

The Online Encyclopaedia Brittanica describes a distilled beverage, liquor or spirit as 'a drinkable liquid containing ethanol that is produced by means of distilling fermented grain, fruit, or vegetables.' For the purposes of this book, the process of distillation involves the application of heat for the separation of ethanol from water and other constituents in alcoholic beverages by virtue of their different boiling points.

Other methods, that don't involve direct heating, can be used for this separation, but are not dealt with in any detail here. Freeze distillation was used by the Mongols and other Asians to produce various distilled drinks, and also by the early American colonialists (to make apple brandy or 'applejack' from cider). It is still used today in certain breweries for the production of high alcohol beers (Section 2.6.13). Likewise, pervaporation using hydrophobic membranes is used in the dealcoholization of alcoholic drinks (Section 2.13.3).

Distillation of alcoholic beverages was originally performed by apocetheries, ecclesiaticals and scientists (alchemists) mainly for medicinal or research purposes. Later, distillation was also conducted on a small scale by farmers and merchants, who recognized it as a profitable way of dealing with surplus cereal, fruit or root crops, or of preserving wine for export. It is only since the latter part of the nineteenth century that large-scale commercial distillation became commonplace.

This chapter deals with the major distilled beverages of the world, their methods of production and their organoleptic and sensory characteristics, related to combinations of flavor components (known as congeners). Chapters 3.2–3.4 deal with cereal based spirits, although some, like akvavit and vodka can be derived from roots, and some Indian whiskies are partially derived from sugar cane. Chapter 3.5 discusses distilled beverages derived from sugar cane and other vegetable sources, as well as aniseed flavored spirits. Fruit based distilled beverages are discussed in Chapters 3.6–3.8, with liqueurs, often composed of spirit from various sources, covered in Chapter 3.9.

3.1.1 A Brief History and Theory of Distillation

For an account of the early history of distillation, the reader is referred to Section 1.2.1. Distillation of alcoholic beverages to make a potable drink, albeit one to be used for medicinal purposes, was first carried out in Europe during the twelfth century, mainly by apothecaries and medical practitioners. These distilled spirits were given the name *aqua ardens* (burning water) in the *Compendium Salerni* from the medical school at Salerno. They were concoctions of spirit and herbs (botanicals) and were used to treat certain human ailments;

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their production method was kept secret by the use of a code system. Later, they were flavored with honey or sugar, in order to make them more palatable, thus developing them into the type of alcoholic beverages now known as liqueurs (Section 3.9.1). Soon enough, they were consumed for enjoyment, as well as for remedial purposes, and their manufacture was taken up by ecclesiastical establishments (and later by secular companies) throughout Europe. At about the same time (the thirteenth to the fifteenth centuries), distillation of alcoholic beverages was becoming widespread throughout Europe and Asia; Irish whiskey, Scotch whisky, vodka and the rice and sorghum spirits of China, Japan, Korea and other Asian countries probably originated in these times.

The earliest stills of the Arabs and eastern Asians were relatively small glass or earthenware pot stills of the alembic type, but did not use flowing water as a coolant. An example of a Korean traditional soju still can be seen in Section 3.4.4. They were originally heated directly by wood or charcoal fires and latterly (in medieval times) by boiling water or steam. Later, copper (and to a lesser extent bronze) became the traditional still material, copper having been produced and used for many centuries and having many advantages over other materials, particularly regarding malleability, durability and heat conductivity. Copper stills could be made to a wide range of shapes and sizes, they could endure several years of use, did not crack when heated directly and caused a more uniform rate of distillation, with minimal charring. It was also known in the nineteenth century that a copper pot still system generally gave a spirit of superior organoleptic properties (as opposed to spirits from say stainless steel stills); this was later attributed to the ability of copper to remove certain malodorous volatile organosulfur compounds from the distillate during distillation (see Sections 3.2.4, 3.3.4, 3.5.3 and 3.8.3). However, on the negative side, under certain unfavorable distillation conditions (Section 3.1.3), the copper material of the still has been implicated in the formation of ethyl carbamate (EC), a noted carcinogen (Section 5.11.5).

The basic alembic (Section 1.2.1) evolved gradually over the centuries into versions of the pot still that are now associated with particular kinds of distilled spirit, such as Cognac (Section 3.6.2), some rum (Section 3.5.3) and Scotch malt whisky (Section 3.2.4), for example. Various refinements and modifications were added, mainly in the nineteenth century and later, in order to give the distiller greater control over the character of the distillate. These refinements included fractionators, dephlegmators and catalytic converters (Section 3.1.2), giving rise to a number of hybrid pot stills and batch column stills that are used in the production of some Armagnac and other grape spirits (Sections 3.6.3 and 3.6.4), some pomace spirits (Section 3.7.2) and some fruit spirits (Sections 3.8.2 and 3.8.3), for example. Other refinements include ethanol (density) or temperature sensors, for tracking distillation progress and, with reagard to batch distillation, for determining the cut off point for distillate fractions.

It is probable that the use of a simple condensing system originated in medieval times. Thaddeus Alderotti, an eminent physician in Bologna during the thirteenth and early fourteenth centuries, in describing cures and medicinal preparations, made reference to a *serpente*, which is believed to have been the coiled tube condenser part of a still. If so, this may be the first reference to this type of cold water condenser system – the worm condenser (a coiled copper tube immersed in a tub of cold water), which can still be seen in a few Scotch malt whisky distilleries (Section 3.2.4), some brandy distilleries (Section 3.6.1) and some rum distilleries (Section 3.5.3). During the twentieth century, in the production of many distilled beverages (e.g. Scotch malt whisky, see Section 3.2.4), the simple worm tub type condenser largely gave way to vertical shell and tube counter flow condensers, which can now be temperature controlled, thus giving the distiller even more control over the nature of his distillate.

The biggest development in distillation equipment came in the early part of the nineteenth century, with the invention of continuous stills (column stills), particularly the Coffey still (Section 3.1.3). Although batch pot still systems were already producing spirit on an industrial scale, the introduction of continuous stills revolutionized distilling by greatly increasing the throughput and minimizing the number of manipulations and amount of manpower needed during the distillation process. Continuous stills rapidly became popular,

so much so that by the end of the nineteenth century, they held top place in the manufacture of most distilled alcoholic beverages, a position that is still held today. There are, however, notable exceptions where pot or batch column stills are prevalent, such as in the production of all Scotch malt whiskies, some other whiskies and grain spirits, some rum, most brandy and some fruit spirits.

The need for distillation in general was originally scientific or commercial; initially in the production of perfumes and medicines from natural (mostly plant) materials and in the alchemist's attempts to turn base metals into gold. Gradually, it became apparent that the process of distillation could be used as a purification technique, the first sample of reasonably pure ethanol probably being recorded by the Arab alchemist Al-Kindi (Alkindus) in the ninth century (al-Hassan, 2009).

In Europe, as distilled alcoholic beverages became more palatable and the focus shifted from purely medicinal purposes to pleasure, so began the gradual increasing commercialization of distilled spirits production. In the sixteenth and later centuries, it was realized that the production of distilled alcoholic beverages was a cost effective way of using surplus perishable crops (e.g. barley, maize (corn), grapes, rice and sugar cane) by making a valuable by-product (e.g. malt whisky, corn whiskey, brandy, rice wine spirit and rum/cachaça respectively). It provided a very desirable extra income for the farmer, by trading it at market, where it was considerably easier to transport then the bulkier fruit or grain crops. Distilled spirits were more durable than the fermented beverages from which they were produced and sometimes, after suitable aging, they were actually an improvement on the original beverage, as in the case of Cognac and Armagnac. Additionally in the eighteenth century, the practice of adding grape spirit to either wine (e.g. Madeira and Sherry) or fermenting must (e.g. Port and vins doux naturels) (see Chapter 2.10) was emerging as an important alcoholic beverage production method.

However, manufacture of distilled spirits has always been in competition with food production from the same crops and there have been numerous occasions in all spirit producing regions when distillation had to give way to the use of crops as food. Also, during the twentieth century, the production of pure (96.5% v:v) ethanol for fuel and industrial uses became an important competitor. Bioethanol, produced from maize, sugar beet, sugar cane and wheat crops, for example, has become an important product in a number of countries, particularly those in North and South America.

To begin a simplified discussion on the theory of distillation, let's consider a near ideal solution of hexane (boiling point 68 °C) in heptane (boiling point 98 °C) at atmospheric pressure (Oxtoby *et al.*, 2002). It is called near ideal, because the forces of attraction between the molecules are relatively weak London dispersion forces. For a given component, the relationship between vapor pressure and composition is expressed by Raoult's Law (Equation 3.1.1), where X is the mole fraction of the component and p_o is the vapor pressure of the pure component. The latter is higher for a more volatile (lower boiling point) component.

$$p(1) = X(1)p_o(1) \tag{3.1.1}$$

The mole fraction is a measure of concentration; for a two component solution, $X(1)$ is given by Equation 3.1.2.

$$X(1) = \frac{n(1)}{n(1) + n(2)} \tag{3.1.2}$$

The vapor pressures of the pure components that make up a solution differ, which means that the vapor phase in equilibrium with the solution will have a different composition: it will be richer in the more volatile (lower boiling point) component.

Consider, at 25 °C, pure hexane, $p_o(\text{hex}) = 0.198$ atm, and pure heptane, $p_o(\text{hep}) = 0.060$ atm, in a solution of mole fraction of hexane $X(\text{hex}) = 0.400$ and mole fraction of heptane $X(\text{hep}) = 0.600$.

$$p(\text{hex}) = X(\text{hex})p_o(\text{hex}) = 0.400 \times 0.198 = 0.0792 \text{ atm}$$

Similarly,

$$p(\text{hep}) = X(\text{hep})p_o(\text{hep}) = 0.600 \times 0.060 = 0.0360 \text{ atm}$$
$$\therefore \text{ total vapor pressure, } p(\text{total}) = 0.0792 + 0.0360 = 0.1152 \text{ atm}$$

If $X(\text{hex})'$ and $X(\text{hep})'$ are the mole fraction of hexane and heptane in the vapor, then

$$p(\text{hex})' = X(\text{hex})'p(\text{total})$$

Hence

$$X(\text{hex})' = 0.0792/0.1152 = 0.688$$

and

$$X(\text{hep})' = 0.312$$

Therefore, the vapor is richer in the more volatile component (the solute, here). Suppose some of the mixture's first vapor were removed by heating and then condensed, on a cold surface to a liquid. The vapor in equilibrium with this new solution would be even richer in the more volatile component: the new solution will boil at a lower temperature than the original one. Continuation of this evaporation/condensation cycle eventually leads to a situation where the vapor is composed entirely of the more volatile component. This is the basic principle of distillation and is best summarized in a temperature/composition diagram (rather than a pressure/composition diagram), since real distillations are carried out at constant pressure (Figure 3.1.1). The diagram is an example of a phase diagram. The curves represent phase boundaries: above the curves only

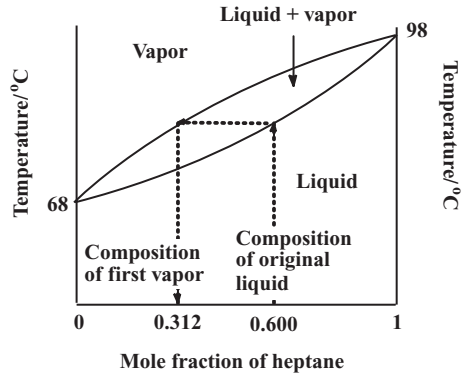

Figure 3.1.1 *Phase diagram for a hexane/heptane solution*

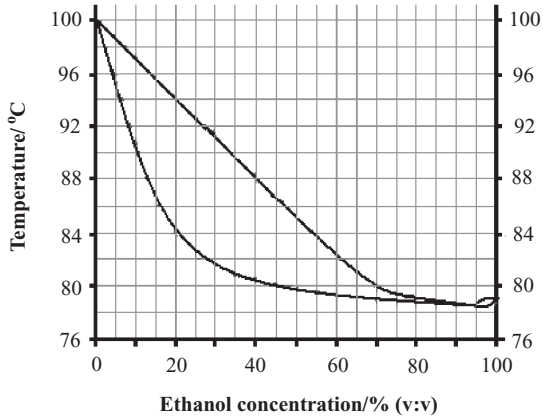

Figure 3.1.2 *Temperature- composition diagram for ethanol–water*

vapor exists (single phase), below only liquid exists, but at the curves liquid and vapor exist in equilibrium, in a two phase system.

Ethanol and water are miscible in all proportions, but do not form an ideal solution because of the relatively strong hydrogen bonding forces that exist between the molecules. Upon distillation, this leads to a more complex phase diagram (Figure 3.1.2), which is characterized by a minimum boiling point at 78.2 °C, corresponding to the ultimate formation a constant boiling mixture (an azeotropic mixture composed of 95.6% ethanol and 4.4% water by volume) when an aqueous ethanolic solution is distilled many times. For convenience, rather than mole fraction, Figure 3.1.2 plots percentage ethanol composition (v:v) (i.e. %ABV) on the *x* axis, because this is the most widely used expression of ethanol content in the alcoholic beverage industry.

If an ethanol–water solution of initial ethanol content of about 8% (v:v) is distilled once, the vapors that condense will possess an ethanol content of about 30%, whereas an initial solution of about 12.5% ABV will yield a first distillate of about 40% (Figure 3.1.2). These correspond to the first distillation (low wines) of whisky and brandy, respectively, ignoring the head and tail 'cuts' (fractions), which are discussed later. If these distillates are redistilled, the result will be distillates of *ca.* 65% ABV and *ca.* 70%, respectively. This corresponds to a double distillation procedure (again ignoring the head and tail fraction management for the time being), used to produce many distilled beverages, such as most malt Scotch whisky, some rum, most brandy and some fruit spirits.

Triple distillation produces distillates of about 80% ABV and over 85% ABV, respectively (Figure 3.1.2) and is used to produce Irish whiskey, a little malt Scotch whisky, some rum and other spirits.

Clearly, if redistillation is carried out many times, the ultimate result will be the azeotropic mixture, containing 95.6% ABV. If this is redistilled, it retains the same composition in the vapor state as in the liquid state and hence no further increase in ethanol concentration can be achieved by simple distillation; this is the upper limit.

3.1.2 Batch Distillation

The multiple distillations described above could be all carried out in batches, by returning the distillate to the (clean and dry) boiler of the same still, or to a fresh still, and redistilling. This is indeed done in the production

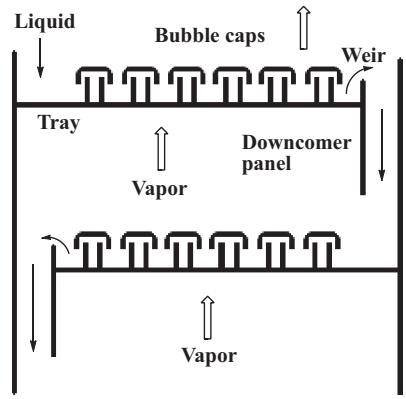

Figure 3.1.3 *Tray and bubble cap system of reflux. Not drawn to scale. [⇑]: vapor flow; [↓]: condensed liquid flow*

of many distilled spirits, as mentioned above, the stills used in this way being known as pot stills. In practice, a certain, but very limited, amount of reflux occurs in simple pot still distillations, depending mainly on the dimensions of the still neck and length of the lyne arm – the length of (usually copper or stainless steel) tubing between the still head and the condenser. A taller neck allows more reflux, thus generally resulting in a somewhat more pure, but rather lighter spirit with somewhat lower levels of congeners.

Greater control of reflux can be provided by the installation of condenser trays into the widest part of the neck, just above the top of the boiler. Reflux is maximized when the trays are in the horizontal position, whereas minimum reflux occurs when the trays are in the vertical position. This version of a pot still is known as a Lomond still. There were once several examples of operating Lomond stills in Scotch malt whisky distilleries, but today, they have all been replaced with conventional stills, although Scapa distillery uses the only surviving example, but only in the capacity of a normal pot still (Section 3.2.4). However, some of the batch column stills described below, used in the production of many fruit and pomace spirits, perform in a similar way to Lomond stills, while being rather more efficient. Examples of pot stills can be seen in Section 3.2.4 (Figure 3.2.8), Section 3.5.3 (Figure 3.5.4), Section 3.6.1 (Figure 3.6.2) and Chapter 3.8 (Figure 3.8.2), whereas simplified diagrams of various stills are given in Sections 3.2.4 (Figure 3.2.9), 3.5.3 (Figure 3.5.3) and 3.6.1 (Figure 3.6.1), as well in Figures 3.1.3 and 3.1.4.

A batch column still has a reflux column fitted somewhere between the head of the boiler and the condenser, so that multiple distillations can be carried out using the same still, in a single run or pass. The reflux column allows a cycle of condensations and vaporizations and in effect acts as a fractionator or rectifier, the efficiency of the column influencing the number of condensations/vaporization cycles, which in turn influences the purity and strength of the spirit emerging from the still.

Various types of reflux columns exist, the most common probably being the bubble cap tray type (Figure 3.1.3). Bubble cap trays are flat and perforated and are fixed to alternative sides of the column internal surface. They have fixed risers above the holes, over which fit the bell-shaped bubble caps. The vapors rise through the risers and bubble caps, and condensation takes place on the column surfaces. Much of the condensed vapors collect in the tray, where eventually sufficient volume will accumulate to cause overflow down the downcomer panel into the tray below. This process continues down the condenser until reaching the boiling liquor. On the way down, much of the condensed vapors will be revaporized, as there is an increasing temperature gradient from column top to column bottom. The cyclical vaporization/condensation processes occurring throughout the length of the column will favor vaporization and collection of lower boiling point components (such as ethanol, esters and sulfur compounds) toward the cooler top part of the column and

significant vaporization of less volatile components (such as acids, fusel alcohols and water) will occur in the hotter lower parts of the column.

Simple sieve trays (without risers and bubble caps) make the most robust tray condensers, but are less efficient compared to bubble cap trays for mass transfer operations and are used when distillations require a smaller number of theoretical stages for separation. Valve tray condensers have perforated tray decks, which are fitted with movable discs or valves to vary the tray open area with changing vapor load.

The reflux column part of a batch column still divides the condensate into fresh distillate, which is collected, and the remainder, which is returned down the column for further reflux. The reflux ratio (returned liquid:fresh distillate) should be high for efficient fractionation. In practice, this means the column still performs best with a lower throughput and a steep temperature gradient in the reflux column. Under these conditions, the residence time of liquid and vapor in the column is long, allowing attainment of equilibrium and lower temperatures at the top of the column, where some of the ethanol/water vapor will run down the condenser as distillate.

Batch column stills, such as those sketched in Figure 3.1.4, are used to make many different types of distilled spirit, including some brandy, much cachaça and rum (Section 3.5.3), most fruit spirit (Section 3.8.3), much North American whiskey (Section 3.3.4) and much pomace spirit (Section 3.7.2).

Even greater control of reflux can be achieved if a cold water condenser is attached to the top of the reflux column or to the still head, or both. This piece of apparatus is known as a dephlegmator; it normally consists of a cylindrical bank of vertical tubes with cold water running through them. A schematic diagram of a batch column still with two dephlegmators can be seen in Figure 3.1.4; one dephlegmator is attached to the top of the boiler, whereas the second is more conventionally situated at the top of the fractionator. Another type of still head condenser, the bowl jacket condenser, is simpler and less expensive than the vertical tube type condensers, but has less control of reflux and sometimes leads to distillations that are both too rapid and too hot (sometimes $> 90\ ^{\circ}C$) (Bruno *et al.*, 2007).

All batch distillations, whether or not reflux columns and dephlegmators are used, need head and tail management, so that high concentrations of potentially harmful or malodorous constituents are removed from the distillate. An account of a typical Scotch malt whisky double distillation using pot stills without either reflux columns or dephlegmators is described in Section 3.2.4, so the account here will focus mainly on batch column distillations.

Fruit and other spirits are frequently produced via a single pass through a column still, as described by Claus and Berglund (2005) for the production of cherry and plum spirits. The 165 l boiler was charged with 150 l of fermented fruit mash and distilled using a temperature probe at the head of the condenser to regulate cooling water flow rate in order to maintain a condenser temperature of 72 °C. The head fraction was discarded and the yield of heart cut (distillate) was up to 7 l when all three trays in the reflux column and a catalytic converter (between the reflux column and condenser) were engaged. The heart cut started at 95% ABV and finished at 58% ABV, giving the distillate an average ethanol content of 77% (v:v). The tail fraction was redistilled with a later batch of mash. The trays were of the sieve type and the catalytic converter was a high surface area copper packing designed to catalyze formation of ethyl carbamate in the converter (rather than elsewhere in the still), where it is separated from the heart cut. The catalytic converter also acted as a fourth tray and so improved rectification, but it also catalyzed the formation of small amounts of acetaldehyde in the distillate by dehydrogenation of ethanol on the catalyst surface.

As expected, congeners with lower boiling points than ethanol (acetaldehyde and ethyl acetate) were more prevalent in the head fraction and early heart cuts, whereas those with higher boiling points (isoamyl acetate and 1-propanol) were more in evidence in the late heart cuts and tails. Methanol, on the other hand, was present in higher concentrations in both the early and late heart cut and at significant levels in the middle hearts. However, careful collection of distillate ensured the methanol content in the product spirit (40% ABV) was below the US regulatory limit of 0.35% v:v ethanol.

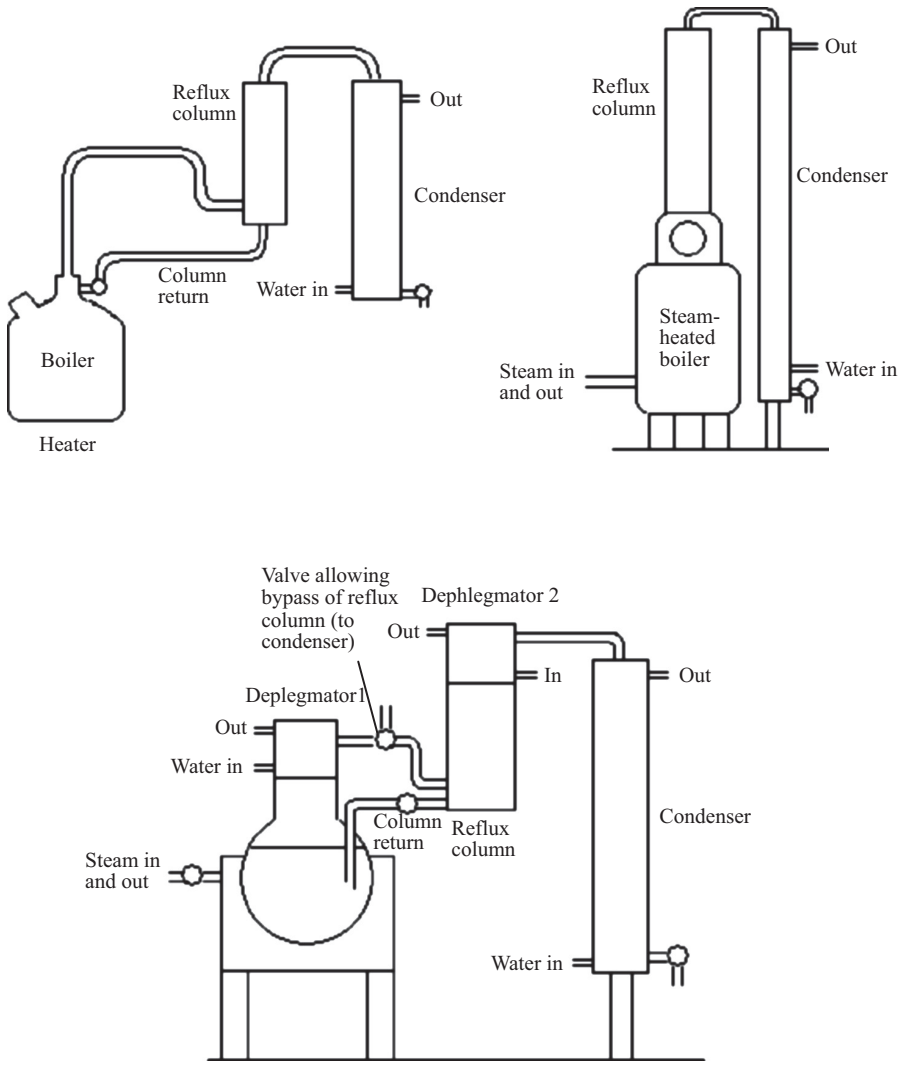

Figure 3.1.4 *Schematic diagrams of batch column still assemblies. Some systems have in line temperature sensors and/or in line catalytic converters (e.g. between reflux column and condenser) for minimizing ethyl carbamate formation. Not drawn to scale*

The distillation of a typical fruit wine to yield a fruit spirit (melon spirit) using a traditional 130 l batch column still (an 'alquitara') for the first distillation and a small (30 l) traditional pot still for the second, has been described by Hernández-Gómez *et al.* (2005). The first distillation was carried out at a rate of 170 ml/min, with the condenser temperature at 21 °C throughout, collecting the distillate until the ethanol content had dropped to 9.2–11.8% (v:v). This gave a distillate of about 18.5–25% ABV and the head fraction was about 200 ml. The second distillation (of 15 l of first distillate) was performed at a rate of 35–40 ml/min. The head fraction (~0.8% of the distillate) was discarded and collection of the heart fraction (second distillate) was stopped at 40% ABV, so giving a final distillate of 58–69% ABV. The fractions from 40–5% ABV were

the tail fractions. The yield of first distillate from 110 l of melon wine (~3% ABV) was 12 l. Analysis of the fractions of the second distillation showed methanol to be distributed throughout the fractions, but most concentrated in the tails, higher alcohols to be fairly evenly distributed between head and heart fractions, and esters to be concentrated in the head fraction, but with significant levels in the heart fraction.

Typical Cognac (Charentais) stills (Section 3.6.2) have boilers of the alembic type, but include a characteristic onion shaped wine warmer that heats the wine before it enters the boiler, as shown in Figure 3.1.5(a). The vapors issuing from the boiler on their way to the condenser heat the wine. Cognac is double distilled, like Scotch malt whisky, the heart cut of the second distillation having about 70% ethanol by volume. Armagnac stills (Section 3.6.3), on the other hand have a pot alembic type boiler, like Cognac stills, but also have a columnar wine warmer and rectifier, and so are intermediate in nature between a pot still and a column still, as can be seen in Figure 3.1.5(b). As there is only one distillation (yielding a spirit of *ca.* 53% ABV), the still can take a continuous supply of wine and hence act as a continuous alembic still. Rectification, however, is

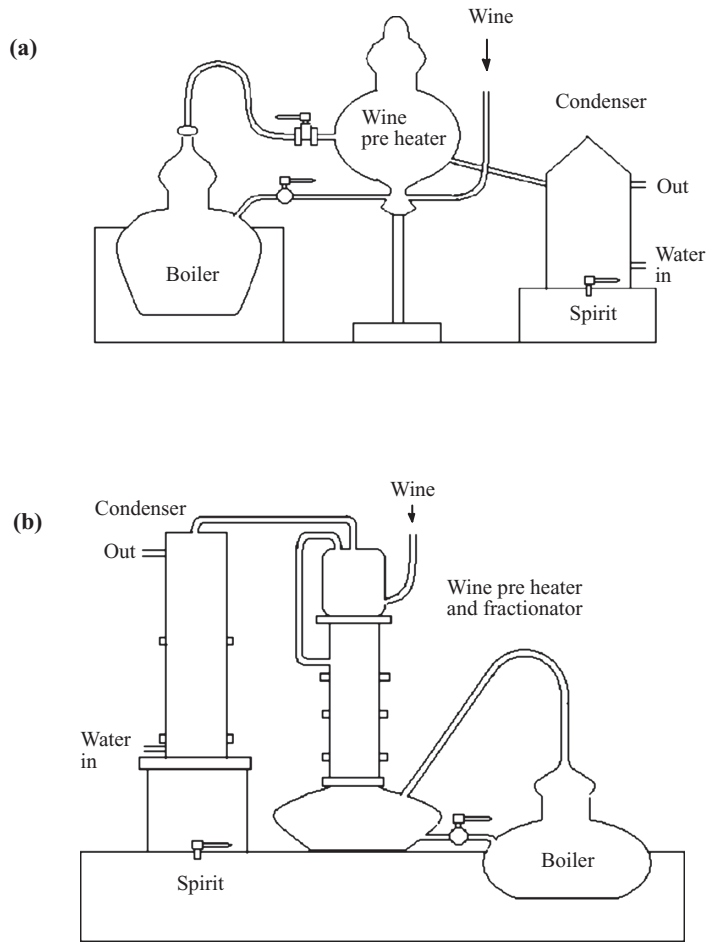

Figure 3.1.5 *Simplified schematic diagrams of (a) Cognac (Charentais) alembic and (b) Armagnac alembic column still. Not drawn to scale*

very limited compared with the continuous column stills discussed in the next section, so that the resulting spirit contains plenty of congeners and has a considerably lower alcohol content than the second spirit from a Cognac still.

Operation of batch stills requires fraction management, so that the desired distillate contains sufficient ethanol, sufficient flavor congeners, but without high levels of undesirable components. For simple pot stills, this means effective separation of the head and tail fractions from the middle fraction, whereas for column stills it means setting the operating conditions to give the optimum spirit. Either way, the distillation needs to be monitored in some way, not only to ensure spirit quality, but also for consistency of quality from batch to batch.

Many distillers monitor distillation progress by manual measurement of distillate density using hydrometers; yet others use in line ultrasonic transmitter/receiver type density detectors (Section 4.6.3) for the same purpose. The former method is the simpler and cheaper of the two, but is open to error if temperature adjustments are not made (see Appendix 2), say in busy periods when a still operator may have to attend to many stills simultaneously. Recently, a 'soft sensor' has been developed for monitoring batch pisco (Section 3.6.4) brandy distillations in Chile (Osorio *et al.*, 2008). The sensor is essentially a computer software programme based on artificial neural networks, involving simple data preprocessing procedures and calibration with laboratory and industrial experimental data. It deduces ethanol concentration from temperature measurements made at four locations within the distillation set up with an accuracy of $\pm 0.6\%$ for small stills and $\pm 1.6\%$ for larger, industrial stills.

3.1.3 Continuous Column Distillation

Continuous column stills, of which patent or Coffey stills are the most common, are designed specifically to distill a pure spirit continuously from a continuous supply of wine or beer. An early version invented by Robert Stein was used at the Cameronbridge grain distillery in Scotland (see Section 3.2.1) in the 1820s. The design was enhanced and patented in 1831 by an Irish excise man, Aeneas Coffey, and it was his version that became popular wherever distilled drinks were produced during the nineteenth century. It is a two column still, the first column (analyzer) has steam rising and wash descending through several levels of perforated fractionating plates. The second column (called the rectifier), carries the alcohol separated in the analyzer from the wash where it circulates until it can condense at the required strength (Figure 3.1.6). Originally, the columns of Coffey stills were of cylindrical design, but modern continuous stills often have rectangular columns, like the ones shown in Figure 3.1.6.

The wash (beer or wine) is fed firstly from a vat or tank through pipes at the top of the rectifier, where it is preheated by steam before being carried from the bottom of the rectifier to the top of the analyzer. There the almost boiling wash cascades through the fractionating plates and meets the rising steam. As the two meet on the surface of the plates, the wash boils and a mixture of vapors of ethanol and other volatile components, as well as uncondensed steam rise to the top of the column. The spent wash (containing less volatile constituents, principally water) runs down the analyzer column and is discharged from the base. The hot vapors now enter the rectifier at the base and as they rise through the column they partially condense on sections of a long coil through which wash is flowing, according to their volatilities (boiling points). The ethanol vapor condenses at the cooler top of the column and is run off through a water cooled condenser to the spirit safe and then on to the spirit receiver. Once the spirit is flowing, it runs continuously until the end of distillation (i.e. until the supply of wash is stopped).

The continuous and rapid nature of the distillation, coupled with the higher concentration of alcohol in the final distillate, are the main advantages over a pot still, or a batch column still, which can only work in batches. Essentially, this means that a pure spirit can be produced more cheaply than with batch columns. The main disadvantage of continuous alcohol stills is that the alcohol produced by them is not entirely free

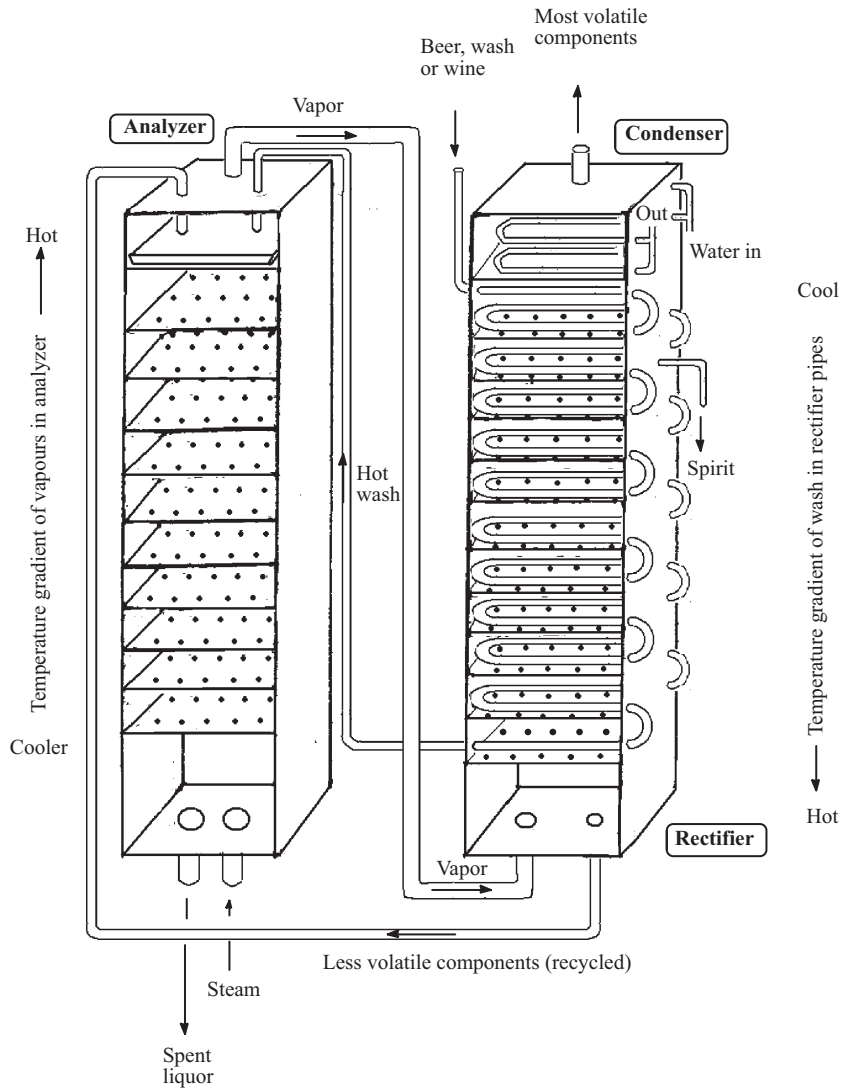

Figure 3.1.6 *Schematic diagram of a continuous still*

of more volatile and potentially harmful components, such as methanol and acetaldehyde, although this situation can be improved by taking the ethanol from just below the top of the rectifier. In this case, most of the acetaldehyde and methanol is taken from the top of the column.

Like batch column stills, continuous column stills yield a relatively pure spirit of high ethanolic strength (up to 95.6% ABV, the strength of the azeotropic mixture), which is low in flavor compounds (congeners: acids, higher alcohols and esters, for example). This is particularly the case with tall column continuous stills, which are able to supply the highest degree of rectification. Grain neutral spirit (GNS) and fuel alcohol ('bioethanol') (produced from any weakly alcoholic wine or wash) are the ultimate examples of pure, almost flavorless spirit. GNS is very important in the production of many spirits, notably gin (Section 3.4.2) and

vodka (where it is further purified by charcoal filtration) (Section 3.4.3), and many liqueurs (Chapter 3.9), where it is frequently used as at least part of the spirit base. The production of bioethanol, although outside the scope of this book, is a booming business in North and South America and is becoming more important in many other countries.

However, in the production of many distilled beverages, regulations require the spirit to be of considerably lower ethanolic strength than that of the azeotropic mixture. For example, the EU stipulates that the distillation of fruit spirits must occur at less than 86% ABV, so that the spirit retains some character of the fruit from which it is derived (Council Regulation (EEC) No. 1576/89 of 29 May 1989, EEC, 1989). Similar regulations pertain to other distilled spirits that are produced using less efficient fractionating systems (Armagnac and some Calvados) or continuous stills (Armagnac).

There has been much discussion in the literature on the relative merits of batch pot still, batch column and continuous column distillation, but as described above, each type has its own character and can produce superlative spirits if operated under proper conditions. Much of the perceived organoleptic differences in pomace spirit (Section 3.7.2) produced by different stills (pot versus column stills) have been ultimately traced to differences in process or operating conditions rather than to fundamental differences in the distillation apparatus (Corich *et al.*, 2005; Cortés *et al.*, 2005). Likewise, differences in the levels of harmful or undesirable components (such as butanoic acid, 2-butanol and methanol) in pomace spirit have been attributed more to process conditions than type of still.

Ethyl carbamate (EC) (Section 5.11.5) formation during distillation is always of concern, because of its potential carcinogenic activity. Bruno *et al.* (2007)in a study of the EC levels in cachaça, produced using a wide variety of still types, concluded that differences in levels were largely due to differences in distillation conditions. The data showed generally higher EC levels for continuous column cachaça, than for alembic (pot still and batch column still) versions, but within each group there were exceptions that could be related to the distillation operating conditions. In particular, low distillation temperatures and high reflux rates gave spirits with minimal EC content, indicating that many of the continuous distillations were either carried out in unsuitable equipment or were performed too rapidly at too high temperatures. Also, insufficient cooling of column still dephlegmators led to high levels of EC in batch column still cachaça.

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3.2
Scotch Whisky

Inspiring bold John Barleycorn!
What dangers thou canst make us scorn!
Wi' tipenny, we fear nae evil;
Wi'usquebae, we'll face the devil!
—Robert Burns, Tam O'Shanter

3.2.1 Introduction and Brief History

Whisky (or whiskey) is produced in many parts of the world by distillation of the beer (called 'wash' in the industry) formed by fermentation of cereal wort (Section 2.6.2). The two words used for these spirits, whisky and whiskey, are both corruptions of the Gaelic name for the spirit, *uisge beatha*, meaning water of life. Thus the derivation of names of this distilled beverage is, like akvavit (Scandanavia) and eau-de-vie (France), from the Latin *aqua vitae*. Whisky is the specific name for spirits produced in Scotland or in countries that have been largely influenced by Scotland, such as Canada, India and Japan (Section 3.3.6). The much less well known products of England, the Isle of Man and Wales also use the word whisky, whereas whiskey is used for the cereal spirits of Ireland and generally for those of the USA (Sections 3.3.2 and 3.3.3).

Scotch whisky refers solely to whisky that has been distilled and matured in Scotland. The bulk of the Scotch whisky today is, as it has been for many decades, a blend of malt whiskies and grain whiskies. Scotch malt whiskies are made from malted barley, using pot stills (Section 3.1.2), whereas grain whiskies come from other malted or cooked cereal grains, such as maize or wheat via Coffey (continuous) stills (Section 3.1.3). A single malt Scotch whisky is the product of only one distillery, although the majority, for the sake of consistency, are blends of malt whiskies from different distillation batches (= different casks) over a period of time. The chief blender, or blending panel, decides on the contributing casks of spirit required for a bottling of a particular malt whisky. The whiskies from these casks are then vatted together for a few weeks before any further processing and bottling. Single malt Scotch whiskies became popular from the 1960s, prior to that time they were often difficult to obtain outside Scotland and most malt whiskies were (and still are) destined for blends. Single grain Scotch whiskies are comparatively rare. Vatted malt Scotch whiskies, produced by vatting (blending) malt whiskies from several distilleries are also available, these usually being the products

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

of whisky blending companies and independent bottlers. The general characteristics of all these whisky types are discussed more fully in Section 3.2.6.

It is not known for certain when the first whiskies were distilled in Scotland, but there is a general consensus of opinion that distillation was well established by the fifteenth century. Like brewing of beer, whisky distillation was essentially a cottage industry until the late eighteenth or early nineteenth centuries. It is probable that the first spirits were produced from local grains, most likely oats and barley. The barley (called bigg or bere) would often be the four rowed type that was indigenous to the west coast, although this was gradually replaced by higher yielding two rowed types (Wilson, 1985a). Even so, the yields (in volume of spirit per mass of grain) would have been low by modern standards (Section 3.2.2) and very much at the mercy of inclement weather. Until well into the eighteenth century in the Western Isles and the Highlands, distillation was a sporadic activity carried out largely by farmers according to yield and in competition with food crops. Nevertheless, whisky became a valuable commodity and production grew, so that by the mid eighteenth century, the highlands and islands had acquired a reputation for their malt whiskies, some of which were exported to the Lowlands. The latter areas, especially around Glasgow and Edinburgh, by this time had comparatively large distilleries producing whisky from grains, especially wheat, maize and barley.

A succession of restrictive laws and taxes caused illicit production of whisky in remote highland and island locations, where their discovery by excise men was difficult. The barley for these early whiskies would have been malted by soaking in burn water and leaving exposed to the sun until germination occurred. Drying of the germinated grains would be over a peat fire and indeed all operations involving heat, including distillation per se, would most likely involve peat, this being the most widely available fuel. Distillation was carried out using a small copper pot still inside a well camouflaged temporary structure (a 'bothy'). By modern standards, these whiskies would have been smoky, fiery and generally rough. Maturation in large oak casks was not widely practiced at that time, the whisky being kept or bottled and consumed according to the circumstances.

The growth of large-scale or industrial and hence legal distilleries began in the eighteenth century, especially after the passing of the Wash Act (1784), which eased certain restrictions on the legal trade and at the same time encouraged illicit distillers to obtain a licence. An amendment to this Act in 1785 allowed two stills of up to 180 l in each parish to be operated by respectable tenants, appointed by local lairds. Many licenses were taken out and the growth of the Scotch whisky industry as we know it can be considered as starting around that time. Growth accelerated and consolidated with the Illicit Distillation Action (1822) and the Excise Act (1824); many distilleries were (legally) founded at this time. The illicit croft based distilling industry began a slow course into decline.

Figure 3.2.1 shows the approximate locations of all the presently (2009) active distilleries, both malt and grain distilleries. Also indicated in Figure 3.2.1 are 'silent' distilleries – those that have been inactive for a number of years – and a brief list of some notable defunct distilleries. Various distilleries claim to be the first legal Scotch whisky distillery, although it is difficult to confirm or refute claims because of a lack of precise records. Also, many famous distilleries were built on or close to sites of former illicit distilleries. Certainly, some of the Glenturret distillery buildings date from 1775 and Bowmore distillery (Islay) was well established at the time of the Wash Act (1784). Other distilleries claiming establishment in the eighteenth century include Balblair (1749), Deanston (1785), Glen Garioch (1798), Highland Park (1798), Littlemill (1772), Oban (1794), Strathisla (1786) and Tobermory (1798) (Jackson, 1989).

Like all alcoholic beverage industries, the Scotch whisky industry has undergone (and is still undergoing) many changes: periods of growth, recessions, rationalization, consolidation, increasing automation, modernization of methods and the effects of legislation (Section 1.2.4). The industry is now a mature industry, generally keeping an open mind on innovation and change, but at the same time respecting past achievements and the pictures of tradition that have been gradually (and successfully) developed since the early twentieth

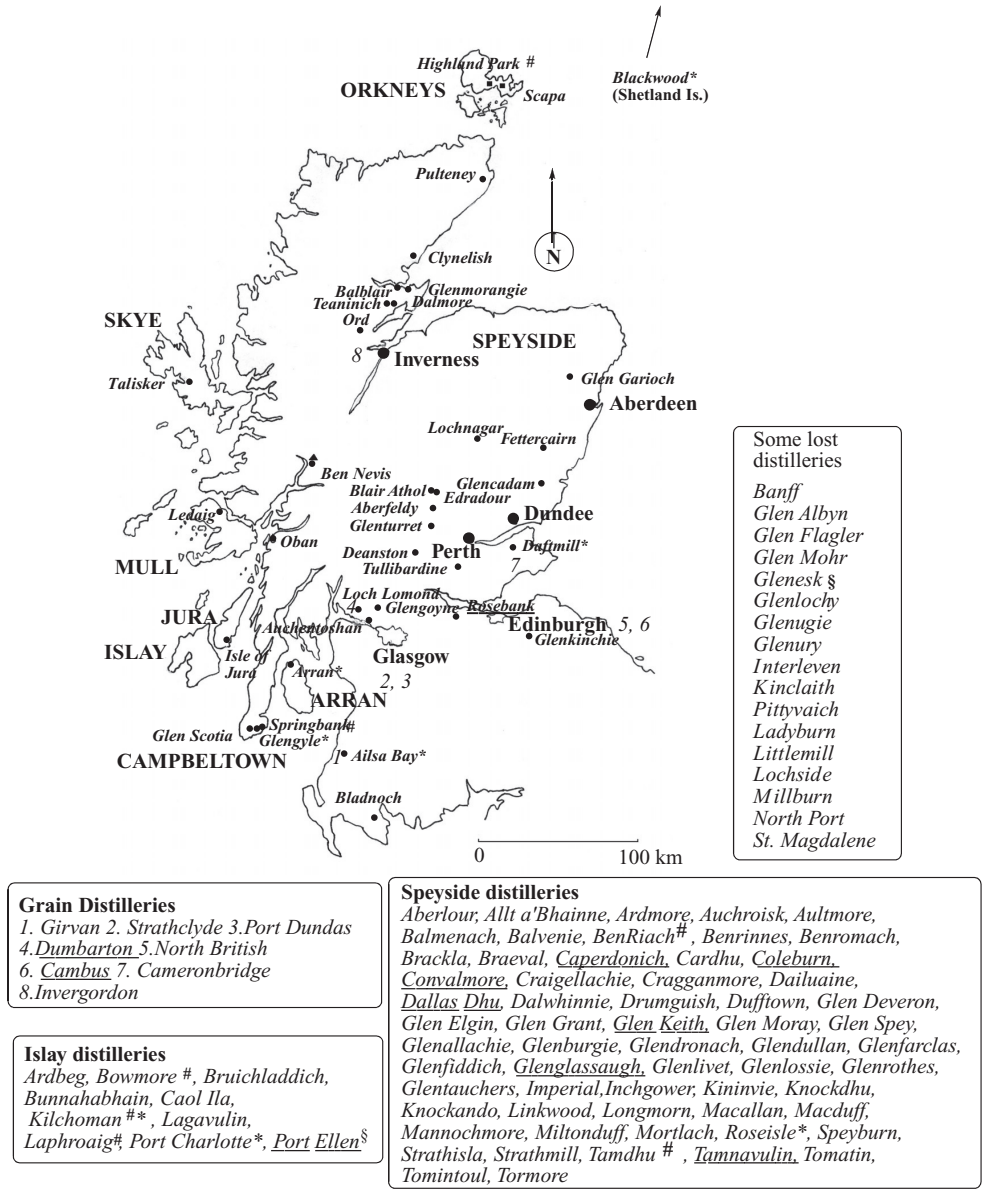


Figure 3.2.1 *The Distilleries of Scotland. Underlined istilleries are silent at the time of writing (2008). Note: some distilleries have alternative names, or produce brands with alternative names - e.g. Longrow, Hazelburn (Springbank), Tobermory (Ledaig), Kilkerran (Glengyle). Lost distilleries are closed down or demolished, but some of their products can be found still. The list of lost distilleries is not exhaustive. Dallas Dhu is a whisky museum. *Relatively new, in some cases single malts not yet available (2008). §Now maltings. #Use own maltings*

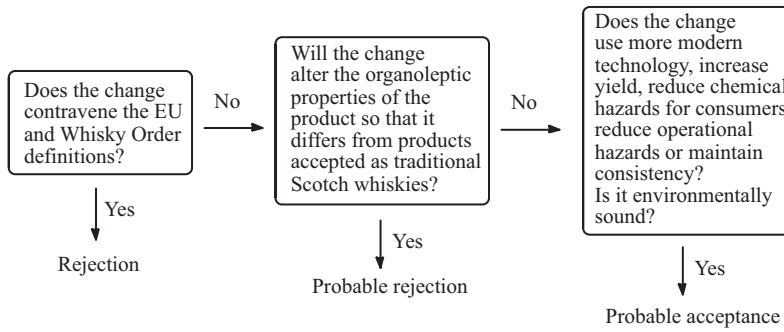


Figure 3.2.2 *General criteria for change within the Scotch whisky industry*

century (Halliday, 2004). Like other alcoholic beverages within the EU, Scotch whisky and whisky in general are protected from counterfeit and fraud by definitions and statements within the EU Spirits Drinks Regulation (1989). This regulation defines the materials from which, and processes by which, whisky must be made (albeit in a rather general way) and also refers broadly to 'traditional practises,' without actually defining them. In particular, the Regulation stipulates that whisky must be matured in casks of less than 700 l capacity for a minimum of three years and the only additive allowed (not counting demineralized water for dilution) is caramel. Additionally, 'tradition practises' must be followed. The Scotch Whisky Order (1990) gives a similar, but more detailed set of definitions and statements, and adds the necessity that Scotch whisky not only must be produced in a distillery in Scotland, but also must be matured in casks in an excise (bonded) warehouse in Scotland.

These protective (and restrictive) legislations leave plenty of room for change and innovations that respect the general picture of tradition (Morison, 2004), as illustrated in Figure 3.2.2. Note that Figure 3.2.2 could be used as a general guide to acceptable and unacceptable change for any protected beverage – inside and outside the EU.

An example of an acceptable innovation is the relatively recent activity of deliberately ageing single malt Scotch whiskies in ex-beer or (more commonly) in ex-wine casks (such as those that had contained Chardonnay, Madeira, Shiraz or Port, for example). This complies with both the law and tradition even though the tradition goes back only to the end of the nineteenth century, ex-Bourbon or ex-Sherry casks being used for this purpose. Ageing in, say, an ex-Port cask will give a different aroma and flavor profile to the spirit, but which is still instantly recognizable as Scotch whisky. In any case, in the early days of cask ageing, some distillers probably used whatever casks were available.

On the other hand, an example of an innovation that would not be acceptable at present is the use of toasted oak chips in the maturation process. Even though aroma and flavor enhancement might well be achieved by this process, it would not be permitted because it is not a traditional process; the spirit could not be called Scotch whisky.

In due course, it is possible that processes like the one described above may become accepted and after a further time become traditional, like cask ageing. Casks were originally used as a convenient storage unit for Scotch whisky, as for most other liquids. In time, it became apparent that longer time in cask certainly benefited the character of the spirit, and so cask ageing became an accepted (traditional) practice, with legislation eventually stipulating a minimum of three years in cask.

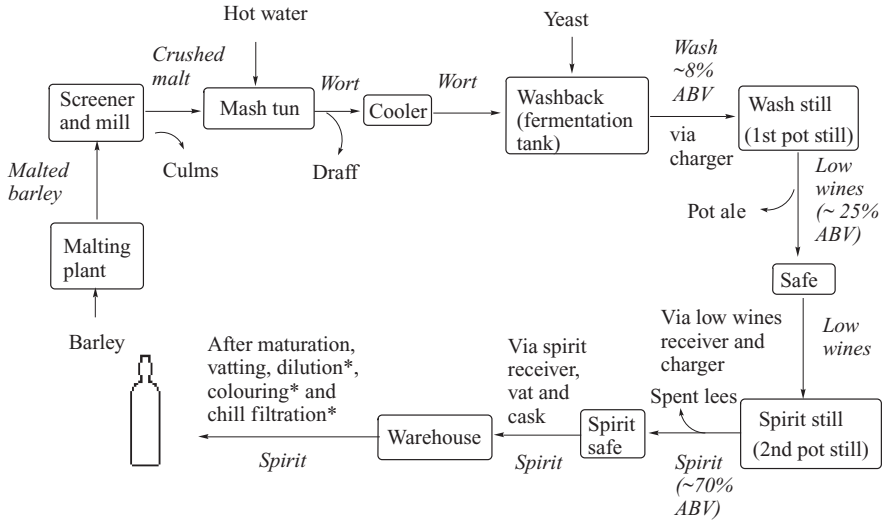
Tradition, then, refers to the accepted ways of doing things at the time; it is forever changing, if only at any one time in subtle details.

Table 3.2.1 *Some twentieth century changes in Scotch whisky production, distillery and allied operations*

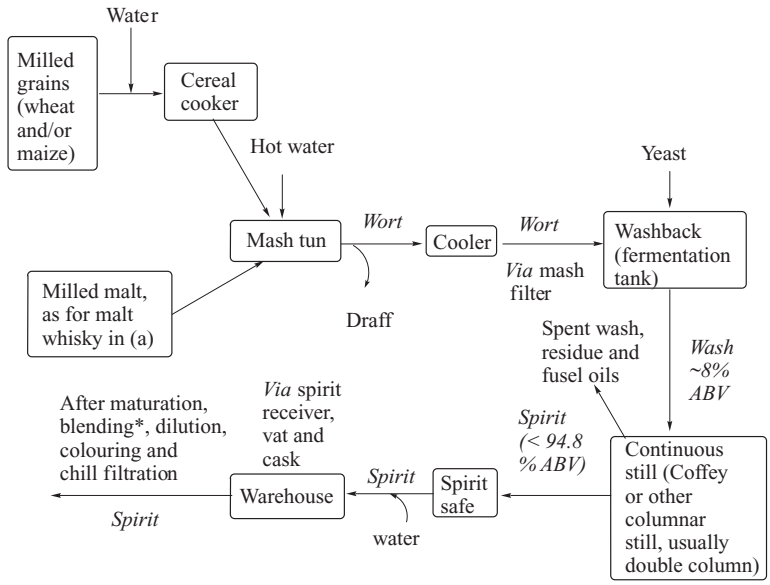
Aspect of production or operation	Major changes and comments
Automation	Extent of automation varies considerably between distilleries and allied industries (e.g. malting), but automation is generally increasing, with fewer personnel involved and much computer control
Barley and other cereals	Breeding and research has produced many improved varieties (with respect to yield, malting quality, spirit yield, disease resistance, etc.) (Section 3.2.2)
Malting	Few distilleries now have their own malting; most malting is done using large drum, saladin box or GKV systems (Section 3.2.3), to distillery specifications, by large malting companies
Fermentation	Improved yeast strains and fermentation control has led to better control of product flavor profile and greater consistency (Section 3.2.3). Some yeasts can produce higher alcohol washes (Section 3.2.3)
Distillation	Steam heating of pot stills has replaced direct heating in some malt whisky distilleries (Sections 3.1.2 and 3.2.4). Vertical shell and tube condensers have replaced worm tubs in many malt whisky distilleries (Section 3.2.4). Alko stills are replacing Coffey stills in some grain distilleries (Sections 3.1.3 and 3.2.4)
Yield of spirit	Yields (in liters of spirit per ton of barley) have increased from ~320 to ~420 over the last 20 years (Sections 3.2.2 and 3.2.4)
Environmental	Water and energy saving operations are in place in many maltings and distilleries, as a result of research and legislation – research is underway to find better and safer use of waste
Maturation	Much Scotch whisky is matured in multistorey palletized warehouses distant from the distillery, as opposed to dunnage warehouses (with earth floors) next to the distillery (Section 3.2.5)
Public relations/tourism	Many distilleries now have visitor centers, with shops selling distillery named goods and related goods, as well as whisky

Scotch whisky production and the ways in which distilleries operate have changed drastically over the last 50 years; some (but by no means all) of these changes are summarized in Table 3.2.1. Also included in Table 3.2.1 are summaries of changes in allied industries, such as malting and agriculture.

The basic processes involved in the production of Scotch malt whisky and Scotch grain whisky are summarized in Figure 3.2.3 ((a) and (b), respectively). The various parts of the overall process depicted in Figure 3.2.3 are discussed in the next sections. The traditional use of pot stills for malt whisky and Coffey or columnar stills for grain whisky is implied in Figure 3.2.3, although in principle these uses can be reversed. There have been experiments using column or hybrid stills (e.g. Lomond stills for the production of malt whisky, and both kinds of stills can be used for Bourbon whiskey (Section 3.3.4), produced from mixed grain (mostly maize) washes. The production of grain whisky, based on wheat (and perhaps some maize), with malted barley up to ~20%, requires a cereal cooking step. Here, the starches of wheat or maize are gelatinized by spending time in the cereal cooker. The resulting soluble starches are then hydrolyzed to fermentable sugars in the hot water mash by α- and β-amylase and limit dextrinase enzymes, released from the malted barley in the mash (Section 3.2.3).



(a) Malt whisky. This scheme is for double distillation. Some malt whiskies are triple distilled. * Not all Scotch malt whiskies undergo these processes. A few single malts are not vatted before bottling (single single malts). Blended spirits from the same distillery give single malt whiskies and from different distilleries give vatted malt whiskies. Many malt whiskies are blended with grain whiskies to give Scotch whisky.



(b) Grain whisky. The vast majority of grain whisky is blended with malt whiskies to give Scotch whisky, but a few single grain whiskies exist. Wheat has been the most important grain for the past three or four decades, prior to that it was maize.

Figure 3.2.3 *Schemes showing production processes of (a) Scotch malt and (b) Scotch grain whisky*

3.2.2 The Raw Materials

The raw materials for making Scotch whisky are cereals (barley alone for malt whisky) and water; yeast is discussed in Section 3.2.3.

Barley is the most important cereal crop regarding Scotch whisky; it the sole cereal for malt whiskies and is used in the production of grain whiskies too. It is the dominant arable crop in Scotland, where the annual yield for 2005–2007 was around 1.7 million tonnes. Almost 50% of this is used in the brewing and distilling industries. The second most important arable crop in Scotland is winter wheat, with an annual production of around 800 000 tonnes for 2005–2007. Again, the Scotch whisky industry is a major consumer of the crop, requiring some 500 000 tonnes annually for the production of grain whisky.

UK barley breeding programmes have tended to develop around certain core varieties, resulting in an elite gene pool and giving rise to cohorts essentially derived from Vada (a nonmalting feedstock variety), and Proctor and Triumph (malting varieties). Selection is according to phenotype, and is based on the following malting, brewing and distilling characteristics:

- High friability (∼90%) (see Section 2.6.2)
- Hot water extractability (∼78%) (amount of soluble starches formed on malting)
- High diastase activity (high levels of starch hydrolytic enzymes)
- High fermentability of wort (for high ethanol yield)
- High β-amylase thermal stability
- Low *epi*-heterodendrin levels
- Low β-glucan and pentosan levels
- Low nitrogen levels.

In addition to these parameters, selection is based on a number of agricultural characteristics, as follows:

- High yield (>72 kg/ha)
- High disease resistance
- Strong, short stems (for use of mechanical harvesting).

The distiller would expect a spirit yield of about 420 l from a tonne of malted barley. Similarly, low nitrogen, low moisture (<14%) and soft endosperm grains are prime characteristics of wheat preferred by distillers.

Cereal plant breeding is carried out by the commercial sector, by companies such as Nickerson, Rothwell, Syngenta, Saaten Union and Twyford. Breeding new barley or wheat varieties is geared toward the needs of both farmers and industries that use these crops: baking, brewing, distilling and animal feed. Practitioners within these industries will generally only accept grain from certain varieties – those that have a maximum number of beneficial attributes for those industries. For distillers, this means high grain yield, good resistance to fungus diseases (minimal fungicide spraying, with minimal crop loss when unsprayed), low nitrogen content, soft endosperm (for wheat), friable grain structure, low β-glucan content (thin cell walls) and low cyanogenic glycoside content.

Under the auspices of HGCA (the cereals and oilseeds sector of the Agricultural and Horticultural Development Board – AHDB), field trials are conducted at various sites and by various companies and institutes. Depending on performance after three years of trials, a new variety can be provisionally recommended, or can be designated for special use by the Scottish Agricultural Colleges (SAC) and the National Institute for Agricultural Botany (NIAB), for example. The HGCA crop evaluation committees are aided by the

Malting Barley Committee, the Scotch Whisky Association and others. Good performance under commercial production leads to recommended status and any new traits (good or bad) are noted for established varieties. Various research institutes, particularly the Scottish Crop Research Institute (SCRI) and NIAB, conduct research that identifies key regions of the barley and wheat genomes that affect desirable and undesirable characteristics and hence determine suitability for brewing and distilling. Knowledge of these genome regions allows development of markers that breeders can use in genotype selection (known as marker assisted selection or MAS). More generally, a better understanding of the genetic factors that determine malting (and hence brewing and distilling) quality and how these are inherited can help scientists and cereal crop breeders alike (Rae *et al.*, 2007). Scientists are seeking molecular markers for major genes or quantitative trait loci (QTL) that control key traits in barley and other cereals, mostly by using doubled haploid (DH) populations specifically targeted to one or more of these traits. Gene markers are available for traits such as resistance to yellow mosaic virus, β-amylase thermostability, hot water extractability, fermentability and nonproduction of *epi*-heterodendrin (Section 5.11.5).

The last named trait is important for distillers, since whisky produced from *epi*-heterodendrin nonproducer barley varieties will have minimal amounts of the carcinogenic ethyl carbamate (urethane) (Section 5.11.5). *Epi*-heterodendrin is a plant defence pheromone – it is toxic to small animals (e.g. rabbits) that attempt to feed on the plant. On disruption of plant tissues, *epi*-heterodendrin is hydrolyzed to 2-hydroxy-3-methylpropanonitrile, a cyanohydrin that decomposes to the toxic hydrogen cyanide (HCN) and 2-methylpropanal (valeraldehyde) (see Section 5.11.5, Figure 5.11.4). In whisky production, HCN reacts with ethanol in the presence of copper during distillation to give ethyl carbamate. The last named compound can also be produced from urea, a byproduct of alcoholic fermentation performed by certain yeast strains. So, careful choice of barley strain for malt and yeast strain for fermentation should give whiskies with levels of ethyl carbamate below limits of detection.

Recommended cereal varieties for brewing and distilling meet with varying success and spend varying lifetimes as recommended varieties. In recent years, Optic (spring) and Pearl (winter) have dominated the production of certified seeds for growing malting barley for whisky production. Previously, Triumph and Golden Promise (both spring types) were favorites, and before them, Tyne and Proctor (1950s). Good winter wheats for distilling are reckoned to be Alchemy, Consort, Glasgow, Istabraq, Riband, Robigans and Zebedee. Some examples of spring and winter barley cultivars and winter wheat varieties that are considered to be best suited to distilling are listed in Table 3.2.2, along with specific weights, crop yield and other data. It can be seen from Table 3.2.2 that yields have generally increased over the years and loss of crop due to lack of fungicide spraying appears to diminish. This latter characteristic is more difficult to assess, as experience has shown that resistance to fungal diseases may not be maintained with time, so such figures may be misleading. Table 3.2.3 lists some of the more successful cultivars of barley, from 1983–2004, for brewing and distilling. Only varieties that have constituted more than 4% of total seed production are included in Table 3.2.3, so that successful varieties like Pipkin (3.4%) and Maris Otter (2.6%) are omitted (Rae *et al.*, 2007).

Before the widespread implementation of intensive arable crop farming, with its use of lime and fertilizers (i.e. prior to the nineteenth century), Scots Bere, a Scottish landrace barley, gave the most important crop for whisky production. In even earlier times, it is possible that oat and wheat crops were also used for the same purpose. Since the nineteenth century, successive cultivars have outperformed Scots Bere in terms of yield and other factors, such as lower nitrogen content. Also, the weak straw of Scots Bere renders it less than ideal for harvesting by combined harvester machines. Nevertheless, interest is growing in Scots Bere, as its malt has a high level of diastatic power (Section 4.6.3), arising from a high level of β-amylase (not α-amylase) (Ellis, 2004). Experimental generation of random inbred lines of plants from a cross between Scots Bere and a modern barley variety is currently underway. Also, one newly proposed malt whisky distillery (Blackwood in the Shetland Isles, whose company already produces gin and vodka) intends to use locally grown Scots Bere for at least part of its source of malt.

Table 3.2.2 *Some barley and wheat varieties for Scotch whisky production. Data from SAC and HGCA (www.hgca.com)*

Cultivar	Status	Type	Date first listed	Yield of grain (t/ha)	Loss of yield through lack of fungicide spraying (%)	Specific weight (kg/ha)	Comments
				Barley			
Publican	P	Sp	2007	9.3	9	69.7	Also for brewing
Appaloose	P	Sp	2006	9.1	12	68.7	
Oxbridge	R	Sp	2005	9.0	9	70.2	
Cocktail	R	Sp	2003	9.0	12	70.2	
Troon	O	Sp	2003	8.5	7	69.7	
Decanter	R	Sp	1999	8.3	8	70.1	
Pearl	S	W	1999	8.6	18	70.8	
Optic	R	Sp	1995	8.4	17	70.4	Also for brewing
				Wheat			
Alchemy	P	W	2006	10.2	15	77.3	
Istabraq	R	W	2004	10.4	22	78.2	
Robigus	R	W	2003	10.0	18	76.4	
Consort	R	W	1995	9.8	27	76.8	

P = provisional; R = recommended; S = specific use variety (SAC and NIAB listings); Sp = spring; W = winter

Scotch grain whisky distilleries relied more heavily on maize than wheat up to the 1980s, but for economic reasons (maize had to be imported), wheat has dominated the industry since that time. As stated previously, wheat is now Scotland's second cereal crop – in 1960, the major cereal was oats, followed by barley and wheat.

Grain whisky distilleries generally use processes originally designed when maize was the major cereal; under such conditions wheat gives an inferior ethanol yield per tonne of grain than maize. Ethanol yield

Table 3.2.3 *Some successful barley cultivars for brewing and distilling, judged by seed production (for period 1983–2004, with >4% of total barley seed production; in order). Information from Rae* et al. *(2007)*

Variety	Type	Variety	Type	Variety	Type
Igri	W	Pastoral	W	Pearl	W
Triumph	Sp	Marinka	W	Fighter	W
Optic	Sp	Panda	W	Golden Promise	Sp
Chariot	Sp	Halcyon	W	Blenheim	Sp
Atem	Sp	Regina	W	Puffin	W

Sp = spring sown; W = winter sown

has been shown to be inversely related to grain nitrogen content and hard wheat can cause some processing problems, so distillers prefer low nitrogen samples from soft wheat varieties. Similarly, wheat varieties whose grains have a mealy endosperm (e.g. Consort), rather than a steely endosperm, release starch easily, but it seems that many genetic factors influence the ethanol yielding potential of wheat (Swanston *et al.*, 2007).

Although there has been very little breeding of wheat varieties specifically for distilling, the possibility exists of combining factors such as high yield, high starch content, low nitrogen content and low cyanogenic glycoside content by choosing complementary parents and selecting progeny with all these desirable characteristics (Swanston *et al.*, 2007).

Recently there has been considerable interest in the assessment of alternative cereals, such as millet and sorghum, for the production of grain whisky (and industrial ethanol). In a comparison of maize, millet, sorghum and wheat for this purpose, Agu *et al.* (2006) showed that, except for high nitrogen wheat, all four cereals gave similar performances with regard to alcohol production and processing. Wheat with high nitrogen content is not generally used by distillers because of its lower alcohol yield and also because of the difficulties caused by its higher viscosity and consequent processing problems (particularly in the recovery of spent wash/spent grains). Agu *et al.* (2006) confirmed these notions and suggested that the higher viscosities of high nitrogen wheat distillation residues were due to higher levels of pentosans in these grains. Levels in maize, millet, sorghum and low nitrogen wheat are known to be similar. Whereas the nitrogen content of wheat greatly influenced the alcoholic yield, this factor appeared to have little influence on the alcohol yield of the other cereals. Greater ethanol yields were achieved with maize and sorghum starches, whereas maize, millet, sorghum and wheat (low N) grains gave similar yields. This suggested that the starches extracted from maize and sorghum can better withstand distillery processing effects (heat and steam stresses) than those of millet and wheat. Millet and sorghum would thus appear to be good alternative cereals for distillation, should unfavorable market conditions overtake maize and wheat in Scotland.

Not counting cleaning, water is used in three ways with regard to Scotch whisky production:

- Steeping of barley grains during malting (Sections 2.6.2 and 3.2.3)
- Mashing – extraction of soluble starches and hydrolytic enzymes from malted barley (and for grain whisky, gelatinized starches from cooked grains) and their conversion to fermentable sugars (Section 3.2.3; see also Section 2.6.2)
- Dilution of spirit to desired strengths prior to bottling (Section 3.2.5).

It can be said that water is the single most important element of Scotch whisky and influences the character of the final spirit, but not in easily defined ways, as there are many other influences (Sections 3.2.3–3.2.5). Most distilleries have their own (jealously guarded) water supply, usually a burn (stream) or loch (lake) and often assisted by a dam or reservoir. Water for distilling is usually untreated, although it must meet standards of purity laid down for potable water (Section 5.10.4). Water that rises from hard rock, such as granite, and has little or no contact with peat is thought to convey a clean softness to the whisky, if no peated malt (Section 3.2.3) is used in its production. Such water is used at Glengoyne distillery in Dumgoyne, a village at the southern foot of the Campsie Hills on the northwestern outskirts of Glasgow (Figure 3.2.1). The water runs off Dumgoyne Hill (427 m, 1404 ft) via Distillery Burn into a dam just behind the distillery. Another example of such water can be found at the Isle of Jura distillery (Figure 3.2.1), which uses water from the dammed Loch a'Bhaile Mhargaidh (Market Loch) to the west of the distillery. However, at Jura, unlike Glengoyne, some peated malt is used. In contrast, water which rises from peat and flows over peat contains much more organic matter and is thought to impart a certain smoky character to the whisky, even if no peated malt is used in its production. However, distilleries located in peat water areas almost invariably use peated malt in producing their whiskies, which are typically smoky. The reasons for this are partly economical and partly traditional. Classic examples of this style can be found in the Islay whiskies (Figures 3.2.1 and 3.2.16), which

draw their water from burns and lochans in the peaty interior of the island, water from the Solan Lochs being used by Lagavulin distillery, for example. Unusually, Laphroaig takes its water directly from the bog to the north.

The water used by many malt distilleries rises from rock and flows partly over rock and partly over peat. This is especially the case with Speyside distilleries (Figure 3.2.1). For example, Glenfarclas and Benrinnes distilleries (Dufftown) draw their water from burns issuing from Ben Rinnes (841 m, 2,759 ft), the highest mountain in Speyside.

Nowadays relatively few breweries and distilleries draw their water from artesian wells, probably because of increasing agricultural pollution and the possibility of insufficient supply for large-scale operations. Nevertheless, Daftmill distillery, a new enterprise near Cupar (Figure 3.2.1) uses artesian well water. Other distilleries that use water from wells or bore holes include Braeval, Glenlivet and Macallan (all Speyside).

Peat contains humic substances such as humic acid and fulvic acid, discussed more fully in Section 3.2.3. Humic acid is a supramolecular aggregation of lignin and tannin-like molecules with carbohydrate and peptide side chains. The hundreds of individual molecules ($<$ 1000 amu) contain many functional groups that can form chelation complexes with heavy metal ions (such as Pb^{2+}) and ions such as Ca^{2+} and Mg^{2+} that contribute toward water hardness. Thus, water that has had prolonged contact with peat is softened to a certain extent and has a lower pH. On the other hand, water that rises from and flows over calcareous rocks contains much higher concentrations of mineral salts and is of higher pH. It is called hard water, the type (permanent or temporary) and extent of hardness being governed largely by the local geology and the extent of water/rock contact (see Section 2.6.2).

The geology of the whisky producing regions of Scotland is such that the majority of distilleries use soft or slightly hard water. This is considered by some to contribute to the unique character of Scotch whisky, but at least two highly respected distilleries, Cragganmore (Speyside) and Highland Park (Orkney) use water that possesses a considerable degree of hardness.

3.2.3 Malting, Mashing, Cooking and Fermentation

The great majority of malting is now carried out in large dedicated malting complexes, some of which belong to the same company as the distilleries. For example, the Diageo Scotland Maltings supply malted barley to distilleries of the former United Distillers, bought by Guinness (now part of Diageo) in 1986. Figure 3.2.4 shows malting sites in Scotland. They are typically located in major barley producing areas: Black Isle (north of Inverness), Grampian (Aberdeen to Inverness), Tayside (around Dundee), Fife and Edinburgh and Ayrshire. The majority use germination and kilning vessels (GKVs) (malting vessels that are used to steep, germinate, dry and kiln the barley) or automated horizontal steeps and Saladin type germinating boxes, with rotating drum dryers and separate kilns. The largest maltings (Burghead) (Figure 3.2.4) produces over 80 000 tonnes of malted barley per year, but all the dedicated maltings shown in Figure 3.2.4 produce over 10 000 tonnes per annum.

At the time of writing (2009), only those distilleries indicated in Figure 3.2.4 malt their own barley: Benriach (Speyside), Bowmore (Islay), Highland Park (Orkney), Kilchoman (new – Islay), Laphroaig (Islay), Springbank (Campeltown) and Tamdhu (Speyside). All but the last named use traditional malting floors; a picture of the Kilchoman Distillery malting floor can be seen in Figure 3.2.5. It is generally agreed that a single large-scale malting complex can produce the same quantity of malt in a more efficient, cost effective way than a large number of smaller traditional floor maltings at distilleries. In the early part of the last century, most Scotch malt whisky distilleries had their own maltings on site; mostly floor maltings, but some, like Benrinnes and Tamdhu, had saladin box maltings. Others, such as Glen Grant and Speyburn (Speyside) had drum maltings. Some distilleries, such as Glengoyne (Western Highlands) converted their malting buildings

Figure 3.2.4 *Malting sites in Scotland. □ Dedicated malting plants; ○ Maltings as part of active distillery*

Figure 3.2.5 *The malting floor at Kilchoman Distillery, Islay. Photo used by courtesy of Kilchoman Distillery, Scotland*

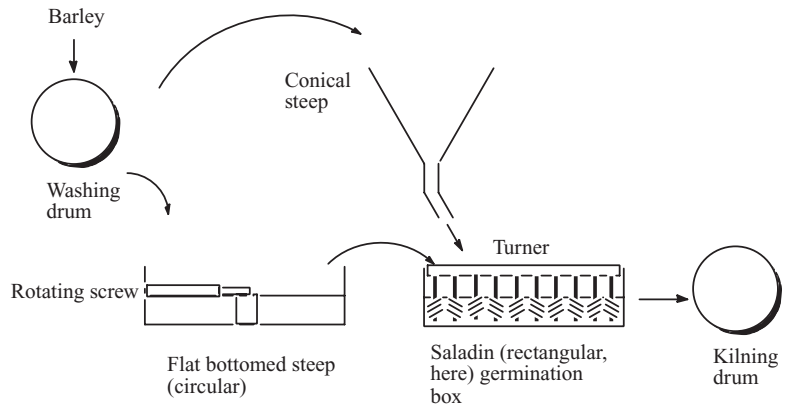


Figure 3.2.6 *Schematic diagram of possible modules in a modern box malting plant*

to perform other functions at an early date, because of lack of space. Some distilleries, such as Ledaig (Tobermory, Isle of Mull) never had a malting house, for the same reason. However, many distilleries gave up malting their own barley only in relatively recent years; these include Ardbeg, Benrinnes, Glen Grant and Glen Garioch.

The malting of barley for distilleries is a similar process to malting for breweries (Section 2.6.2), although there are two major differences. Firstly, the distiller needs only lightly kilned malts; colored and roasted malts are of interest only to the brewer. Secondly, some distillers require peat fire dried malt, to give a smoky flavor to some or all of their products. A few brewers also use some smoked malts for specialist beers (Section 2.6.13), but the vast majority of malts destined for beer production are hot air dried.

The biological and chemical changes that occur during malting of barley are described in Section 2.6.2, so the following paragraphs are limited to a discussion of modern malting plants and the role of peat.

The traditional floor malting process is outlined in Section 2.6.2. The majority of modern malting plants use highly automated processes that can give annual outputs of over 100 000 tonnes of malt, with more economical use of labor and space, and with reduced water and energy consumption. Modern plants are either of the box or tower types, with the malting processes of washing, steeping, germination and kiln drying being essentially horizontal in the former and vertical in the latter. Figure 3.2.6 depicts possible arrangements of modules in a box malting plant. One version of a tower malting plant is described in Section 2.6.2. In Figure 3.2.6, the rotating drum washer allows barley to absorb *ca.* 25% of its own weight of water (in 30 min) ready for the steeping phase. Washing removes fungal spores and although intensive, uses only 0.8 m^3/tonne of water. The barley is then transferred to a flat bottomed steep or a series of conical steeps. The former typically hold around 500 tonnes of barley, with the rotating screw supplying the steeping water, discharging the spent steeping water, giving forced aeration during the wet phase and extracting CO_2 during the dry phase. Conical steeps hold around 50 tonnes of barley each and several are arranged in parallel for dealing with large batches. Again, there is forced aeration during wet steeping and CO_2 extraction during the dry phase.

Some malting plants carry out the entire malting process in large automated containers known as germinating and kilning vessels (GKVs), thus washing/steeping, germination and kilning all occur in an accurately controlled single vessel. This, and the procedures mentioned in the previous paragraph are expensive to install, but increased malt output together with reduced water, energy and labor costs leads to greater cost effectiveness compared with traditional floor maltings.

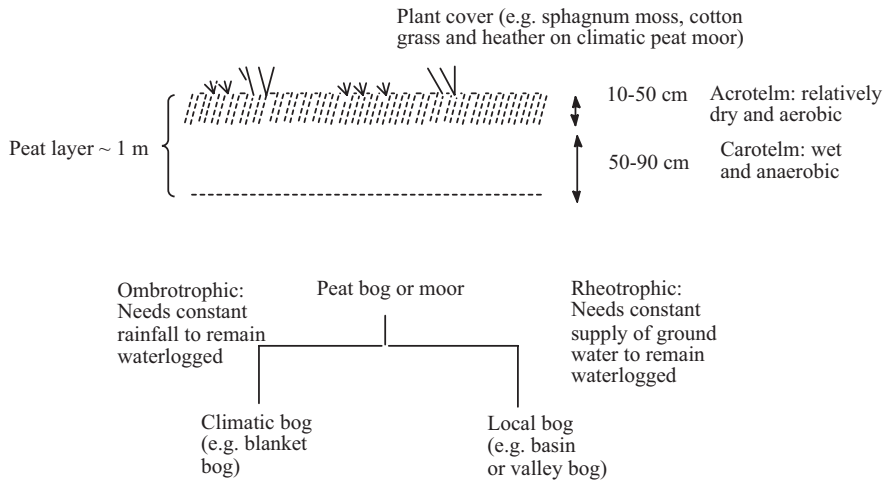


Figure 3.2.7 *Simplified anatomy of a peat moor and classification of peat bogs that supply peat for the smoking of malted barley in the production of Scotch malt whisky*

Scotch malt whiskies vary greatly in smoke character, from the highly smoky products of Islay (and the islands in general) to certain nonsmoky, cleaner malts of the Highlands (e.g. Glengoyne) and the Lowlands (e.g. Auchentoshan). Some distilleries produce malt whiskies of both types: Springbank distillery, for example, produces a smoky malt known as Longrow, besides its normal products.

Smokiness is derived from the smoke emanating from peat fires used to dry or kiln the malt after germination has proceeded far enough. For that reason, malt whiskies made using smoked malt are often known as peated malts. Up to the instalment of hot air kilns, all malted barley was dried over peat or wood fires, so that all malt whiskies prior to the late nineteenth century were smoky in character.

Peat moors and bogs cover a substantial area of Scotland, especially western and northern regions, including islands such as Islay and mainland Orkney. Peat is defined as the sediment formed by the decomposition of dead plant material under waterlogged conditions. It takes about 1000 years to form a 1 m thick layer of peat, which can be formed from almost any plant source – mosses and other bryophytes, grasses, shrubs (like heather and bilberry) and trees. Peat moors and bogs can be classified broadly as climatic bogs (such as blanket bog of the western isles) or local bogs, as outlined in Figure 3.2.7. Thus the plant species that have produced peat over two centuries vary widely. The humic substances (derived from specific plant materials by decomposition under specific geological, climatic and microbiological conditions) will differ from area to area. Their pyrolysis products (in the smoke of burning peat) will likewise vary, although there will be many common products, irrespective of the origin of the peat. Indeed, it has been possible to differentiate peats from various locations using infrared spectroscopy and analyzing the data using principal component discriminant function analysis (Harrison *et al*., 2006).

The humic substances of peat include the water insoluble humic acid and the water soluble fulvic acid; although written in the singular, they both contain a very large number of chemical compounds. They are formed by the oxidative degradation of biomacromolecules derived from plant tissues, such as carbohydrates, lignins, lipids and proteins. Humic material is a large supramolecular structure of relatively small molecules ($<$1000 Da molecular mass) held together by weak dispersive forces (hydrogen bonding, dipole–dipole forces, π–π forces, CH–π forces and van der Waals forces) (Fiorentino *et al.*, 2006; Piccolo, 2002). Humic acid is composed of large supramolecules, whose individual molecules contain phenol-like, lignin-like, quinoline

core units, with short carbohydrate, fatty acid and peptide chains. The core units are highly oxidized, the oxygen being present in carboxylic acid groups, phenolic OH functions, ether linkages and ketone (and quinone) functions (Peña-Mendez *et al.*, 2006). The fulvic acid fraction appears to be a supramolecular mixture of smaller polyphenol, carbohydrate and peptide molecules (Gonzalez *et al.*, 2003).

The burning of peat leads to the formation of pyrolysis products (thermal degradation products when there is inadequate supply of oxygen for combustion) that are typical of the humic fractions; humic acid, fulvic acid and humin. Typical pyrolysis products of humic acid include phenolic compounds, such as 2,4-dimethylphenol, guaiacol, 4-methylcatechol and 4-vinylphenol, benzoic acid derivatives, such as benzoic acid itself, syringic acid and vanillic acid, reflecting the polyphenol/lignin character humic acid acid molecules (Gonzalez *et al.*, 2003). Other pyrolysis products, such as 2-acetylfuran and pentanedione can be considered as being derived from carbohydrate units in humic acid. Typical carbohydrate pyrolysis products, such as furfuraldehyde, furanone, hydroxydihydropyranones and levoglucosenone were more prevalent in the pyrolysis of fulvic acid. Products indicating the presence of polyphenol/lignin moeities and peptide moeities in fulvic acid were also observed (González *et al.*, 2003). See Section 3.2.6 for further discussion of pyrolysis compounds in relation to the organoleptic character of Scotch malt whiskies.

The peat for use in kilning is dug in narrow strips and the briquettes are stacked in small pyramid formations indoors to dry. Increasing production of Scotch whisky over the years has led to some peat moor sites being exhausted, so that some distilleries, such as Springbank (Campbeltown) have to import peat from Islay or other areas. Like many other industries, the Scotch whisky industry pays attention to environmental issues, so there are in operation several methods that either increase the efficiency of the smoking of malted barley process and/or cut down the quantity of peat used in the smoking process.

Three distilleries on Islay have malted their own barley using somewhat different smoking techniques to this effect. In the 1980s, Laphroaig used peat smoke for drying malt over rather more than half the kilning time; the rest of the time the malt is dried with hot air (Wilson, 1985b). At around the same period, Bowmore distillery, on the other hand, used dried peat that was ground to a coarse powder and exposed to a smokeless fire used to dry the malt. The small particle size of the peat increases its smoke yield (Wilson, 1985b). In both uses, however, much of the smoke escapes through the pagoda roof and contact time with the barley will be relatively short.

The more traditional approach to smoking the malt is exemplified by the new Kilchoman distillery, which at the time of writing (uniquely) actually grows and malts its own barley, which makes up about one third of the grain bill, the remainder coming from the Port Ellen malting plant. At Kilchoman, the germinated barley, containing 30–35% moisture, is transferred from the malting floor (Figure 3.2.5) to the kiln, where it is exposed to a peat fire that was started 8 h previously. The malt lies on a perforated floor above the fire and the smoke is drawn through the bed of malt by a fan. This continues for 8 h, after which time the peat fire is extinguished and the malt is dried for a further 30 h by warm air. The malt now has 4–5% moisture content with 18–23 ppm phenolic content from the peat smoke; it is normally used for production within six weeks.

Probably the most efficient smoking of malt can be achieved using rotating drum kilns at larger malting plants, such as Port Ellen (Islay). Here contact time between malt and smoke is much longer, the drum kilns providing a relatively closed environment.

The next stage in Scotch whisky production is mashing, and for grain whiskies this includes cereal cooking. Mashing is the process whereby amylase and limit dextrinase enzymes hydrolyze most of the soluble starch molecules (which are extracted from the crushed grains by the hot water) to fermentable sugars. Other reactions occur during mashing. These, and details of other aspects of mashing are described in Section 2.6.2, so discussion here is limited to details that are more specific to Scotch whisky production.

In the case of malt whisky, crushed malt and water are the only ingredients of the mash, which in some distilleries is still carried out in cast iron vessels with perforated false bottoms and turning rakes. Some malt whisky distilleries now use water jacketed stainless steel mashing vessels (e.g. Balmenach, Speyside). In the

production of grain whisky, the mash is a slurry of milled malt (10–20%) and a cooked slurry of milled grains of wheat and/or maize. Traditional cereal cookers are large horizontal, cylindrical pressure cookers, operating with continuous batches of cereals. The malt grist supplies some of the soluble starch and all of the diastase enzymes, whereas most of the soluble starch comes from the cooked cereals. Cooking consists of heating a slurry of milled grains and water (approximately 3:8 w:w) slowly to 85° C and then at *ca*. 140 °C under pressure for about 15 minutes. This process gelatinizes the starch cells in the grain flour via disruption of the cell walls, making the starch molecules more amenable to hydrolysis by diastase enzymes. The cooking slurry is then transferred to the mash tun, where at a predetermined initial mashing temperature (see Section 2.6.2), the grist of high diastase malt is added and mashing begins.

The cooking conditions in grain whisky distilleries are essentially those designed for maize, even though wheat is now the major cereal. Under these conditions, wheat delivers less alcohol per tonne of grains than maize. It has been found that on a laboratory scale, a lower cooking temperature (85 °C, as opposed to142 °C) of wheat led to a large increase in potential ethanol yield, irrespective of whether high or low nitrogen wheat was used (Agu *et al*., 2008).

In many malt whisky distilleries, the mashing program involves firstly a residence at 65 °C, followed by draining of the wort and subjecting the grist to two further residences, with fresh water at 75 °C and 85 °C. The worts from the first two residences are mixed, cooled and fermented, whereas the wort from the last stage is used to start the next mashing programme, being too low in fermentable sugars. Other malt whisky distilleries and grain whisky distilleries mash at a single temperature (∼65 °C) for about 90 minutes and then sparge with water at ∼75°C.

Unlike in the brewing of modern beers (Section 2.6.2), the wort for whisky production is not boiled prior to fermentation and so is nonsterile. It contains diastase and other enzymes, many of which may be active well into fermentation. Also, the wort contains a range of thermophilic microflora whose activity lowers the mash pH toward the end of the mashing period and into the fermentation period. In a Japanese malt whisky distillery, it was found that the lactic acid concentration at the beginning of wort transport (to the wash back or fermentation vessel) was only half of that at the beginning of sparging (520 μg/l) (Takatani and Ikemoto, 2004). Likewise, the level of acetic acid was much higher at the start of sparging (185 μg/l, as opposed to 15 μg/l at the start of wort transport).

The presence of microorganisms in the wort, lower wort pH and the presence of starch hydrolytic enzymes (especially limit dextrinase) in the wort all have important influences on the organoleptic character of the new make spirit (Takatani and Ikemoto, 2004) and on the fermentability (ethanol yield) of the wort (Takatani and Ikemoto, 2004; Bryce *et al*., 2004). Laboratory-scale malt whisky mashes acidified with either lactic acid or acetic acid (reducing the pH from ∼5.9 to ∼5.5) showed slightly improved sugar yield and a higher amino acid content. The resulting worts fermented more rapidly than conventional worts (Takatani and Ikemoto, 2004).

Fermentability of the wort is of prime importance in the production of Scotch whisky and indeed more generally in the production of spirits from cereals. Even a slight increase in fermentability, leading to increased spirit yield, is of high economic importance to a distillery. Oligosaccharides that have α-(1→6)-glycosidic branches (see Figure 2.6.4, Section 2.6.2) are not easily metabolized by yeasts and hence are essentially nonfermentable. These sugars are known as branched α-glucans or branched (limit) dextrins and are formed during mashing by the action of α- and β-amylases on the branched starch amylopectin (Section 2.6.2). Of the diastatic enzymes, only limit dextrinase can hydrolyze α-(1→6)-glycosidic linkages and hence full activity of this enzyme is vital for maximum fermentability of the wort and consequently for maximum spirit production.

Bryce and coworkers (2004) have shown that limit dextrinase activity in laboratory-scale mashes was higher at pH 4.4 than at the normal mash pH of 5.5. Furthermore, limit dextrinase activity of wort at pH 5.5 increased as fermentation proceeded with a drop in pH from 5.5 to 4.0. A comparable fermentation of

wort buffered at pH 5.5 also showed increased limit dextrinase activity, but not to the same extent as the unbuffered fermentation. Limit dextrinase in malt is mostly protein bound and inactive. Furthermore, the purified enzyme is inhibited by two natural barley proteins at pH 5.0–7.0, but not at pH 4.5 (MacGregor *et al.*, 1994). It thus appears that reduced mash or fermentation pH activates 'free' limit dextrinase from its bound form and prevents protein derived inhibition.

Support for the above findings comes from the common practice in grain distilleries of adding 'backset' (stillage or spent wash) in place of 40% of the water used to cook the wheat during mashing. Some distilleries also add 'backset' to the wash back. The reasons for this are to reduce water consumption and energy expenditure. Stillage is basically the wash stripped of ethanol and most other volatiles. It has considerable acidity. Experiments with laboratory-scale grain mashing (wheat:barley = 9:1) and subsequent fermentation showed that addition of 40% backset generally enhanced the mash activity of limit dextrinase (Cooper *et al.*, 2004). α-Amylase activity was adversely affected in the 40% backset wash, whose pH was *ca.* 4.5. Limit dextrinase peak activity was lower during fermentations of wort with 40% added backset (pH 4.5–4.0) compared with fermentations of control wort at (pH 6.0–4.0). However, in the latter case, significant enzyme activity occurred during only about half the fermentation period, reaching maximum values when the pH had dropped to 4.0–4.5. With added backset, enzyme activity was present over the entire fermentation period.

After the mashing period (up to 1.5 h), most malt whisky distilleries transfer the wort and sparges to the wash back (fermentation vessel) by filtration through the spent husks and grains of the coarsely crushed malt. Nearly all distilleries now use a heat exchanger (cooler in Figure 3.2.3) to cool the wort and sparges to suitable fermentation temperatures. Edradour distillery still uses an open cooling vessel (a coolship – see Section 2.6.3). Grain distilleries mill their grains for cooking and mashing to powder using hammer mills. This gives maximum conversion and extraction during mashing, but requires the use of a mash filter to remove the majority of suspended solids before addition of the yeast for fermentation.

Like breweries, distilleries possess their own strains of yeast, grown from pure strains in sterile conditions. Distiller's, brewer's and baker's yeast are all used, with some distillers using several strains for the fermentation in order to balance yield of spirit against flavor. Most fermentations begin at temperatures around 20 °C, increasing to over 30 °C in some malt whisky distilleries where no temperature control is exercised.

Brewer's yeast is thought to influence the organoleptic qualities of malt whisky both directly, by producing a range of flavor active metabolic end products and indirectly, by its influence on other microorganisms, especially lactic acid bacteria (LAB). Distillates of brewer's yeast fermented malt worts have been reported to have higher concentrations of fruity esters, fatty methional and sweet (2*H*)-furanones than distillates from distiller's yeast fermentations (Wanikawa *et al.*, 2004). These were considered to be direct flavor contributions from yeast metabolism. Fermentations of malt wort with brewer's yeast were shown to give washes richer in certain esters (e.g. ethyl 9-decanoate and ethyl 2-methyl butyrate) than those fermented with distiller's yeast. The same authors (Wanikawa *et al.*, 2004) have reported that brewer's yeast fermentations, in the presence of LAB, give higher concentrations of 10-hydroxypalmitic acid and 10-hydroxystearic acid in the wash than corresponding fermentations with distiller's yeast and LAB. These hydroxy acids, derived from fatty acids such as oleic acid, are thought to be precursors of γ-lactones, such as γ-dodecalactone, that are responsible for sweet, fatty notes in malt whiskies.

Because whisky production involves the fermentation of unboiled (nonsterile) wort, microorganisms that survive the mashing regime will be transferred in the wort to the wash back and will be present during fermentation, upon which they may have considerable influence. Although thermophilic bacteria, such as *Streptococcus thermophilus* are present, the most important from the point of view of fermentation are LAB, particularly those of the genus *Lactobaccillus*. Lactic acid bacteria (discussed more fully in Chapter 2.3) are either of the homofermentative (they convert sugars to lactate) or heterofermentative type (they convert sugars to lactate, acetate and CO_2). The former can metabolize only hexose sugars, whereas the latter can metabolize both hexoses and pentoses. *Saccharomyces cerevisiae* cannot utilize pentoses, hence these sugars,

though in a minority in malt wort, provide a ready source of carbon for the growth of heterofermentative LAB in the early and middle stages of fermentation (Priest *et al.,* 2004). The principal LAB at these stages are *Lactobacillus brevis*, *Lb. fermentum* and *Lb. ferintoshensis*, but populations of these will normally be low, so *S. cerevisiae* dominates. As fermentation proceeds, the *S. cerevisiae* cells die off, pH drops, temperature increases and then drops, and populations of LAB change. Toward the end of fermentation, homofermentative LAB, particularly, *Lb. paracasei*, *Lb. acidophilus* and *Lb. delbruckii*, actually dominate. For example, after 120 h of fermentation, the population of *S. cerevisiae* are $\sim 10^4$ cells/ml (especially if a brewers' yeast, rather than distiller's yeast strain is used), whilst LAB populations may be above $\sim 10^8$ cells/ml (Wanikawa *et al.*, 2004). This means that any influence of LAB on the organoliptic character of the spirit is likely to be more pronounced when lengthy fermentation periods are used.

Priest *et al.* (2004) identified lactic acid bacteria in late fermentation samples of 23 Scotch malt whisky distilleries by partial sequencing of the CRNA gene. They were strains of *Lb. brevis*, *Lb. fermentum* and *Lb. paracasei* mostly, but strains of *Lb. pentosus* and *Lb. plantarum* were also found, along with a new species, named *Lb. ferintoshensis* (after Ferintosh, an early brand of whisky). The more detailed fingerprinting technique of random amplified polymorphic DNA fingerprinting (RAPD) showed the strains of specific species (say *Lb. paracasei*) from different distilleries to be generally different – each distillery has its own distinct bacterial flora. The same group also found that the floral spectrum isolated from samples of Glenkinchie (Figure 3.2.1) wash remained the same over 11 months of sampling. It also appears that changes in raw materials (e.g. change in malt) have little impact on the strains present in the distillery (Simpson, 2004).

Lactic acid bacteria may influence the sensory properties of scotch malt whisky in several ways, by production of specific metabolites that survive distillation and maturation, production of metabolites that are precursors for flavor active compounds in the mature spirit, alteration of the chemical processes occurring during distillation, as a result of lower wash pH and the presence of enhanced levels of acetate and lactate in the wash.

Examples of the first kind include the production of higher levels of certain esters and phenolic compounds, such as vinylguaiacol and 4-vinlyphenol (by decarboxylation of ferulic acid and coumaric acid, respectively), in new make spirit (Priest *et al.*, 2004). Likewise, enhanced levels of β-damascenone were found in the new make spirit from LAB-containing fermentations (van Beek and Priest, 2000). All the above components are also found in mature whiskies, where β-damascenone in particular is considered to be important (floral, herbal notes, sometimes tobacco-like). Also hydroxyalkanoic acids produced in the wash of fermentations in the presence of LAB are thought to be precursors of γ-lactones (sweet, fatty or waxy notes) (Wanikawa *et al.*, 2004).

In traditional Scotch whisky production, spirit yield, savings in energy and materials, and dispersal of waste have long been items of interest. Yield of spirit is related to both yeast activity and effective conversion of starch to fermentable sugars during the cooking and mashing stages. Energy savings have been achieved to a limited degree by using heat exchange systems (Section 3.2.4), but the most energetically costly processes of cereal cooking, mashing and distillation are essentially unaltered since the middle of the twentieth century. Consequently, there has been interest in developing yeast strains that are able to secrete diastase enzymes to hydrolyze grain starch and ferment the resulting sugars to produce a wash for grain whisky in a single low temperature step. This process would cut out the mashing step at least, the cooking step still being needed to produce gelatinized starch. *S. cerevisiae* does not, except for *S. cerevisiae* var. *diastaticus*, secrete diastases and the addition of exogenous enzymes is not permitted in the production of Scotch whisky.

Early attempts at achieving starch hydrolysis and fermentation in a single step using a whisky yeast *S. cerevisiae* var. *diastaticus* cross (or hybrid) had only limited success, because of the low levels of diastatic enzymes released by this hybrid (Pretorius, 1997). *Lipomyces kononenkoae* is a fungus that efficiently degrades raw starch, so four whisky yeast strains (and a commercial wine yeast strain, as control) were transformed

with integration plasmids containing the LKA1 and LKA2 α-amylase genes from *L. kononenkoae* and tested on starch containing media (La Grange-Nel *et al.*, 2004). These fermentation trials indicated that the GMOs produced up to 66% of the theoretical ethanol yield, but those based on wine yeasts (such as VIN13) may be better candidates for the development of starch degrading whisky yeasts than contemporary whisky yeasts.

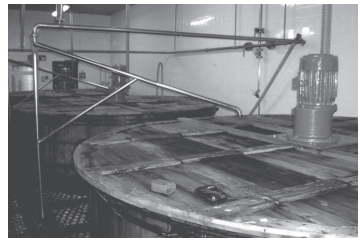
Although there are obvious economic advantages that could be achieved by the development and use of starch degrading SMO yeasts, quite aside from the general resistance toward the use of GMOs, it would need to be shown that the resulting spirit is organoleptically indistinguishable from traditional Scotch whisky before such yeasts could be accepted by the industry.

3.2.4 Distillation

The copper pot still is central to Scotch malt whisky production. It is also central to the traditional images of malt whisky distilleries and their products, along with the pagoda roof of the maltings, although this is now merely a decoration in all but a handful of Scotch malt whisky distilleries.

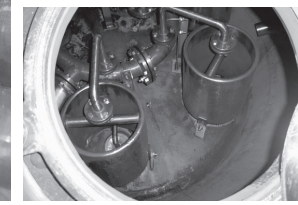
As with brewery kettles (Section 2.6.3), pot stills were originally constructed of copper because of the malleability, ductility and superior heat conduction of that metal. Unlike breweries, however, copper is still very much in evidence in Scotch malt whisky distilleries, despite some disadvantages such as erosion (pitting) and difficulties of maintenance. In most distilleries, working at less than a seven day per week production, most stills will last 10–20 years before needing replacement. The bottom half of a wash still will normally last twice as long as the top half (∼20 and 10 years respectively) and the reverse is true for a spirit still. Naturally, a more intensive production year after year would shorten the useful lifetime of both wash and spirit stills. In the nineteenth and early twentieth centuries, engineering works manufactured pot stills in a wide range of dimensions and many distilleries have taken pains in the past to add or replace stills with the same dimensions as the old. However, Scotch malt whisky distilleries nowadays have a much more uniform range of modern pot stills, although there is still plenty of diversity, especially in capacity, as can be seen in Figure 3.2.8. Small capacity stills can be seen at the Edradour, Macallan and Oban distilleries, whereas the Imperial and Glenmorangie distilleries possess some of the largest pot stills in Scotland. The simplified anatomy of a pot still is shown in Figure 3.2.9. Most Scotch malt whisky is double distilled, distillation firstly being carried out in the wash stills and then continued in the spirits stills (Figure 3.2.8), so that in many distilleries the stills are arranged in pairs. Wash stills are generally of rather greater capacity than spirits stills, those at Glengoyne distillery (16 780 l or 3729 gallons; 5000 l or 1111 gallons, respectively) being typical. Imperial distillery (Speyside) had 36 000 l wash stills, while in contrast, Macallan distillery (also Speyside) has 11 000 l wash stills and 3600 l spirit stills) (Figure 3.2.8). The height of the still (the distance of the still head from the boiler bottom) influences the nature of the spirit. A tall still allows a greater number of condensation/evaporation (reflux) cycles to occur during distillation, giving a somewhat more pure spirit, with fewer fusel alcohol and ester congeners. A short still, on the other hand, tends to produce a more heavily flavored spirit. A few distilleries once possessed Lomond stills, which had rectifying plates in the wide still neck. These could either be filled with cold water or left empty. When the plates were rotated into the horizontal position, refluxing action was maximized and a cleaner spirit resulted. The opposite situation applied when the plates were in the vertical position, whence a heavier spirit was produced. Such distilleries made varying styles of whisky, some of which are still available (see Section 3.2.6).

Some pot stills have a cooling system on the lyne arm (or lye pipe) (Figure 3.2.9), but all have cooled condensers. Most distilleries now have vertical shell and tube counter flow condensers attached to the lyne arm, where the vapors and liquids are cooled by cold water flow in the inner tubes – rather like a multiple Liebig condenser in reverse. Shell and tube condensers can be seen in Figure 3.2.8. Some distilleries employ automated temperature control of the condensers, for consistent distillate character.



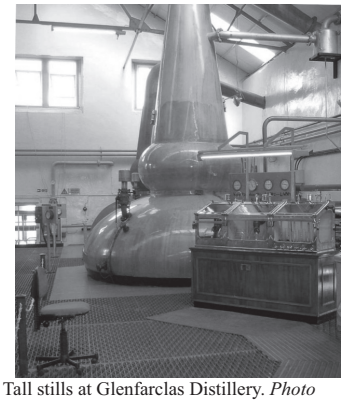
Oregon Pine washbacks at Glengoyne Distillery.

Spirit still (far side) and wash still (near) at Glengoyne Distillery.

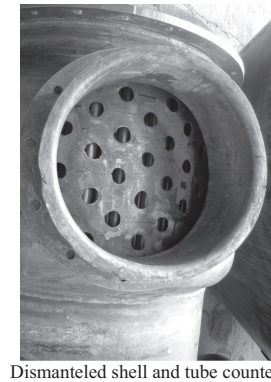


Steam heating pipes in still at Glengoyne Distillery.

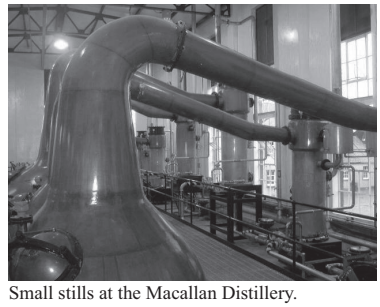
Duncan at the spirits safe at Glengoyne Distillery. *Photograph by courtesy of Glengoyne Distillery.*



Tall stills at Glenfarclas Distillery. *Photo by I.S. Hornsey*.



Dismanteled shell and tube counter flow condenser. *Photo by I.S. Hornsey*.



Small stills at the Macallan Distillery. *Photo by I.S. Hornsey.*

Figure 3.2.8 *Stills and other equipment at Scotch malt whisky distilleries. Photo courtesy of Glenoyne Distillery. Photos courtesy of Dr. I.S Hornsey*

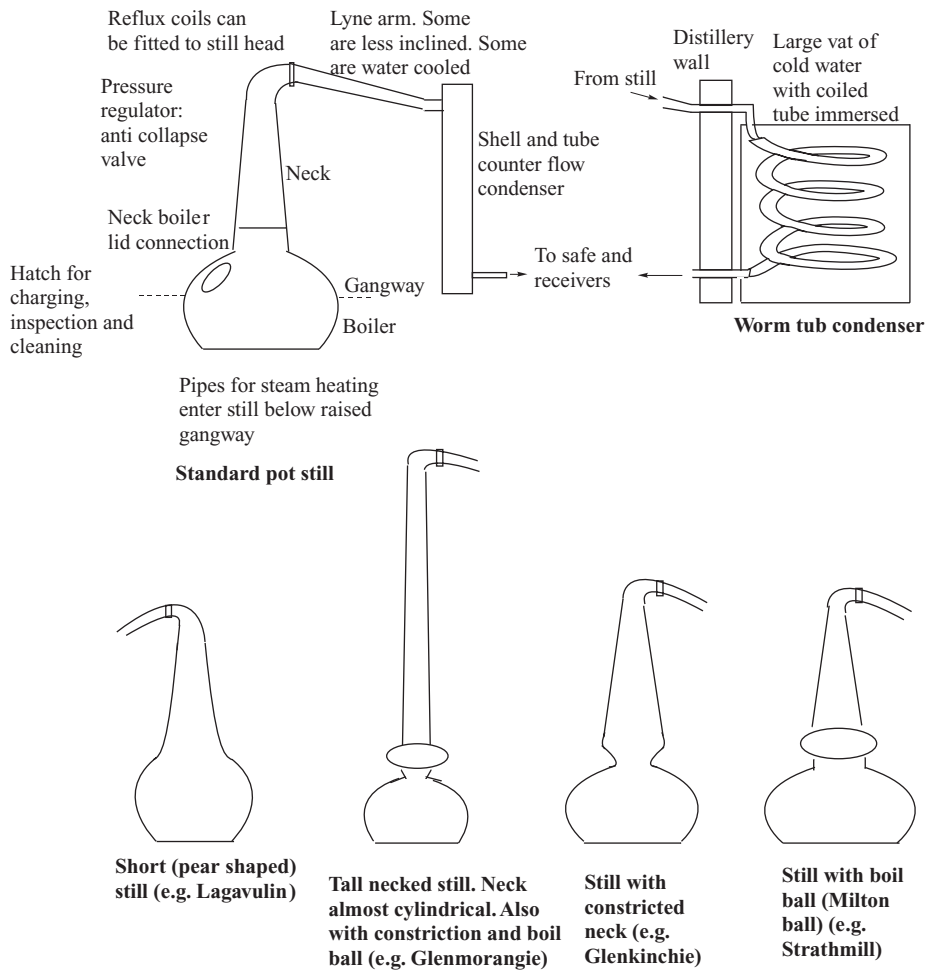


Figure 3.2.9 *Schematic diagrams of standard pot still components, and a number of spirits still variants. Stills not drawn to same scale*

Many distillers are of the opinion that the geometry of the lyne arm and the type of condenser strongly influence the nature of the spirit, and is likely that a particular distillery's stills are the result of much trial and error. In some cases, configurations are partly decided by circumstances – e.g. short lyne arms in cramped distilleries, such as Oban and Ledaig.

The original type of condenser is known as a worm tub condenser. Here the tube from the lyne arm is formed into a coil (the worm), which is immersed in a vat (the tub) of cold water, often standing against the distillery's outside wall (Figure 3.2.9). Distilleries where worm tub condensers are in operation (2009) include Balmenach (Speyside), Benrinnes (Speyside), Edradour (central Highlands), Mortlach (Speyside), Oban (western Highlands), Pulteney (northern Highlands) and Talisker (Skye) (see Figure 3.2.1). Worm tub condensers can also be seen in some rum and cachaça distilleries (Section 3.5.3).

Originally, pot stills were heated by peat or wood fires, which gave way to coal in many distilleries in the nineteenth century. Coal firing in turn gave way to heavy oil or gas firing in the twentieth century. Nowadays,

the majority of pot stills are heated indirectly with steam generated by the heating of water in external oil or gas fired boilers. The steam most likely heats the still via internal coils, such as those illustrated in Figure 3.2.8.

Because of the large amount of heat required for distillation, some distilleries operate heat exchange or recycling systems. In the 1980s, Bowmore distillery (Islay), operated a system whereby some of the hot water from the condensers (produced by condensation of the hot vapor) was led to a water/air heat exchanger, where it supplied the hot air for kilning the malt (Wilson, 1985b). Bowmore is one of the few distilleries that still has its own malting house, in this case a traditional floor maltings. Some of the hot condenser water was also used to produce steam (at reduced pressure) to heat the still and mashing water. Processes like these, although they require considerable capital investment, are claimed to reduce fuel costs by 50% with payback on investment in around three years (Wilson, 1985b).

The Glen Garioch distillery at Old Meldrum, near Aberdeen, in the 1970s employed a waste heat recovery system that provided heat for growing tomatoes and other plants in greenhouses built adjacent to the distillery. The produce was sold locally, and although the scheme was reasonably successful, it was terminated in the 1980s. Schemes such as the ones outlined above are useful in lowering the temperature of the water when it is ultimately discharged into streams and rivers.

Environmental regulations governing factory (including distillery) discharge water temperatures are likely to tighten, so that distilleries with no heat exchange schemes may need to lower the temperature of the condenser water by refrigeration, which expends more energy and creates CO_2 emissions. On a more positive note, refrigeration of the input condenser water will maximize the spirit yield and with controlled flow rate and temperature should assure distillate consistency (Halliday, 2004).

Sets of two pot stills are used in the big majority of malt whisky distilleries to give double distilled spirit, but a few, such as Auchentoshan (Lowlands) produced triple distilled spirit from sets of three pot stills. With reference to Figure 3.2.3(a), the first distillation in a double distillation process occurs in the 'wash still,' whereas the second distillation occurs in the 'spirit' or 'low wines still.'

The wash still is charged with wash (~8% ABV) and slowly brought to the boil. The vapor, containing about 25–30% ethanol (v:v), condenses to liquid in the condenser, whence this first distillate is run into the low wines safe. Here, it is monitored for strength and quality and is then run into a vessel known as the low wines and feints receiver.

This first distillate, called low wines, is first mixed with feints and foreshots from a previous running of the spirit still. Then this mixture is transferred to the spirit still and brought to the boil for the second distillation. The second distillate is monitored carefully at the spirit safe, as follows. The first spirits to arrive at the spirit safe (the first 15% or so of the run) are known as foreshots or leads; this fraction is not considered fit for consumption, so it is diverted via the spirit safe to the low wines and feints receiver to be redistilled with the next batch of low wines.

The next fraction to arrive at the spirits safe, after about 20 minutes of distillation and composing around 15% of the total distillate, is the spirit or middle cut. This fraction is collected in the spirits receiver while it has an ethanol concentration of 68–73% (v:v), as monitored by the still operator at the spirit safe.

When the ethanol content of the middle cut suddenly drops to about 60% (v:v), the next (final) fraction, known as feints, is diverted (about 40% of the total distillate) to the low wines and feints receiver to join the foreshots and low wines already there. This mixture will be redistilled later in one of the spirit stills. After this, the distillation is stopped and the residual liquid (spent lees) is allowed to cool and then discharged to waste.

The spirit still is charged with a new batch of low wines, foreshots and feints, which are distilled to give a fresh middle cut fraction to the spirits receiver. The middle cut fractions from several batches of distillations are combined in a vat before being transferred to casks to be matured in a warehouse (Section 3.2.5).

Scotch grain whisky is traditionally made by continuous distillation in a Coffey or similar still. These stills normally consist of two columns, the 'analyzer' and the 'rectifier,' although a third column can be added for the production of neutral spirits, but not for Scotch whisky. The Coffey still is described in Section 3.1.3

(Figure 3.1.6), where it can be seen that the analyzer and rectifier correspond very broadly to the wash still and spirit still of a pot still system. The continuous still is a multiple distillation system capable of producing a much purer (and higher strength) spirit than double distillation using pot stills.

The wash, after being preheated in the rectifier column, is introduced at the top of the analyzer column, while at the same time, steam enters the bottom of the column. As the wash proceeds downs the column, via the perforated plates, it is heated by the rising steam. The aqueous ethanolic vapor, called low wines, rises to the top of the column and is fed into the rectifier. The residual liquid reaching the bottom of the analyzer (spent wash or stillage) consists of water and dissolved solids mainly, and is discharged to waste.

The low wines are redistilled many times in the rectifier column, where the less volatile fractions reach the bottom and are pumped back to the analyzer for recycling. The spirit fraction is run off from the top of the rectifier through the spirit safe to the spirit receiver. It is frequently monitored for alcoholic strength, which is much higher than the ∼70% (v:v) strength of spirit from standard twin pot stills. Nevertheless, according to Council Regulation (EEC) No. 1576/89, the alcoholic strength must be less than 94.8% (v:v). New make grain whisky is diluted with pure water to ∼68% (v:v) before being transferred to cask for maturation in the warehouse (Section 3.2.5).

3.2.5 Maturation, Blending and Other Postdistillation Processes

All Scotch whisky by law must be aged for a minimum of three years in wooden casks. In practice, the benefits of long (but not too long!) cask maturation are well recognized and most Scotch whiskies spend more than five years in cask; 15–21 years are often considered optimum cask maturation times for many malt whiskies. Depending on the character of the whisky and the quality of the cask, much longer than 21 years in cask can be detrimental, leading to a wood dominated palate. Some distillers maintain that cask maturation and blending make the biggest contribution to the whisky's final character. Certainly, the importance of these two processes should not be under-rated.

In the days before 1824, when much of the malt whisky in the highlands was produced from illicit stills, large casks may not have been widely used, being too expensive and too conspicuous for an illegal product. Widespread use of casks probably started at the lowlands grain distilleries and in the highlands after 1824, when legal distillation became a more financially attractive business. Initially, a wide variety of casks would have been used – refill Burgundy, claret, Sherry and Port casks, since in those days much wine from Europe was shipped to the British Isles in cask. The improvement in quality arising from time in wood soon became apparent and Sherry, especially ex-oloroso casks gradually became popular with some malt whisky distilleries, during the twentieth century. Indeed, distilleries like the Macallan have rows of oloroso casks in Jerez bodegas (Section 2.10.2) earmarked for shipping to Scotland once their contents have been bottled. Additionally, since US law requires Bourbon whiskey to be matured in new charred oak casks (which must not be reused for ageing Bourbon) and there are many connections between Scotch whisky Bourbon whiskey distillers, there is a steady supply of ex-Bourbon casks for the maturation of Scotch whisky.

Nowadays casks used for Scotch whisky ageing are of either (first fill or refill) American oak (Bourbon casks), (first fill or refill) European oak (usually Sherry casks) or rejuvenated oak (Halliday, 2004). Casks of rejuvenated oak are made from selected staves of used casks. It is common practice to ship once used Bourbon cask staves to Scotland, where they are built into casks with larger (non-Bourbon) heads, thus increasing their capacity from 180 l to 250 l (a 'hogshead') (Delevante, 2005). Sherry butts are of 500 l capacity. It will be discussed later that whisky ages more rapidly in smaller casks. By law, Bourbon casks must be charred, just enough to singe the internal surface of the staves and cause tiny cracks to appear. Both these factors influence the character of Scotch whisky aged in ex-Bourbon casks. Many Scotch whisky distillers prefer ex-Bourbon

casks to ex-Sherry casks, because they feel the former allow more of the distillate's own character to shine through. Many malt whiskies are vatted (blended) from components out of both kinds of casks.

The changes that occur to Scotch whisky during its long sojourn in oak casks are summarized below:

- Oxidation and subsequent reactions
- Leaching of substances from the cask material
- Adsorption of substances onto the cask material
- Loss of ethanol.

The first two can be considered as contributing to mature flavor and masking of immature flavor, whilst the first and third may contribute to loss of immature or undesirable flavors.

These processes, including their rates, are dependent on a number of factors, the most important of which are listed next:

- Temperature of storage
- Humidity of storage
- Size (capacity) of the cask
- Configuration of stored casks (on their sides or on end)
- Nature of the wood – American or European oak
- Cask history – Bourbon (charred) or Sherry (etc.) (first fill or refill)
- Alcoholic strength of the new make whisky.

An important part of the ageing process is oxidation. Ingress of oxygen through the pores of the wood promotes slow oxidation of new make spirit components such as fusel alcohols, ethanol and aldehydes to carboxylic acids in some cases, which react slowly with alcohols to produce fruity esters. The flavor contribution of these esters in the mature spirit may not be high, however, depending on the other components of the whisky matrix (Steele *et al*., 2004). As ageing proceeds, wood lignins are hydrolyzed to phenolic adlehydes, which are released into the whisky. Also, oak tannins, such as those of ellagic and gallic acids, and lignins are leached from the wood during maturation. The tannins and lignins are strongly antioxidant and probably prevent excessive oxidation of alcohols and aldehydes, including phenolic aldehydes (Conner *et al*., 2004). However, some of the latter are oxidized to flavor active compounds like syringaldehyde and vanillin during ageing.

Another important factor in the whisky maturation process is the leaching of hydrolyzable tannins (Section 5.8.6), lignin hydrolysis products and pyrolysis products from the wood. The last named include the whisky lactones (*cis*- and *trans*-β-methyl-γ-octalactone), formed during the charring of Bourbon casks or the toasting of wine casks. Also included here are colored compounds, which give the finished whisky a mature appearance; a deep or dark golden color is expected in a mature whisky. As Bourbon casks are used and reused, the overall quantity of substances leached from the wood diminishes and the depth at which leaching occurs increases. In some experiments with cask staves prepared from American White oak (*Querous alba*), it was found that repeated exposure to whisky led to less leaching. New charred (unused) Bourbon staves had more extractable lignin hydrolysis products, whisky lactones and colored substances than either used Bourbon or first fill Scotch staves, and 'exhausted' Scotch staves had the least (Conner *et al*., 1992). It has been suggested that the concentrations of lignin derived guaiacyl and syringyl compounds could be used as an indicator of cask exhaustion (Conner *et al*., 1993).

Apart from aroma and flavor changes, ageing is responsible for improved 'mouthfeel' – a smooth and harmonious texture, with no rough edges. Tannins are believed to contribute to mouthfeel (as in red wines – see Section 2.9.2), but if their concentrations are too high, the whisky may have bitter, harsh overtones

(Delevante 2005). Smoothness or mellowness of palate in malt whiskies is thought to be characterized by clusters or aggregates of ethanol and water molecules (e.g. $16C_2H_5OH{:}12H_2O$ (Nose *et al*., 2004). Tanaka *et al*. (2002) showed that total polyphenol concentration increased with increasing maturation time in oak. Results of titrations at pH 7 and pH 12 suggested that extracted polyphenols were oxidized during extended maturation and these oxidation products were better able to stabilize the ethanol–water clusters. More recently, Koga *et al*. (2007) have shown that reactive oxygen scavenging activity (free radical scavenging activity, H_2O_2 reduction activity under peroxidase coculture and peroxidase-like activity) of Japanese and Scotch malt whiskies increased with increased ageing in oak. About 20% of this activity was attributed to ellagic and tannic acids (derived from tannins) and lyoniresinol (derived from lignins). Koga *et al*. (2007) proposed that the reactive oxygen scavenging activity of mature whisky components prevent excessive stimulation of taste receptor cells in the mouth and the epidermal mucous membrane in the oral cavity. This excessive stimulation by reactive oxygen species (including hypothiocyanite ($OSCN^-$) ion produced by peroxidase oxidation of saliva thiocyanate) is thought to give harsh residual sensations associated with new make or young whisky.

As the whisky impregnates the wood, via the pores and the cracks caused by charring, certain aroma active compounds are adsorbed, especially if the cask is a new or first fill Bourbon cask. This can have beneficial and detrimental values. For example many organosulfur compounds have objectionable odors (e.g. bad egg or rotten cabbage notes) at high concentrations, but at levels close to their odor threshold values (OTV) (see Section 4.7.3) they can provide pleasant background aromas. Some, like certain thiophene derivatives, have plant-like or nutty aromas; others give rise to fruity, floral or solvent notes, more typical of carboxylate esters (Steele *et al*., 2004). Methyl 2-methyl 3-furyl disulfide (MMFDS), with an OTV of 0.015 μg/l (or ppb), is responsible for meaty notes, which can be very attractive in certain full bodied whiskies (Steele *et al*., 2004). A happy medium would be adsorption of sufficient quantities of organosulfur compounds, leaving residual levels that can contribute to the complexity of flavor. Although new make malt whisky has comparatively high copper ion content, this tends to decrease during ageing, as Cu^{2+} ions are bound strongly to ligands (probably lignins and tannins) in the wood (Adam *et al*., 2002).

Most Scotch malt whiskies are casked for ageing at about 68% ABV, but some, such as those at Glengoyne distillery start their maturation period at just under 64% ABV. Scotch grain whiskies are generally casked at around 68% ABV. At these alcoholic strengths, casks have a greater porosity for ethanol than for water, meaning that evaporation of ethanol is more rapid than evaporation of water and consequently the whiskies decline in alcoholic strength as ageing progresses. This loss of ethanol is known as the 'angels' share' and it has been calculated to amount to 11 million l per annum in the 1970s (Lichine, 1982).

Bourbon whiskeys and some other spirits start their aging at alcoholic strengths close to 55% (v:v) (Delevante, 2005). These, like the fortified wines Madeira, Port and Sherry (and others) (Chapter 2.10) of 17–20% ABV actually gain in alcoholic strength as maturation proceeds; at these alcoholic contents the cask wood is more porous toward water than ethanol.

The four factors contributing to Scotch whisky ageing (described above) are influenced by many storage parameters, other than cask origin or history (also described above). European oak is less dense than American oak, so that the whisky penetrates the former more quickly, leaches lignin hydrolysis products and tannins more rapidly and is exposed to rather more oxygen during ageing. Hence maturation in European oak casks tends to be more rapid and color development is faster, especially if ex-oloroso Sherry casks are used. Ex-Bourbon single fill casks also give good color development, because of the specific charring that is required by US law (Section 3.3.4).

Ageing rates also depend on cask capacity: the smaller the cask, the more rapid the ageing process. Bourbon is aged in 180 l casks (Section 3.3.4), but the standard cask sizes for Scotch whisky maturation are ∼250 l ('hogshead') and ∼500 l ('butt'). Ex-Bourbon casks are sometimes broken up into separate staves in the USA and are then shipped to Scotland, where they are used to construct larger casks. Other cask sizes are used to

mature Scotch whisky, but are comparatively rare. Maturation in general is known to proceed more rapidly in hot climates – especially when there are distinct variations in day and night temperatures. Scotch whisky must be matured in Scotland, which has a moist, temperate oceanic climate. Under these conditions, the oxidation, condensation, esterification and other reactions, and the leaching and hydrolysis of wood materials are slow – even necessitating warehouse heating in winter. Hence malt whiskies normally have a minimum of 10 years ageing, with 15–21 years often being regarded as optimum, whereas for rum matured in hot climates, 10 years is often considered to be more than sufficient.

The optimum temperature for whisky ageing has never been established. In experiments involving the maturation of Scotch whisky in 1.2 l mini casks (to aid rapid ageing – see next paragraph) at different temperatures, it was found that the lignin extractive profile of the whisky after eight weeks of ageing did not vary significantly with temperature (5–45 °C) (Steele *et al*., 2004). Only the synapaldehyde concentrations of warm matured whisky (45 °C) were significantly higher than those of cooler matured whisky (5 °C, 35 °C).

Warehouse humidity, as well as temperature, has an influence on the way Scotch whisky matures. In traditional dunnage warehouses, with earth or cinder floors, casks stacked on their sides three high on wooden runners mature their contents reasonably evenly. The humidity in such warehouses is usually high because of the permeable nature of the floor, especially if the warehouse is close to the sea, as in some highland distilleries, or adjacent to the sea, as in most of the Islay distilleries. Figure 3.2.10 shows casks of malt whisky in the traditional warehouse at Kilchoman distillery, Islay. In a humid atmosphere, evaporation from the casks is less, so that at the end of the maturation period there is less ullage (smaller air pockets) in the casks. Many distillers believe that malt whiskies aged in humid surroundings are softer and more rounded than those stored in drier warehouses, although they may need slightly longer maturation times. Much Scotch whisky is now transported in tankers to warehouses in central southern Scotland where they are transferred to oak casks. The casks are either stacked on their sides in high steel racks 12 or so high or on their ends on pallets six or so high. Although this is more efficient in space and manpower usage, it does raise some questions regarding maturation. In such warehouses, with concrete floors and high roofs, the casks experience

Figure 3.2.10 *Casks of malt whisky in the maturation warehouse at Kilchoman distillery, Islay.* Photo courtesy of Kilchoman Distillery, Scotland

different air circulation, humidity and temperature conditions according to their positions in the stack. Those at the top will experience the warmest and least humid conditions; the opposite to those at the bottom. Casks in the centre of a stack will experience less circulation of air. Ingress of oxygen, reaction rates and evaporation rates – in other words maturation rates – will vary somewhat according to the location of casks in the stack. Furthermore, modern warehouses are generally less well insulated than traditional stone walled and slate roofed dunnage warehouses, and are less humid. Both these factors favor more rapid ageing, but many distillers and blenders (who can sense subtle differences) claim that a malt whisky aged in a modern warehouse in the ways described above will be rather harsher and less rounded than the same whisky aged in a traditional warehouse, despite the practice of cask rotation in the former.

The production of just about all Scotch whisky, like most other spirits, involves blending as an important step. In most cases, even a single malt whisky will be a blend of malt spirits from the same distillery – a vatting of several casks of different distillation batches. These contributors can come from different seasons, but the label on the bottle must specify the age of the youngest spirit in the blend. Thus Springbank 10 year old indicates that the youngest whisky in the bottle has had 10 years in oak casks; this will be the major component, but there could well be 11 or 12 year old (or even older) components present.

Some Scotch malt whiskies are made up entirely of components distilled in the same season; this fact is sometimes indicated on the bottle label (e.g. Bowmore, distilled in 1966). At the other extreme, some distilleries offer limited quantities of malt whiskies bottled from single casks (i.e. unblended). These are known as 'single single malts,' and they too display a distillation date on the label – Glengoyne distillery produces an unusually wide range of these specialized items, which are named 'single cask.'

A blend of malt whiskies from several distilleries is known as a 'vatted malt.' These are not so common, but the independent bottlers (see Section 3.2.6) produce pleasant examples that are meant to typify the region of origin of their components. For this reason, they are often given names like 'Pride of Orkney.' Others have less descriptive names, such as Glenleven and Strathconon, but whatever the name, a vatted malt will be indicated on the label as (Highland) malt Scotch whisky, the word 'single' will be missing.

Scotch whisky per se (without words 'single' or 'malt' on the label) is a blend of grain whiskies and malt whiskies. Usually it will be a complex blend of spirits of varying ages, but as ever, the age of the youngest component must be quoted on the bottle label. Cheaper Scotch whiskies will include younger spirits (e.g. five year old) and a greater proportion (up to ~60%) of grain whiskies. More expensive versions (usually called 'de luxe' blends or given special names like 'Blue Label') are composed of more aged spirits (e.g. 12–27 year old) and have a higher proportion of malt whiskies (sometimes exceeding 70%).

In all the above examples, except single cask malt whiskies, success depends upon the skill of the blender. Indeed, the enormous growth in the export of Scotch whisky during the last century was as much a result of skilled blending as skilled marketing. The blenders are much prized individuals in distilleries and each company has its own closely guarded styles and methods of blending. The blenders' main job is to ensure high quality and uniformity (consistency) of the distillery's regular products. In a malt whisky distillery, he or she will advise on which casks are to be vatted for producing say, the 15 year old version, from year to year. For Scotch whiskies, the blender will decide on the various proportions of the various casks of malt whiskies that will be blended with the grain whiskies. In either case the blenders base their decisions on fine sensory evaluation skills and experience of what the company and its customers expect of the products.

Sensory evaluation of Scotch whisky is carried out via aroma only – by sniffing or 'nosing' the sample, usually after it has been diluted by pure water to 20% ABV. The dilution reduces the pungency due to the presence of ethanol and also helps release aroma compounds. Medium and long chain esters have more limited solubility at 20% ABV, so that volatile esters originally held in agglomerates are released into solution and/or into the headspace (Conner *et al.*, 1994). Evaluation can be performed by a single person, the head blender, or more likely these days, by a team of trained panellists, in which case standard procedures for sensory evaluation are used (Jack, 2003). In these procedures, special attention is paid to general preparation

Figure 3.2.11 *Bill McDowall nosing a sample at Glengoyne Distillery.* Photograph by courtesy of Glengoyne Distillery

and presentation of the sample, control of temperature and sample sharing, as well as experimental design, training of panelists and sensory room design. The panelists give scores for predetermined aroma descriptors and the results are collected and processed using sensory evaluation software, often being presented as spider diagrams (Section 4.7.4).

Nosing is carried out in the distiller sample room at around 21 °C, with the sample held in a tulip glass no more than 25% full, and equilibrated to room temperature (Figure 3.2.11). The chief blender will nose the whisky at different stages: as new make spirit, as aged spirit, prior to vatting and just prior to bottling. New make and many other spirit samples, as well as aroma evaluation standards, are stored in the sample room for comparison and reference purposes.

In most recent years, there have been attempts to correlate sensory and analytical data, the objective being to use analytical data to predict and control aroma/flavor character, thus enabling greater consistency. This is especially important, as new make malt spirits destined to be components of blended Scotch whiskies are often sold prior to maturation, with sensory character being the major quality criterion. Experiments focusing on the 'sulfury' character of a large number of examples of new make malt spirits from several distilleries showed a good correlation between predicted sulfury character (from GC analytical data) and sensory panel scores for sulfury character (Jack and Fotheringham, 2004). Partial least squares regression (PLS) analysis was used to compare the levels of all 36 identified organosulfur components ('routine sulfur data') and sensory scores, but the better correlation was obtained by including the levels of 20 unknown sulfur compounds in the analysis.

Most Scotch whisky is diluted, color standardized (by addition of caramel) and chill filtered prior to bottling. The first two processes are used for the sake of consistency and customer expectation, whereas the third is used to prevent the formation of chill haze and is traditionally carried out after the first two. New

make malt spirit enters the cask at *ca*. 68% ABV. After ageing 12 years, this will have dropped to around 59%, depending on the cask and conditions of maturation; the ethanol contents of spirits of the same age from two different casks are never exactly the same. Hence vatting and addition of pure water (which must conform to potable water standards) is carried out for most whiskies to a final ethanol content of 40%, 43% and 46% (v:v).

Likewise, the color development of ageing whisky proceeds at different rates in different casks, so vatting of the matured spirits with addition of caramel (as well as dilution) ensures consistency of color. Some distilleries bottle some of their malt whiskies without vatting, dilution or addition of caramel (Section 3.2.6). Filtration of Scotch whisky is permitted by regulation only to remove physical impurities and components that may lead to the formation chill haze (chill floc) (Halliday, 2004). Most Scotch whisky is chill filtered after vatting, dilution and coloring, prior to bottling. Some malt whiskies are bottled unfiltered, in which case they are likely to develop a haze at low temperatures (Section 3.2.6).

The processes of dilution, coloration and filtration outlined above are carried out in various ways, depending on the distillery. In some, they are largely manually controlled, in others, especially larger distilleries, there is considerable or full automation. Consistency of alcoholic strength of the final product, after blending and dilution, is important for both payment of excise duty and expected organoleptic character. The ethanol content of whisky is monitored at all stages in its production, by a variety of in line and off line techniques, including gas chromatography (Section 4.3.2), ultrasound sensors (Section 4.6.3), hydrometry (Section 4.6.3) and ebulliometry (Section 4.6.3).

Packaging of whisky is still very much biased toward glass bottles. The traditional bottle is ~70 cl capacity, made of colorless glass, cylindrical with a bulge in the neck (Figure 3.2.12) and often enclosed by a metal screw cap. Some of the lower capacity bottles are of the squat, flat type. Other sizes (e.g. 1 l, 50 cl, 350 ml down to 5 cl for miniatures) are available and many malt whiskies can be found in bottles enclosed with a flanged cork stopper and finished with an attractive foil cap (Figure 3.2.12). A few whiskies, such as Glenfiddich malt whisky are offered in green glass bottles and some whiskies are offered in bottles with plastic screw caps. Bottling lines at central warehouses or bigger distilleries are often fully automated, coping with up to

Figure 3.2.12 *Standard range of single malt whiskies from Glengoyne distillery. Many malt whisky distilleries produce whiskies of different ages, as well as speciality products - see Section 3.2.6.* Photograph by courtesy of Glengoyne Distillery

600 bottles per minute, including depalletization of empty bottles, loading onto conveyors, filling, enclosing, labeling, capping and palletization of filled bottles. Sophisticated computer control on some bottling lines allows fast bottle format and size change with parallel label and capper change.

Scotch whisky is seen by many consumers as a more traditional drink than, say, gin or vodka, so the glass bottle continues in use for the great majority of whiskies. Although the traditionally minded consumer will always be important, there is a growing need for greater consumer accessibility for Scotch whisky. Greater breadth of format (e.g. premix in kegs, tetrapaks and sachets) should help the industry to target nontraditional consumers, as has already happened in other spirit industries (Halliday, 2004).

Waste in the Scotch whisky production process, although not entirely a postdistillation phenomenon, is discussed as the last topic of this section.

Figures 3.2.3(a) and 3.2.3(b) indicate the points in the production process where waste occurs. The two major wastes are spent grains from the mash and spent wash from the distillation (also the pot ale from the wash still in malt whisky distilleries). In the USA, traditionally, the spent grains (draff) are dried and sold as distillers dried grains (DDG) and distillers dried grains with soluble (DDG-S) to farmers for use as livestock feed. As they are low in starch, but high in protein and fiber, DDG and DDG-S are valuable in this respect. Recently, there has been interest in developing alternative uses for DDG and DDG-S derived from maize (corn) (Saunders *et al.*, 2008). A possibility is their use in the diets of people suffering from diabetes or Coeliac's disease. Processes making flour from these grains and the possibilities of modifying their color, odor and baking functionality are being investigated. In the USA corn ethanol plants now produce over 3.5×10^9 l of ethanol via fermentation/distillation, so alternative uses for waste are worthy of investigation. In Scotland, however, the majority of spirits are distilled from malted barley or wheat wash rather than maize wash and so spent grains would not be suitable for Coeliac's disease sufferers, because of the presence of allergenic proteins (prolamins) (Section 5.11.3).

Spent lees, and in malt whisky distilleries pot ales, are wastes that can only be discharged to the environment, although some is used as 'backset' (Section 3.2.3) in mashing/cereal cooking and fermentation in grain whisky distilleries. However, spent lees from malt whisky distilleries contain high levels of copper compounds (including soluble organic copper complexes) - up to 20 μg/l - and pot ales contain up to 0.7 μg/l (Adam *et al*., 2002). Although a little of this copper (∼3%) comes from the barley, the majority is derived from the copper pot stills, as indicated by the generally very much higher copper content of malt whiskies over grain whiskies (Adam *et al*., 2002). It is estimated that during distillation, about 1 kg of copper is released daily to waste with the pot ale and spent lees from a distillery such as Talisker (Adam *et al*., 2002). This can be taken as a representative value and illustrates why distilleries ensure adequate dilution of pot ale and spent lees is carried out before the waste is discharged into the environment.

Also a problem is the discharge of hot water from the condenser coils (cooling waste water). Some continuously working distilleries have or have had heat exchanger or recycling schemes, whereby hot cooling water is used for mashing or for heating the next batch for distillation or (as at Bowmore distillery, Islay) is used in a water/air exchanger, the hot air being used to dry malt in the kiln. Thus recycling saves fuel, although optimum benefits are only felt during continuous distillery operations. A less busy distillery, as Imperial (Speyside), used a canal system for cooling the water prior to discharge into a local burn.

3.2.6 The Flavor and Styles of Scotch Malt Whiskies

Some aspects of Scotch whisky flavor have been discussed briefly in Sections 3.2.3 and 3.2.5, with regard to the contributions arising from peated malt and maturation in oak, respectively. The purpose of this subsection is to examine more closely certain aspects of flavor, particularly those relating to molecular origin and influences of distillery practices or conditions.

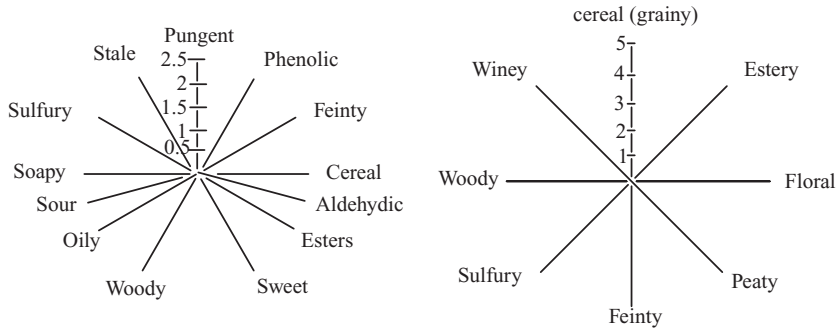


Figure 3.2.13 *Scaled flavor profile templates used to describe Scotch malt whisky. The arbitrary scale applies to all spokes of the wheel. The panel gives a mean score for each flavor descriptor, then the scores are joined by lines to give a spider web pattern describing the flavor profile*

There are several flavor vocabularies that have been proposed for the description of whisky flavor (Simpson *et al*., 2004). The terms in these vocabularies range from 13 to 43, and although flavor descriptors used by the Scotch whisky industry have not been universally agreed, there is a general consensus of opinion favoring the use of 13 terms to describe various whiskies, such as malt, blended or new make. Flavor profiles for Scotch whiskies can be built using these terms in flavor wheel or spider web diagrams (Jack, 2003) (Figure 3.2.13) – these are discussed more fully in Section 4.7.3. Thus, the basic flavor descriptors commonly used for Scotch malt whiskies are aldehydric (sometimes grassy or green is used here), cereal, estery (fruity can be used here), feinty, oily, phenolic, pungent, soapy, sour, stale, sulfury, sweet and woody.

In reality, there are over 250 known flavor compounds in Scotch whisky, which may contribute to one or other of the flavor attributes mentioned above. The vast majority of these flavor components are common to many distilled beverages and it is their relative concentrations that allow differentiation of a malt whisky from, say, a dark rum. Nevertheless, there are some components that are probably unique to Scotch whisky or are more strongly associated with that product. These include some phenolic compounds, characteristic of peated malts, whisky lactone, common to all whiskies, and certain organosulfur compounds, with various flavor impacts.

Many of the flavor descriptors mentioned above can be subdivided into related terms: phenolic, for example, can be described as burnt, medicinal or smoky. Likewise, the term estery, can be given the qualifying descriptors fruity or solvent – particular fruit may even be invoked, such as apples or bananas. Olfactometric-GC ('sniff GC' or GC-O) analysis usually leads to a wider range of descriptors, now relating to individual components as they are eluted from the gas chromatograph (Section 4.7.3). Terms such as mushroom, spicy or curry can be used to describe certain individual elements (Steele *et al*., 2004). Thus individual odorous components, often with quite specific aroma descriptors, mingle in the bulk sample of Scotch whisky and combine to give a more general aroma/flavor profile that is characteristic of Scotch whisky, despite the fact not one of the individual component's odors can be described as whisky.

The contribution each component makes to the overall flavor depends on its odor activity, which in turn depends on its odor threshold value (OTV) and concentration, or more specifically its activity coefficient. The latter parameter for a specific component depends on the identity and quantity of cosolutes, which influence aroma intensity by affecting the headspace spirit partition coefficient (Conner *et al*., 1999) for that component (matrix interactions). For example, the leeching of phenolic compounds from oak during maturation was found to reduce the headspace spirit partition coefficient of long chain ethyl esters (such as ethyl decanoate), which are generally believed to confer soapy or waxy, immature notes to Scotch whisky. This result was

used to explain the decreased impact of immature aromas in matured whiskies and was linked to the greater formation of ethanol–water aggregates in oak-aged spirits (Conner *et al*., 1999) (Section 3.2.5). Thus some components are not flavor active, but contribute nonetheless to the overall odor background or by affecting the spirit headspace partition of flavor active compound.

As with other alcoholic beverages, it is possible to relate flavor descriptors for, say, malt whisky, to the presence of certain types of aroma compounds. Thus the terms grassy, green or aldehydic can be attributed to aldehydes, estery to esters, phenolic to phenols, sulfury to organosulfur compounds and so on. Although this is useful, it is by no means universally correct, because the aromas of a set of structurally diverse molecules can be described by the same flavor descriptor (or similar flavor descriptors). Thus estery aromas can also arise from certain organosulfur compounds, some phenols have aromas best described as spicy, rather than phenolic, and some esters do not have typical estery aromas (Steele *et al*., 2004).

Many esters have been found in Scotch whisky, as in other spirits (Demyttenaere *et al.*, 2003; Câmara *et al*., 2007; Campo *et al*., 2007). These are formed during fermentation (e.g. see Sections 2.2.11 and 2.6.4) and are subsequently concentrated by distillation. Some esters may be formed in slow reactions between ethanol and carboxylic acids, during maturation. Short chain esters such as ethyl acetate, 2-methylpropyl acetate, ethyl 3-methylbutanoate, *iso*-amyl acetate and medium chain ethyl esters (such as ethyl hexanoate) possess aromas that can be described as estery, fruity or solvent-like. Longer chain esters, such as ethyl hexadecanoate have aromas more reminiscent of soap or wax and some mixed aliphatic/aromatic esters, such as 2-phenylethyl acetate, have floral odors. Although many of the short and medium chain esters have low OTVs, their presence may not contribute greatly to the overall whisky aroma. This could be because of low concentrations in the spirit (and hence in the headspace) or possibly because of low headspace levels caused by the presence of long chain esters trapping them in the liquid phase (an example of matrix effects, described previously). Certainly, there appears to be no correlation between concentrations of esters in the liquid and in the headspace; increased ester levels in a whisky will not necessarily be perceived as increase in estery or fruity character (Steele *et al*., 2004). At least two studies have shown that long chain fatty acid ethyl esters, such as decanoate, dodecanoate, tetradecanoate and hexadecanoate, are amongst the most abundant esters in both the liquid and headspace of Scotch whisky, judging by relative peak areas in GC chromatograms (Demyttenaere *et al*., 2003; Câmara *et al.*, 2007).

A recent multidimensional GC/MS study has focused on the presence of esters with particularly low OTVs in alcoholic beverages, including wine, Port, Sherry, brandy and two Speyside malt whiskies - Cardhu and Knockando. The esters, ethyl cyclohexanoate, ethyl 2-methylpentanoate, ethyl 3-methylpentanoate and ethyl 4-methylpentanoate, were found in many of the beverages, but their odor activity values (OAVs) (= concentrations/OTVs) were generally higher in fortified wines and spirits, including the two malt whiskies (Campo *et al*., 2007). It was concluded that these powerfully odorous esters made a major contribution to the fruity notes of the beverages, especially certain fortified wines, brandies and the two malt whiskies. Similarly, in an analysis of volatile components in blended Scotch whiskies, on the basis of OAVs (Section 4.7.2), ethyl decanaoate, ethyl dodecanoate, ethyl hexanoate, ethyl octanoate, isoamyl acetate, 3-methylbutan-1-ol, 2-methylpropanol, decanoic acid, hexanoic acid and octanoic acid were considered to contribute most to aroma (Caldeira *et al.*, 2007). The most odor active component was ethyl octanoate (fruity, sweet), followed by isoamyl acetate (banana, fruity), 2-methylpropan-1ol (winey, solvent), ethyl decanoate (floral, soap) and decanoic acid (fatty).

Phenolic compounds make important contributions to Scotch whisky flavor, either directly, as in the case of volatile phenols, or indirectly, as in the case of nonvolatile phenols. Volatile phenols are derived from:

- Barley (via hydrolysis of lignins during mashing (fermentation)
- Peat smoke (via pyrolysis of lignins and polyphenols)
- Oak (via hydrolysis of wood lignins).

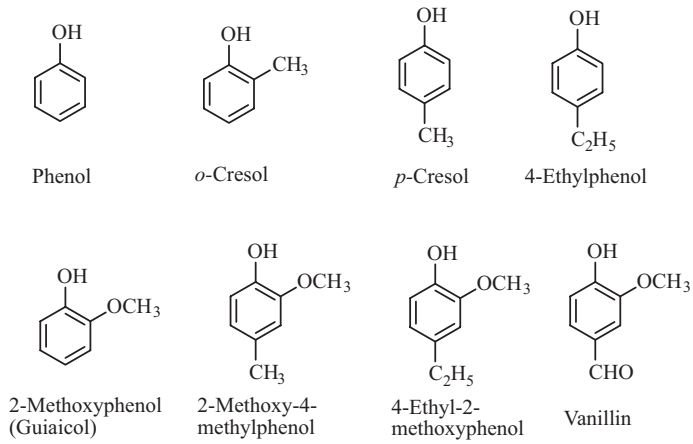


Figure 3.2.14 *Some volatile phenols found in Scotch malt whisky*

Several, mainly simple substituted phenols, have been found in malt whiskies (Steele *et al*., 2004; Câmara *et al*., 2007). These include the compounds shown in Figure 3.2.14. Phenolic notes (burnt, medicinal, smoky) are most noticeable in peated malt whiskies, where some or all of the malted barley has been kilned using the hot air derived from burning peat. The smoke, containing pyrolysis products of lignins and polyphenols (Section 3.2.3) permeates the malt, and the compounds deposited there tend to survive the whisky production process into the mature whisky. Most of the volatile phenols of peated malt whiskies have been found as pyrolysis products of the humic acid and fulvic acid fractions of peat (González *et al*., 2003) (Section 3.2.3).

The more highly peated malt whiskies, such as those of Islay and the islands, show flavor characteristics that can be related to the concentration of phenols. However, GC-olfactometry shows that relatively few of the phenols are present at high enough concentrations to provide a distinctive aroma (Steele *et al*., 2004). Many are present at levels below their OTVs and will not themselves be of sensory significance. Even so, these may synergistically contribute to the overall phenolic notes of a malt whisky, and this, combined with the differing levels of flavor active phenols, may account for the differences in peaty/phenolic aroma perceived in malt whiskies such as Highland Park, Lagavulin, Laphroaig, Ledaig, Longrow and Talisker.

Matured nonpeated malt whiskies and new make spirit from nonpeated malt also possess a portfolio of phenolic compounds, although generally their direct influence on flavor is much less profound than in the case of peated malt whiskies. This suggests that some volatile phenols are derived from the cask and raw materials (barley) in all malt whiskies, with peated spirits also having phenols derived from the peat smoke.

As with all spirits and wines aged in new or almost new oak casks, phenols such as 4-ethylphenol, guaiacol, 4-ethylguaiacol and 4-methylguaiacol, hydrolysis or pyrolysis products of wood lignins, are leeched into the malt whisky during maturation. This is true whether the malt whisky is aged in charred ex-Bourbon casks or in toasted ex-Sherry casks, although different levels of essentially the same phenols lead to differences in flavor profiles (Steele *et al*., 2004). Malt whiskies aged in ex-Bourbon casks tend to have more pronounced floral and sweet toffee notes, whilst those aged in ex-Sherry casks tend to possess nutty, burnt sweet and vinegar notes.

Low concentrations of phenols have been found in new make spirits. For example, vanillin (Figure 3.2.14) was present in 53 out of 59 new make malt spirits at levels ranging from 20–170 μg/l (Steele *et al*., 2004). The most likely origin of vanillin in new make spirit is via hydrolysis of barley endosperm lignins during

mashing. Although levels are low, it is possible that these phenols make some contribution toward mature character during aging.

Apart from the direct contributions to flavor profile outlined above, the presence of phenols in general in malt whisky is thought to influence the partition coefficients of volatile constituents, thus affecting the flavor profile in an indirect way. For example the volatilities of ethyl esters of long chain fatty acids (responsible for soapy immature notes) are subdued by the presence of phenolic compounds, such as those derived from oak aging (Conner *et al*., 1999). Also, as discussed previously, other contributions from phenols toward mature character, may include stabilization of ethanol–water clusters and reactive oxygen scavenging ability (Koga *et al*., 2007) (see Section 3.2.5).

Organosulfur compounds are present in malt whiskies and many are considered to be important flavor components. They are generally present at low levels and at such levels their flavor contributions are positive, having a range of descriptors (using GC-O analysis) from burnt match, through vegetal, nutty, floral, fruity to meaty (Figure 3.1.15) (Steele *et al*., 2004). At higher concentrations, however, their flavor impact may be less beneficial, although this is uncommon in copper pot still spirits in general (see Section 3.5.3) because of some decomposition caused by reaction with Cu at distillation temperatures.

The origins of organosulfur compounds can be traced back to fermentation (Sections 2.2.11 and 2.6.4). Methional (3-thiomethyl propanal) is produced from the α-amino acid methionine (Section 2.10.6) by yeast metabolism during fermentation. Methional concentrations in cereal wash have been shown to correlate with dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) levels in spirit from Coffey stills (Prentice *et al*., 1998). Thus DMDS and DMTS appear to be distillation products of methional and hence in the production of grain whisky, the methional concentration of the wash needs to be regulated, so that extraneous DMDS and DMTS concentrations are not formed. Fermentation of the wort with low sulfur compound producing yeasts is beneficial here, and also particular operation of the Coffey stills can modulate final DMTS levels. Such considerations are not usually necessary in the production of malt whisky, because of the role played by the copper still material in preventing high levels of organosulfur compounds from entering the distilled spirit. In grain whisky production, it may be possible to incorporate a portion of copper into the otherwise stainless steel Coffey stills, as has been suggested for cachaça (Faria *et al.*, 2004).

The ‘aldehydic’ flavor descriptor (Figure 3.2.13) can be given the alternative name of ‘green’ and can be subdivided into secondary descriptors ‘leafy’ and ‘floral.’ Green notes can be important in unpeated lowland and highland malt whiskies, such as Auchentoshan and Glengoyne, respectively. Multidimensional GC-MS-olfactometry of Japanese malt whiskies has demonstrated two aldehydes and three alcohols (mostly unsaturated) as contributing significantly to the green notes of those whiskies (Figure 3.2.15) (Wanikawa *et al*., 2002). Malt whisky samples with high green note organoleptic scores contained higher levels of some of these compounds than whiskies with low scores. These compounds, (2*E*),(6*Z*)-nonadienal, (2*E*)-nonenal, 1-octen-3-ol, 4-hepten-1-ol and nonan-2-ol are thought to be formed during fermentation by the action of yeast lipoxygenases on barley lipids. At the time of writing (2009), the configurations of the double bond in 4-hepten-1-ol and of the chiral centres in 1-octen-3-ol and 2-nonanol are not known, nor is it known the identities of several other compounds that gave a green note in olfactometric analysis.

The great wines of the world are generally classified according to their region of origin. So it is with Scotch single malt whiskies. The regions were originally devised for the regulation of licenses and excise duties, but in general they do each give certain broad characteristics of flavor and style to their whiskies. Naturally, within each region, there are variations in style, just as in, say, Haut Medoc, the wines of St Estéphe differ in detail from those of Margaux, even though they have a family resemblance that sets them apart from red wines of a different region, say the Northern Rhône.

The Scotch single malt whisky regions are generally reckoned to be the Lowlands, Campbeltown, the Highlands (which includes Speyside) and Islay, as summarized in Figure 3.2.16.

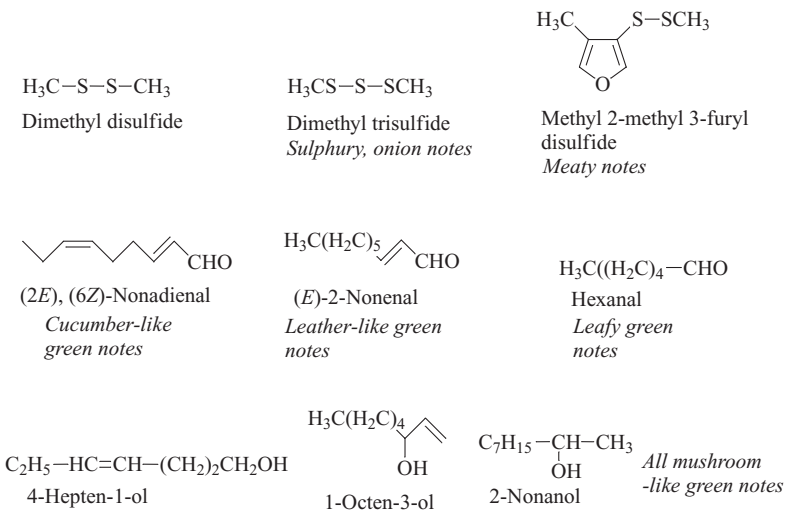


Figure 3.2.15 *Organosulfur compounds of wide sensory character and some compounds responsible for 'green' or 'aldehydic' notes in malt whiskies*

Campbeltown is sometimes considered as a district of the Lowlands region, but its whiskies are so distinctive as to deserve their own Appellation. In Figure 3.2.16, it can be seen that the very diverse Highland region possesses the big majority of working distilleries, with Speyside being the most important (in terms of output of single malt whiskies) of all districts.

The demarcation line between the Lowland and Highland regions follows the old county boundaries between the Clyde and Tay estuaries. Except for Speyside, both regions are geographically very diverse, matching the diversity of their single malt styles, particularly with respect to the Highlands. Rather less variation is observed for the single malts of the geographically more restricted regions of Campbeltown and Islay, along with the Speyside district of the Highland region. The next paragraphs discuss the general characteristics of style and flavor of the single malt whiskies from the four regions in terms of methods and practices of production. Where appropriate, characteristics of the products of individual distilleries are discussed in terms of specific aspects of their own production.

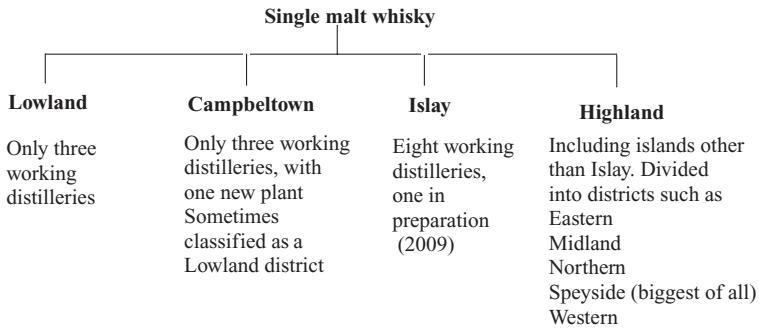


Figure 3.2.16 *Scotch single malt whiskies classified according to region*

The Lowlands region is geographically large, stretching from a line joining the Clyde and Tay estuaries to the border with England. Unfortunately, the number of Lowland malt distilleries presently stands at just four: Ailsa Bay, Auchentoshan, Bladnoch and Glenkinchie. These are well separated, in the west, south and east of the region, respectively, but all produce light bodied, mellow (even subtle) malt whiskies with a fresh grassy or citrus character. Lightness of body and freshness arise from the use of unpeated malt or lightly peated malt and (sometimes) triple distillation, the traditional mode of pot still distillation in the Lowlands. Triple distillation produces a new make spirit of around 76% ABV (as opposed to ~68% ABV for double distillation), with rather lower concentrations of flavor compounds (congeners), especially if a narrow 'middle cut' is taken from the final still. The use of refill ex-Bourbon casks rather than ex-Sherry casks for aging ensures that the whisky does not take up too much vanilla or Sherry character, allowing the malt character to shine through.

The Lowlands region has witnessed the demise of several malt distilleries since 1983: Interleven, Kinclaith, Littlemill, Rosebank and Saint Magdalene. Just before that time, in the 1970s, the region lost Ladyburn distillery, adjacent to the Girvan grain distillery (Figure 3.2.1). The owners of the Girvan grain distillery (W. Grant of Glenfiddich fame) built Ailsa Bay malt distillery, on the site of the Girvan complex. It started production in September 2007 and it is expected that the first malts will be available for blending in 2012.

Campbeltown, rather isolated at the southern end of the Mull of Kintyre peninsular, once had 30 distilleries. Nowadays (2009) there are only three, with the youngest (Glengyle) being owned by an existing local distillery company that owns Springbank distillery (J. and A. Mitchell). Glen Scotia is the other working Campbeltown distillery and its eight year old product, along with partially triple distilled Springbank 10 year old can be regarded as the epitome of Campbeltown single malts. The latter whisky is produced using one wash still and two low wines (spirits) stills, but the second low wines still receives charges (~20–25% ABV) directly from the wash still, as well as the feints (heads and tails ~30–35% ABV) in about a 1:4 ratio, the middle cut from the final still being collected at *ca.* 71% ABV. These whiskies have the lightness of body typical of Lowland malt whiskies, but at the same time a fresh, briny character is evident on the nose and palate that is rarely found so clearly in other whiskies. These characteristics, along with a remarkable smoothness and depth of flavor for such young malt whiskies is considered to be the result of aging in warehouses that are close to the sea, so that a damp, salty atmosphere prevails.

Although the eight and 10 year old products mentioned above have subdued or no discernable smoky character (due to the use of lightly peated or unpeated malt), Springbank distillery also produces a smoky style of malt whisky. This is known as Longrow, and as it is double distilled using entirely heavily peated malt, it has a pronounced smoky/phenolic character on the nose and palate, although the characteristic saltiness is still there in the aftertaste. Springbank distillery has its own traditional floor malting house, but the peat for smoking the malt comes mostly from Islay. The same distillery also produces (since 1999) a triple distilled single malt called Hazelburn, after a now defunct nearby distillery. Also, the distillery company has renovated the Glengyle distillery next door to Springbank to produce a malt whisky known as Kilkerran. The first distillation was in 2004, with the limited release of a five year-old spirit in 2009, with subsequent limited releases until the 12 year old is ready for bottling in 2016.

The Arran distillery at Lochranza on the Isle of Arran started producing single malt whiskies in 1995. In style, its products are somewhere between a typical Campbeltown malt, such as Glen Scotia, and Western Highland malt, such as Oban.

The Highland region possesses the huge majority of distilleries (Figure 3.2.1), with the greatest diversity of styles and flavors in its single malt whiskies. There are only four distilleries in the western districts – Ben Nevis in Fort William, Oban on the coast, Glengoyne, northwest of Glasgow, and Loch Lomond at Alexandria, north of Dumbarton. The latter two produce light/medium bodied, fragrant whiskies, whilst Oban makes a peaty, smoky malt whisky, somewhat akin to the island whiskies.

Glengoyne is situated in Dumgoyne, a hamlet just over the Highland line; in fact the whiskies are distilled in the Highlands and are matured in the warehouses across the road, in the Lowlands. Glengoyne distillery produces an unusually broad range of malt whiskies, apart from the 10, 12, 17, and 21 year olds (Figure 3.2.12), there are single cask malts of a wide range of ages, as well as vintage malts. Some single cask malts are aged in special sherry hogsheads that previously held amontillado or palo cortado Sherry. All the Glengoyne malt whiskies are produced from unpeated malt.

The Lomond distillery has both pot stills and stills incorporating a rectifying column, which can be adjusted to produce whiskies of varying heaviness. A range of malt whiskies has been produced by their distillery, as well as vatted malts and various blends. The best known malt whisky is probably Inchmurrin.

Further north, the Oban distillery produces a 14 year old malt whisky that has some of the peaty/smokiness of some of the island whiskies. The stills are small (the distillery is rather cramped), with small lyne arms leading to worm tub condensers (Section 3.2.4). The island distilleries of the Highland region: Isle of Jura, Ledaig (Tobermory, Mull), Talisker (Skye), Highland Park and Scapa (both mainland Orkney) are all well established producers of malt whiskies of varying peatiness. Scapa distillery uses unpeated malt, but peaty water. Its malt whiskies are aged in ex-Bourbon casks and are characterized by a salty grassiness, with attractive bitter chocolate and caramel notes. Scapa's neighbor, Highland Park uses peated malt from its own floor maltings (Figures 3.2.1 and 3.2.3). Consequently, its malts have a smoky character, but also heathery notes that are a characteristic of other northern Highland malt whiskies. It may be that the local shallow beds of peat, with a plant cover largely of heather, make some contribution to those aroma and flavor notes. At the time of writing, there are intentions of setting up a malt distillery called Blackwood in the Shetland Islands. Blackwood is a company already producing a range of spirits, and although plans are not well advanced (2009), the establishment of a malt distillery on Shetland would make it the most northerly.

Of the three west coast island distilleries (ignoring Islay for the moment), Isle of Jura, in Craighouse, on the southeastern coast of the island, makes the mostly lightly peated malt whisky. It uses water from Loch a' Bhaile Mhargaidh that flows mostly over rock. The stills at Jura have tall necks and thus produce a relatively clean spirit, with only a hint of peatiness, but with a characteristic dry island saltiness.

Ledaig and Talisker, the other two west coast island distilleries, produce intensely pungent smoky malts, more in the style of certain of the Islay malt whiskies. The former distillery, situated on a very cramped site by the harbor in Tobermory, has had a chequered history of production and there were many silent periods up to the 1990s. Talisker, on the other hand is owned by Diageo and its distinctive malt whiskies are included in that company's Johnnie Walker and other Scotch whisky brands. Talisker malt is also a component of the vatted malt whisky Poit Dubh (Black Bottle), produced on Skye. The distillery, situated at Carbost, on the west coast of the island, is one of the few with worm tub condensers, the worm tubs being kept full of cool water from Carbost Burn.

The Highland region contains clusters of distilleries (16) in the east (three), the midlands (six) and the north (seven, not counting Orkney) (Figure 3.2.1). Although generalization inevitably leads to oversimplification, common characteristics, apart from some peatiness, of the northern malt whiskies are heatheriness, salt and spice, whereas the midland and eastern malts can often be described as fruity and nutty, and often have a creamy palate.

The most northerly mainland distillery, Pulteney (near Wick and not far from John O'Groats) produces its malt whiskies from unpeated malt and these are thus the least peaty of the northern Highland whiskies. All the distilleries of this group are close to the sea, but Pulteney is on the coast and the fresh, salt-like character typical of coastal whiskies is more pronounced in Old Pulteney malts than in any of the other products of this group of distilleries. Pulteney is also interesting in that at the time of writing (2009), production of its malts is entirely nonautomated and worm tubs are still used for condensation of the spirit.

Clynelish distillery at Brora is the next along the coast from Pulteney. Its malts also have a salty 'coastal' or 'island' character, but are less delicate and more robust than Pulteney, with some spicy notes on the finish. Stills with shorter necks than some of those at Pulteney may account for some of the differences.

At the southern end of the northern Highland group of distilleries can be found Balblair, Dalmore, Glenmorangie and Teaninich, quite close together around the Dornoch Firth (Balblair and Glenmorangie) and Cromarty Firth (Dalmore and Teaninich). The first two generally produce the lighter spirits. Glenmorangie distillery possesses the tallest necked stills in Scotland (over 5 m high). This, the use of lightly peated malt, ex-Bourbon casks and the distillery's estuary location all contribute to the light, spicy character of the 10 year old product, with its faint, but distinct whiff of the sea. Older malt whiskies are often given some time in ex-oloroso casks to finish their maturation time. Glenmorangie, along with Glenfiddich and Glen Grant (both Speyside), was one of the first distilleries to promote its malt whisky aggressively in and outside Scotland; it is now the market leader of single malt whiskies in Scotland. Balblair has a claim to be Scotland's oldest distillery (1749), although the present buildings date from the 1870s only.

Dalmore and Teaninich further south, on the Cromarty Firth, produce rather heavier spirits, with spicy, Sherry and earthy, spicy, smoky notes, respectively. Single malts of the former distillery possess components aged in ex-Sherry casks, whereas most of the malt whiskies from the Teaninich distillery are components of a number of blended Scotch whiskies.

The midland and eastern Highland distilleries (Aberfeldy, Blair Athol, Deanston, Edradour Fettercairn, Glencadam, Glengarioch, Glenturret, Lochnagar and Tullibardine) tend to produce malt whiskies with citrus or fruity notes overlaying the malt character, provided ageing in ex-Sherry casks has not been too extensive. Many of these malts are also characterized by a smooth, creamy palate. Edradour distillery (owned by Pernod Ricard) is the smallest in Scotland. Situated close to Pitlochry, it takes some pride in its 'farm distillery' status and traditional equipment, including mainland Scotland's smallest stills, with worm tub condensers. Some ageing in ex-oloroso casks is practiced for its 10 year old malt, which can be detected on the nose, along with spice, smoke and mushroom. Despite the small stills, the spirit is relatively light, but after 10 years maturation, develops a remarkably smooth, creamy palate.

Glenturret distillery, on the banks of the river Turret near Crieff, is also very small and has a good claim to be Scotland's oldest, with some of its buildings dating from 1775. The distillery was revived in 1959 (after closing in the 1920s) by James Fairlie as a craft malt distillery showpiece – a venture that was very much against the grain at that time. The result has been a remarkable range of malt whiskies, in age, style and alcoholic strength. The over-riding character seems to be malt, toffee, toasted oak and nuttiness, but some versions have pronounced Sherry notes and most are characterized by a smooth, creamy finish, like Edradour. Deanston distillery is interesting in that it is partly housed (the warehouse) in a former cotton mill designed by Richard Arkwright and built in 1785. The original mill was water powered and the present distillery uses water powered turbines; a major reason for the distillery's location is the abundant water supply. Deanston's spirit stills are characterized by sloping lyne arms, which promote reflux during distillation thereby producing a lighter, cleaner spirit.

The biggest concentration of malt whisky distilleries in Scotland is to be found in the area known as Speyside, a hilly region with a network of rivers and burns, stretching from Tomatin distillery in the west to Glenglassaugh in the north, Glendronach in the east and Balmenach in the south (Figure 3.2.17). Many burns rising in granite hills and flowing over granite and peat to the many river valleys (glens) in which the distilleries are located, provide the water for a wide range of whisky styles. The major rivers are (west–east) the Findhorn, Lossie, Spey, Avon, Livet, Fiddich, Dullan, Isla, Deveron and Bogie, with the greatest number of distilleries being in the Spey valley (Figure 3.2.17).

Differences in methods of production (e.g. malt peatiness, types of stills, distillation procedure, types of casks used for ageing, etc.) probably contribute more to the variation in style than geographical location of the distillery (and its water). Having said that, the distilleries close to the Avon or Livet (several include

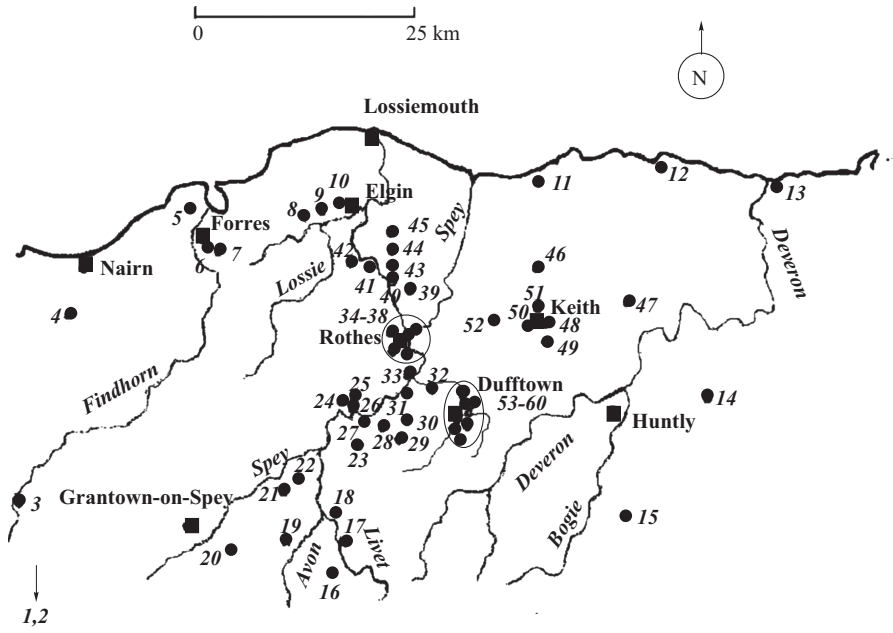


Figure 3.2.17 *Speyside distillery sketch map. Key: ■ Towns ◇ Distilleries. 1. Dalwhinnie 2. Drumguish (Speyside) 3. Tomatin 4. Brackla 5. Roseisle 6. Dallas Dhu (museum) 7. Benromach 8. Glenburgie 9. Miltonduff 10. Glen Moray 11. Inchgower 12. Glenglassaugh (silent) 13. Macduff (Glen Deveron) 14. Glendronach 15. Ardmore16. Braeval 17. Tamnavulin (silent) 18. Glenlivet 19. Tomintoul. 20. Balmenach 21. Tormore 22. Cragganmore 23. Glenfarclas 24. Tamdhu 25. Cardhu 26. Knockando 27. Imperial 28. Dailuaine 29. Benrinnes 30. Glenallachie 31. Aberlour 32. Craigellachie 33. Macallan 34. Glen Spey 35. Glenrothes 36. Glen Grant 37. Caperdonich (silent) 38. Speyburn 39. Coleburn (silent) 40. Glen Elgin 41. Mannochmore 42. Glenlossie 43. Longmorn 44. BenRiach 45. Linkwood 46. Aultmore 47. Knockdhu 48. Glentauchers 49. Glen Keith (silent) 50. Strathmill 51. Strathisla 52. Auchroisk 53. Convalmore (silent) 54. Glenfiddich 55. Balvenie 56. Glendullan 57. Mortlach 58. Dufftown 59. Allt a' Bhainne 60. Kininvie. Although 1 and 2 are well to the south, they are both in the upper Spey valley*

Glenlivet in their names) – Braeval, Cragganmore, Glenlivet, Tamnavulin, Tomintoul and Tormore – are noted for their delicate, light, but complex malt whiskies. Such is the reputation of Glenlivet, several distilleries (e.g. Dufftown and Longmorn) situated a long way from the valley of the Livet sometimes use Glenlivet as a subscript to their own names on bottles of their products. The glen itself is deeply set in the hills and its burns of very cold water travel good distances underground from their sources on the hillsides.

Glenlivet distillery rather unusually obtains its water, which has a degree of hardness, from an artesian well. About one third of Glenlivet distillery's whisky is aged in ex-Sherry casks, which is evident on the nose and palate of some of its wide range of products. Likewise, smokiness is evident from the use of some peated malt, but like the Sherry character, this does not overwhelm the complex, delicate combination of fruity, flowery and spicy notes. Of the Livet valley malts, those of Glenlivet distillery are the most assertive, whilst those of Tamnavulin are the lightest and most delicate. Tomintoul and Tormore malt whiskies are of the same mold, but perhaps a little fuller in body than Tamnavulin. The highly automated Braeval (Braes of Glenlivet), some 355 m above sea level now (2009) markets a single malt as an official bottling.

At the other extreme, the full bodied, high Sherry character Speyside style of malt whisky is typified by the products of Aberlour, Balvenie, Glenfarclas, Linkwood, Longmorn, the Macallan and Mortlach. Although

most of these distilleries are along either the river Spey or Fiddich/Dullan, it is probably the production methods rather than geographical location that accounts for their full bodied character, as many neighboring distilleries (e.g. Dufftown, Glenallachie, Glenfiddich, Glen Spey and Tamdhu) produce lighter malts. The Macallan distillery in Craigellachie produces some of the most respected of all single malt whiskies, the emphasis being on sherry character - indeed, the distillery owns casks holding oloroso wines (2.10.2) in Jerez bodegas. Once the casks have been emptied they are shipped whole (i.e. not dismantled) to Scotland for ageing Macallan whisky.

The smallest stills on Speyside can be found in the Macallan distillery (Figure 3.2.8), which must (at least in part) account for the heaviness of the spirit, although Glenfarclas distillery (further up the Spey valley - see Figure 3.2.17) produces malts of similar style and reputation, but slightly lighter, using the biggest stills on Speyside (Figure 3.2.8). This distillery uses new (unsherried) casks, as well as ex-Sherry (first fill and refill) casks, which could account for the somewhat muted Sherry character of its malts, compared with those of the Macallan distillery.

Both the Macallan and Glenfarclas were early in marketing their single malt whiskies, but they were not the pioneers – that accolade goes to Glenfiddich distillery, near Dufftown in the valley of the Fiddich. Glenfiddich Special Old Reserve was vigorously promoted outside Scotland in the early 1960s, the characteristic black, gold, red and white label showing the company emblem (a stag's head) on its distinctive dark bottle soon became a familiar sight in bars and wine shops. Glenfiddich distillery is the only Speyside distillery to have a bottling line on site; indeed, the only other malt distillery having on site bottling facilities in Scotland is Springbank distillery, Campbeltown. Next door, the Balvenie distillery is also owned by William Grant and Sons, but produces malts with much more Sherry character – particularly the 'Classic' version.

Elsewhere in Speyside, the malt whiskies from the distilleries around Keith (Auchroisk, Aultmore, Glen Keith, Glentauchers and Strathisla) are noted for their firm, robust constitutions. Strathisla distillery probably has the best claim as Speyside's oldest – indeed, it has a good claim to be Scotland's oldest malt distillery. Its wide range of malts is characterized by firm body, maltiness, Sherry notes and a long, smooth aftertaste. These whiskies are produced in small stills using local semi-hard spring water and lightly peated malt.

All of the distilleries on the periphery of Speyside (Tomatin, Brackla, Benomach, Glenburgie, Glen Moray, Miltonduff, Knockdhu, Glendronach, Ardmore and Balmenach), as well as those on the coast (Inchgower, Glenglassaugh and Macduff/Glen Deveron) produce characterful malts, many of which are highly regarded by blenders. Glendronach, once belong to Teachers, is now owned by the same management as Benriach, the only Speyside distillery to have its own floor maltings (Tamdhu distillery on the River Spey has saladin box maltings). The distillery was bought from Chivas Brothers (who closed it in 2002) by the present management in 2004. The malts from the coastal distilleries possess some fresh, grassy, salty character that sets them aside from most other Speyside malts. This character is more pronounced in the Inchgower and Glenglassaugh products than in Glen Deveron's (Macduff's) malts. However, Glenglassaugh is closed at the time of writing, but was bought by the Scaent group in 2008.

A majority of Speyside distilleries are owned by Diageo, who inherited United Distillers when the company was formed from the merger of Grand Metropolitan and Guinness (1997), the latter company having bought United Distillers in 1986. Like most malt distilleries, much of their production goes into blended Scotch whiskies, in this case brands such as Bell's, Haig, Johnnie Walker and White Horse, and deluxe blends such as the Antiquary, Dimple and Old Parr. The single malt whiskies of many of these distilleries are more easily found as independent bottlings (notably those of Gordon and Macphail) than as official bottlings. Indeed the products of Allt a'Bhainne (Dufftown, Chivers/Pernod Ricard), Kininvie (Dufftown, W. Grant & Sons) and Mannochmore (Lossie, Diageo) have been available as official bottlings only recently – Kininvie in 2006.

A surprising number of Speyside distilleries were either built or extensively reconstructed (not counting extensions) in relatively recent times: Allt a'Bhainne, Aultmore, Benromach, Brackla, Caperdonich, Cardhu, Craigellachie, Drumguish, Glenallachie, Glen Deveron, Glendullan, Glen Elgin, Glenglassaugh, Glen Keith,

Glen Spey, Glentauchers, Glenury, Kininvie, Linkwood, Mannochmore, Tamdhu, Tamnavulin, Tomintoul and Tormore. Many were built or reconstructed during the 'boom years': 1950s–1970s. Many closed during the recession years of the 1980s, including Brackla, Dallas Dhu, Glenglassaugh, Glentauchers, Imperial and Knockdhu. Several closed for good – Dallas Dhu became a whisky museum, whilst others, such as Banff, Glen Albyn, Glen Mohr and Millburn have been demolished. Caperdonich, Coleburn and Convalmore are still mothballed, but the others were gradually brought back into circulation in the 1990s. Mannochmore works only in alternate years at the time of writing (2009) and shares it workforce with the nearby Glenlossie distillery. Nevertheless, it has produced a single malt called Loch Dhu. At the time of writing, Diageo is building a malt distillery at Roseisle next to the Burghead/Roseisle malting plants. It is anticipated that the new distillery's output will go largely into the company's many Scotch brands. It is a large distillery, with an intended output of 10 million liters of malt whisky per annum.

The malt whisky production processes used by the Speyside distilleries vary enormously in their degree of automation and extent of modernization. The majority of distilleries have experienced some degree of modernization and the newer ones, such as Allt a'Bhainne and Braeval (Braes of Glenlivet) are fully automated, employing very few staff. However, it was another Speyside distillery, Glen Keith (Isla – see Figure 3.2.17) that was the first to use gas fired stills and it was also the first to computerize part of its production process. In other distilleries where modernization has been extensive, it is still possible to see the occasional piece of traditional equipment still in use – worm tubs, for example, are still used to condense the distillate at the Benrinnes and Mortlach distilleries. At the other extreme, Balmenach distillery (Cromdale – see Figure 3.2.17) still uses a cast iron mash tun and Douglas fir wash backs, as well as worm tubs. It is owned by Inver House Distillers (who also own Pulteney Distillery), a company that prides itself on tradition.

Traditional appearance of the distillery is usually considered to be something of importance, as witnessed by the presence of malting house ventilation pagodas in even the most modern of distilleries, such as Braeval. All Speyside distilleries apart from two, Benriach (Ben Riach) and Tamdhu, receive all their malt either from big nearby malting complexes (often owned by the parent companies of the distilleries) or from suppliers outside the region, so the pagodas are entirely decorative. Ben Riach uses traditional floor maltings (unique on Speyside) for some of its malt and Tamdhu distillery uses Saladin box maltings for all its malt.

Islay is an important producer of highly distinctive malt whiskies; it has been said that there are very few Scotch whisky blends that do not have at least a small Islay malt whisky component. The production area is naturally compact (Figure 3.2.18), and although there is a wide range of styles (particularly in degree of smokiness or peatiness), all the malts share the 'island character' of saltiness (or brine) found in other island or coastal malt whiskies.

The smoky character comes from the use of peated malt (Section 3.2.3) and is most pronounced in the products of the distilleries on the south coast, close to Port Ellen – Laphroaig, Lagavulin and Ardberg. Port Ellen (Diageo) ceased distilling in 1983 (although its malts can still be found as independent bottlings), but malting is still carried out in the large drum malting house next door. Malt is supplied to Diageo's two Islay distilleries, Lagavulin (mentioned above) and Caol Isla, on the northeastern coast of the island and to other independent distilleries. Those two distillery's floor maltings ceased production when the Port Ellen malting complex was built in 1973. Floor malting, however, is still carried out at Bowmore, Kilchoman and Laphroaig distilleries, satisfying around 50% of their malt requirements, the remainder coming from the Port Ellen maltings, mainland Scotland or further afield. The kilning of malt (Section 3.2.3) for Ardberg, Lagavulin and Laphroaig distilleries involves the greatest use of smoke from peat furnaces. Consequently, the malt whiskies from these three distilleries are the most powerfully smoky of all the Islay products. This, coupled with their salty island character, makes for some of the most pungent of Scotch malt whiskies, along with Ledaig, Longrow and Talisker. Terms like 'iodine,' 'medicinal,' 'disinfectant,' 'tar' and 'seaweed' have been used by tasters to describe the aroma and flavor of some of these malt whiskies. Using the flavor wheel shown in Figure 3.2.13, these malts would have high scores on the pungent and phenolic spokes of the wheel.

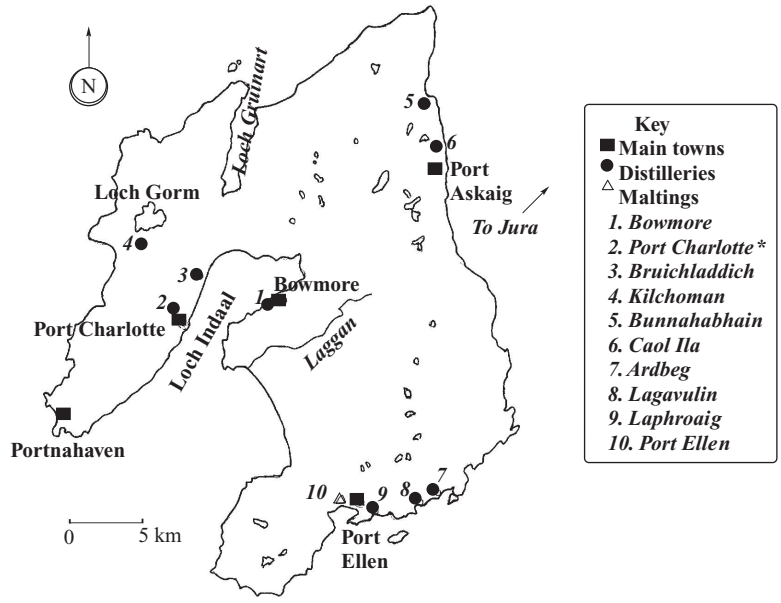


Figure 3.2.18 *Islay malt distilleries. *In preparation (2009)*

The lightest of the Islay malt whiskies are those of the Bunnahabhain and Bruichladdich distilleries - which is no criticism, as they are both well rounded and elegant products with lots of island character. The latter distillery uses stills with long necks, which probably accounts for some of the distillate's relative lightness. Intermediate between these two extremes are the malt whiskies of Bowmore and Caol Isla. The former distillery uses both ex-Bourbon and ex-Sherry casks, so a Sherry character is often discernable in some of its many versions of malt whisky. The products of both distilleries are characterized by a good balance between maltiness, saltiness, smokiness and Sherry character.

The distillery at Kilmochan, just south of Loch Gorm (Figure 3.2.18) distilled its first whisky in November 2005, some of which will be bottled and ready for sale in 2009. It is aiming at an annual production of 90 000 l (see Table 3.2.4 for selected malt whisky production figures), fashioning its distillate in part from home grown and home malted barley (using traditional floor maltings). Kilchoman is now the most westerly distillery in Scotland. At the time of writing (2009), Bruichladdich distillery is helping to prepare a new distillery in Port Charlotte, using equipment from the now demolished Interleven distillery. The new distillery is on the site of the former Lochindaal distillery.

The lowland grain distilleries were amongst the first licensed (and therefore legal) distilleries and for a long time were much more industrialized and technically advanced than their (mostly highland) malt distillery counterparts. Originally producing their distillates using pot stills, they rapidly switched to Coffey stills in earlyto mid nineteenth century. Until the 1980s, maize was the major grain, the switch to wheat being economically driven.

The usual cycles of economic growth and recession with their concomitant rationalization programmes have left just a few (see Figure 3.2.1) large grain distilleries, which are in the hands of the larger companies, such as Diageo and Pernod Ricard. They are mostly in the industrial belt around Glasgow and Edinburgh, with one (Invergordon) to the north and another (Girvan) to the south. Almost all of their whiskies are blended with malt whiskies to produce the large number of brands of Scotch whisky that are available worldwide. Larger

Table 3.2.4 *Approximate annual production figures for selected malt whisky distilleries and grain whisky distilleries**

Distillery	Production of spirit ($\times 10^6$ l per annum)	Distillery	Production of spirit ($\times 10^6$ l per annum)
Balmenach (Speyside)	1.8	Glenmorangie (N. Highlands)	4.0
Benromach (Speyside)	0.25	Bowmore (Islay)	2.0
Clynelish (N. Highlands)	3.4	Kilchoman (Islay)	0.1
Cragganmore (Speyside)	1.6	Knockdhu (Speyside)	0.9
Glengoyne (W. Highlands)	1.2	Macallan (Speyside)	6.0
Glen Grant (Speyside)	5.9	Cameronbridge* (Diageo)	30
Glenlivet (Speyside)	5.9	Girvan (W. Grant)*	15

companies have their own grain and malt distilleries, with malt whiskies from particular distilleries featuring in particular brands, the grain whisky acting as a base for blending. For example Glen Elgin, Linkwood and Lagavulin malts are important components of the White Horse brand (Diageo); Dufftown and Inchgower of Bell's whisky (Diageo); Highland Park and Tamdhu of the Famous Grouse blend; Dalmore and Fettercairn of Whyte and Mackay blends.

Grain whiskies are subject to the same regulations as malt whiskies and the age statement on a bottle of blended Scotch whisky must refer to the youngest whisky in the blend, be it malt or grain whisky. Most blended Scotch whiskies have around 60% grain whisky in their composition, but this can drop to 40% or less for deluxe brands. As with other alcoholic beverages (see for example, Sherry Section 2.10.2) blending is an art, but the grain whisky is normally meant as a backdrop to the more pronounced character of the malt whisky components. Rather few single grain whiskies have been released over the years, the best known probably being Cameronbrigg, from Diageo's Cameronbridge distillery in Fife.

For many years now, various malt distilleries have sold bulk new make spirit to a number of companies (usually via brokers or distributors), ranging from grocers, department stores, supermarkets to wine and spirit merchants and independent bottlers/blenders. Over the years these have included (and many are still in business) Adolphi, Berry Brothers and Rudd, Cadenhead, Gordon and MacPhail, Harrods, Reid Wines (Bristol), The Scotch Whisky Society, Signatory, Duncan Taylor, J.G. Thomson (Glasgow), the Wine Society (Stevenage) and various supermarket chains.

Some of these companies, such as Gordon and MacPhail, mature the whiskies in their own warehouses and bottle them when they are considered to be at their best. This can be done by the vatting of several casks of the same distillate to ensure continuity of style (this is the tendency of Gordon and MacPhail) or from individual casks (which is the tendency of Cadenhead). Amongst these independent bottlers, there is also a variation in prebottling dilution, as well as in bottling techniques. Thus Cadenhead tends to dilute its products to 46% ABV, whereas Gordon and MacPhail tend to bottle at 40% ABV and J.G. Thomson specialize in cask strength (~58% ABV) malts (the 'As We Get It' series). Bottling is sometimes performed without chill filtration, as at Cadenhead.

All the variations mentioned above, plus the variety of casks in use, has naturally led to wide variations in the character of single malt whiskies from particular distilleries that have been matured and bottled by independent bottlers. This has sometimes resulted in considerable controversy, as some distilleries are unhappy about their products appearing for sale in a form over which they have had no control. For example, for many years the distillers William Grant and Sons have not released whisky to independent bottlers for

blending under the Glenfiddich name. However, there can be no doubt that the independent bottlers perform a valuable service to the malt whisky enthusiast, especially in the following respects.

They provide an extended range of malts from distilleries that bottle only one or two versions of their own whiskies. For example, for a time Strathisla distillery released only a 12 year old 40% ABV version of its malt, but 8, 15 and up to 35 year old versions were available from Gordon and MacPhail, whereas Cadenhead provided a 20 year old 46% ABV version.

They provide malts from distilleries that have either never or rarely bottled their own. Examples here include Ardmore, Benrinnes, Glencadam and Teaninich.

They provide malts from mothballed or demolished distilleries, such as Banff, Coleburn, Convalmore, Glen Mohr, Glenugie, Interleven, Kinclaith, Ladyburn, Littlemill, Lochside, North Port, St Magdalene and Tamnavulin.

They provide special bottlings of malts produced in certain distilleries from stills that are no longer in operation. Thus, Gordon and MacPhail have provided malts from now defunct Lomond stills in Glenburgie and Miltonduff distilleries. These versions were named 'Glencraig' and 'Mosstowie,' respectively. They provide a range of 'regional' and other vatted malts and blends.

Many independent bottlings have acquired a high reputation and have become well known, examples being the Scotch Malt Whisky Society bottlings, the Signatory range, the 'As We Get It' range, Gordon and MacPhail's Connoiseur's Choice range and Duncan Taylor's Peerless range. Two independent bottlers have direct links with the industry (other than as customers for some of its whiskies): Gordon and MacPhail own the small Benromach distillery near Forres (Speyside) and Cadenhead are owned by the Springbank distillery owners (J. and A. Mitchell).

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3.3
Whiskeys

Too much of anything is bad, but too much of good whiskey is barely enough.
—Mark Twain

3.3.1 Scope

The main purpose of this chapter is to review distilled beverages that are distilled from fermented cereal mash and take the name whisky (apart from Scotch whisky, see Chapter 3.2) or whiskey. Also discussed here are similar drinks, known as Korn or Kornbrand, popular in German speaking countries. The Council Regulation (EEC) No. 1576/89 of 29 May 1989 of the (now) European Union (EU) defines whisky or whiskey in the following way:

- A spirit drink produced by the distillation of a mash of cereals saccharified by the diastase of the malt contained therein, with or without other natural enzymes
- Fermented by the action of yeast
- Distilled at less than 94.8% (v:v), so that the distillate has an aroma and taste derived from the raw materials used
- Matured for at least three years in wooden casks not exceeding 700 l capacity.

Hence for a distilled beverage to be sold as whisky or whiskey, its manufacture must comply with the above details. Korn or Kornbrand has a similar definition, except that the cereals are actually specified (wheat, barley, oats, rye or buckwheat), the regions are broadly specified ('Germany and in regions of the Community where German is one of the official languages provided that this drink is traditionally produced in these regions and if the grain spirit is obtained there without any additive') and there is no mention of maturation.

Within and outside the EU, the governments of individual countries superimpose additional or impose their own specific regulations governing the production of specific distilled beverages. This is necessary to protect the product from fraud and counterfeit and to ensure consistently high quality. Thus, Irish whiskey must be produced and matured in Ireland according to specified regulations (Section 3.3.2), Bourbon must

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
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be produced and matured in the USA according to its own set of regulations (Section 3.3.4), and so on. Most whiskies produced in different parts of the world are grain spirits and can be exported to countries (e.g. to the EU or the USA) where this definition is in place. However, certain inexpensive Indian whiskies are produced from molasses spirit or from a mixture of molasses and grain spirit, and so cannot be exported to such countries under the name of whisky or whiskey (Section 3.3.6).

3.3.2 Irish Whiskey

The debate on whether it was the Irish or Scots who first distilled whisky is probably as old as the spirit itself. Although there is a distinct lack of written evidence, it is probable that regular distilling of whisky was established at about the same time in Ireland and Scotland. However, in the early part of the nineteenth century (and indeed, up to the end of that century), it was Irish whiskey that had the greater reputation abroad. Scotch malt whiskies at that time were largely consumed in Scotland, being thought too strongly flavored for the English and other foreign palates. This view was still widely held in the 1950s/1960s when distillers like W. Grant made the first serious drives to market single malt whiskies, such as Glenfiddich in England and elsewhere. Grain whisky from the lowlands (in the early nineteenth century, a pot still spirit) was the main Scotch for export and Irish whiskey was considered by many to be generally superior to this.

Later in the nineteenth century, especially following the advent of the Coffey still, blended Scotch whisky was the main competition for Irish whiskey abroad. Different economic circumstances, rather than differences in quality or character caused the eclipse of Irish whiskey in favor of (blended) Scotch whisky. General difficulties under British rule, the struggle for independence, economic depression and two World Wars (the Republic of Ireland was neutral in the latter) led to mergers, closures and general rationalization of the industry, so that the number of distillers diminished alarmingly in the twentieth century.

In 1966, John Power and Son (Dublin), John Jameson and Son (Dublin) and Cork Distillers (Midleton) merged to form the Irish Distillers Group. The Dublin distilleries were closed and a new facility was built adjacent to the old distillery at Midleton (which is now a visitors' center). The new distillery houses no less than thirteen 75 000 l pot and column stills (the pot stills are the largest in the world) and uses a combination of stills to produce all the old brands, as well as some new ones. The whole process is highly automated and highly computerized, with sensor devices on the column stills to control distillation. The production capacity is 19 000 000 l per annum. For a comparison with the annual output of Scotch distilleries (see Table 3.2.4, Section 3.2.6). In 1972, Bushmills Distillery (County Antrim, Northern Ireland) joined the group, keeping its distillery. However, weakened by an unsolicited takeover bid by Allied-Lyons, Grand Metropolitan and Guinness, (rather in the style of the acquisition of United Distillers by Guinness in 1986), Irish Distillers was bought by Pernod Ricard in 1988. Bushmills was sold to Diageo in 2005.

From 1972 until the early 1990s, all Irish whiskey was produced by the Irish Distillers Group (later Pernod Ricard), but Cooley Distillery was set up 1987 in a former vodka plant in County Louth on the Cooley Peninsular and now provides some much needed competition.

In many ways, Irish whiskey is similar to Scotch whisky: barley and other cereals (maize and wheat) are used, both pot stills and continuous stills are used, and both spirits are aged in small oak casks. However, there are three major differences: Scotch malt whisky is mostly double distilled from all malted barley, usually at least part of which (peated malt) has been exposed to peat smoke. Irish whiskey, on the other hand can be made with unmalted barley as well as malted barley, traditionally all unpeated and triple distillation (as for some Lowland and a handful of Highland Scotch malts) is the traditional method. Nowadays there are exceptions to the Irish tradition of unpeated malt and triple distillation, the first examples, significantly, being provided by Cooley Distillery, the only independent distillers in Ireland.

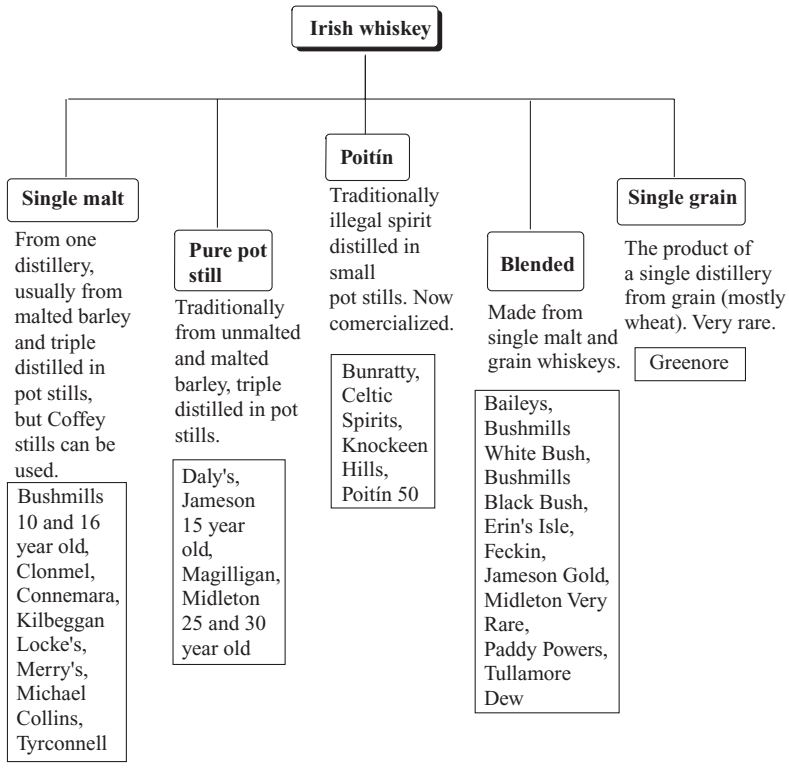

Figure 3.3.1 *Five basic categories of Irish whiskey*

Irish whiskey can be subdivided into five categories as shown in Figure 3.3.1. Of these, the blended types have the biggest share of the Irish whiskey market, with the greatest number of brands. The blend names often allude to now defunct, but once famous distilleries or their brands. The single malt and single grain categories have essentially the same meaning as for Scotch whisky (Section 3.2.1). Poitín (Potcheen) is the name for an originally artisanal and, for a long time, illegal spirit, the Irish 'moonshine.' Poitín was once widely produced in many small private pot stills in farmsteads and villages dotted about the countryside. Increased taxation and attempts by various British governments to suppress private distillation forced the production of poitín underground, as in Scotland. It was recently legalized for consumption in Ireland and there are now several commercial versions, some of which have 80% alcohol by volume. Single grain whiskey (like its Scots equivalent) is very rare, the huge majority of grain whiskeys being used in blends. At present, there is only one brand, Greenore, produced by the Cooley Distillery.

The pure pot still whiskey category is unique to Ireland, the 'green' or unmalted barley giving the spirit a distinct grainy/spicy character. The ratio of unmalted to malted barley varies between brands, but the Jameson 15 year old has an unmalted:malted barley ratio of 60:40. According to Irish law, any whiskey distilled using pot stills can be called 'pot still whiskey,' hence it is not a legal requirement for the Irish whiskey distiller to include unmalted barley in the grain bill. In a sense this is a pity, because a purely Irish characteristic remains unprotected at a time when virtually every indigenous style of alcoholic beverage in the European Union has been protected by a recognized geographic name of origin.

The next paragraphs give a brief account if the Irish Whiskey distilleries and their products.

Irish Distillers Group

Now owned by Pernod Ricard, the IDG runs the big distillery at Midleton, near Cork, producing a wide range of whiskeys, of which the Jameson brands are the biggest selling worldwide and the Powers brands are Ireland's best sellers. Other blends produced are Midleton Very Rare, Redbreast and Tullamore Dew. Pure pot still whiskey is also made here, Jameson's 12 year old, Midleton 25 year old and Midleton 30 year old being some of the oldest Irish pot still whiskeys available. Production at the new distillery began in 1975, but the old distillery was established in the early seventeenth century.

Grain whiskey, distilled at the Midleton site goes into the many IDG blends and some is sold to the Bushmills Distillery in Northern Ireland. Additionally, highly rectified spirits from Coffey stills are used to make Cork Dry Gin (Ireland's best selling gin) and Huzzar Vodka (the second best selling vodka brand).

Old Bushmills

Now owned by Diageo, the Bushmills Distillery, in the village of that name in County Antrim, is the oldest licensed whiskey distillery. The license was obtained in 1608 from King James I and in 1784 Old Bushmills Distillery became an officially registered company, in this respect predating Scotch malt whisky distilleries by several decades. The distillery produces two blends: Original ('White Bush' or 'White Label') and Black Bush. The latter contains a high proportion of oloroso cask-matured single malt whiskey in its blend. The Bushmills Distillery was the first (1980s) to introduce an Irish single malt whiskey, the 10 year old, matured in ex-Bourbon casks, but nowadays several single malts are available at varying ages – 12, 16 and 21 year old. These malt whiskeys generally undergo mixed cask maturation, for example the 21 year old spends time in firstly ex-Bourbon casks, then ex-oloroso sherry casks and is finished in ex-Madeira casks before bottling. All are triple distilled using pot stills.

Cooley

This is the only independent all Irish owned distillery company. It has two distilleries, the original one on the Cooley Peninsular near Drogheda, set up in 1987, and the recently (2007) renovated Locke's distillery/museum at Kilbeggan in County Westmeath. The Kilbeggan distillery has its origins in the eighteenth century and so is one the oldest in the world. In 2007, a nineteenth century pot still at the distillery was rejuvenated and put into operation after 50 years of silence, making it the oldest operating pot still in the world. A limited number of miniatures of spirit from this still of varying degrees of maturity were released in 2009 in advance of the release of the first (three year old) spirit. The first releases from the Kilbeggan distillery are expected to be in 2010, but at present Locke's Single Malt Whiskey, Locke's Blended and Kilbeggan brands are distilled at Cooley, but the malt whiskey is matured at Kilbeggan. The Cooley distillery has modern automated, computer controlled facilities for making both malt and grain whiskeys. Included are pot stills with long, wide necks that allow for some reflux during distillation, thus producing a lighter, cleaner spirit. A number of Cooley's products break away from Irish whiskey tradition; double rather than triple distillation is carried out in pot stills for the single malt brands and Connemara Peated Single Malt is the only Irish whiskey produced using wholly peated malt. Also, Greenore (15 year old) is the only single grain whiskey commercially available. Cooley Distillery has progressed remarkably since its birth and has accumulated numerous international awards, most recently (2010) being named 'Distillery of the Year' by the US whisky magazine, 'Malt Advocate.'

All of the above distillery companies produce whiskeys for international drinks companies, supermarkets and food companies (for use in confectionery and baked products for example).

The Cooley and Midleton distilleries are two of the most modern in the world. The former, originally much smaller, is expanding as demand grows, but its production monitoring system allows for expanding facilities, as well as enabling tuning of the manufacturing processes, quality assurance/control, preventative maintenance and early warning of process failure. At the present time (2009), there are no artisanal or craft distillers of Irish whiskey.

3.3.3 The Whiskeys of America

The alcoholic drinks of the early American and Canadian settlers and postrevolutionary Americans did not include that much whiskey; cider and beer (and later, fruit wines) were the main fermented drinks, whilst rum, fruit spirits and applejack were the major distilled drinks. The last mentioned drink was often made by pouring off the alcohol enriched liquid from frozen cider. Whiskey became the staple spirit of the pioneers who, from the closing years of the eighteenth century pushed west from the original American states through to the fertile lands in Kentucky, Illinois, Indiana, western Pennsylvania, Maryland and Tennessee. Here, the land was ideal for growing cereals – barley, maize, rye and wheat in particular – and the water was clear, with little organic matter, but contained dissolved salts (hardness), since it ran over chalk or limestone in many of these areas. High yielding native corn (maize) and barley predominated in Kentucky, Tennessee and Virginia, whereas rye was the main crop in Pennsylvania. Given that many of the new settlers were of Scots-Irish or German descent, the remoteness of these areas with respect to the richer eastern states and the poor road system (often impassable after heavy rain), it is understandable that most farmers chose to distil part of their cereals crop into a valuable, less bulky, more easily transported product, whiskey, just like their ancestors in Ireland and Scotland.

Whiskey had ousted rum as the major distilled spirit in the USA in the latter half of the nineteenth century, by which time production was running at around 374 000 000 l per annum. Up to that time, the big majority of American whiskey distillers had been using copper pot stills, like their malt whisk(e)y producing ancestors in Ireland and Scotland, but column stills (made of copper and wood) began replacing many of these in the 1890s, until today, the big majority of American whiskeys are produced using column stills. Also, definite styles were well established by that time: corn whiskey, rye whiskey, sweet mash, sour mash, charcoal filtration, aging in charred casks were all in progress and Kentucky Bourbon County whiskey had taken a high profile since about the time of the Civil War. In the twentieth century, the fortunes of American whiskey took a roller coaster ride between the lows of two World Wars, the Depression and Prohibition (1920–1933) and the boom period a few years after the Second World War, when in 1951, production peaked at ~767 000 000 l (Lichine, 1982). Since then, production of American whiskey more or less stabilized at ~400 000 000 l – about the level of production just before World War I – for many years.

Nowadays, consumption of American whiskey of all types makes up about 13% of spirits consumption in the USA, with Canadian whisky close behind on 10%. In 2001, the total whisk(e)y consumption in the USA was 29% of all distilled spirits; about half of the 1975 figure (Corrigan, 2004), but still a substantial portion. The ground has been lost principally to gin, rum, tequila and vodka, which are used mainly in cocktails, 'long' alcoholic drinks and latterly, 'ready to drink' beverages. The prohibition period damaged the American whiskey industry, not only by diminishing the number of distilleries (many of those in Kentucky and Tennessee, the heart of American distilling, never reopened after Prohibition ended), but also by influencing public taste. During Prohibition, people acquired a taste for blended Scotch whisky and Canadian whisky smuggled into the country, both of which generally had milder characters than pre-Prohibition Kentucky, Tennessee or rye whiskeys. Canadian whisky is still consumed more than American 'straight' whiskeys (see next paragraphs), although consumption of Scotch whisky has dropped, except for Deluxe blends and single malts (Section 3.2.6), whose consumption has increased markedly (Corrigan, 2004).

Table 3.3.1 *Categories of American whiskey*

Whiskey	Cereal-base	Comments
Bourbon	≥51% corn (maize), the rest usually rye and barley.	Other restrictions are given and discussed in 3.3.4. Most Bourbon is of the "straight" category.
Corn	≥80% corn	No cask-aging requirement, but in practice, often aged in used or uncharred casks.
Rye	≥51% rye	Much is blended, but there are examples of straight rye and blended straight rye whiskeys.
Straight	As above	Must be distilled at no more than 80% ABV and aged at least 2 years in charred oak casks (except straight corn whiskey).
Blended Straight	As above	A blend of two or more straight whiskeys. Used to make blended whiskeys.
Blended	As above	A blend of at least 20% straight whiskey (50% ABV) and either other whiskey or, more likely, grain neutral spirit.
Bonded (or Bottled in Bond)	As above	Refers to straight whiskey (usually Bourbon) (50% ABV) from a single distillery, from a single season and aged in government-supervised "bonded" warehouses for at least 4 years.

The production of modern American whiskeys is governed by legislation overseen by the US Food and Drugs Administration (USFDA or simply FDA) and supported by such commercial bodies as the American Distillers Association and the Kentucky Distillers' Association. However, labeling, like that of wines and beers, is regulated by the Treasury Department's Alcohol and Tobacco Tax Bureau.

American Whiskey can be made using any grain (but with certain restrictions for specific whiskeys, like Bourbon), distilled to less than 95% ABV (above which it becomes grain neutral spirit), aged in oak casks at not more than 62.5% ABV and bottled at no less than 40% ABV. These requirements govern the production of all whiskeys, with additional requirements for some, such as Bourbon and rye whiskey and modifications for others, such as corn whiskey (which is not required to be aged in casks). Further details are given under the appropriate whiskey heading, in Sections 3.3.4 and 3.3.5.

By law, American whiskey is classified into several categories, according to production history, although one of these categories (Blended Straight Whiskey) is of little importance to the consumer as it serves as the base from which other categories are constituted. They are summarized in Table 3.3.1.

Nowadays, the big majority of American whiskey distilleries are located in Indiana, Illinois, Kentucky, Ohio, Tennessee and Virginia, with many others dotted about in various states, such as California, Colorado and New York. Some of these are microdistillers (craft or artisanal distillers) producing a range of spirits, rather than just whiskey. Tuthilltown Distillery in New York makes corn and Bourbon whiskey, Peach Street Distillers in Colorado produce Bourbon whiskey, Templeton Spirits (Iowa) makes Bourbon, two Californian companies, St. George Spirits and St. James Spirits produce malt whiskey and whisky, respectively. A full list can be found in the Directory of Craft Distillers (2009).

3.3.4 Bourbon and Tennessee Whiskey

Bourbon whiskey (sometimes the Scottish variant whisky is used) takes its name from the old Bourbon County of Kentucky, to the west of Virginia, itself named after the French Bourbon dynasty that did much

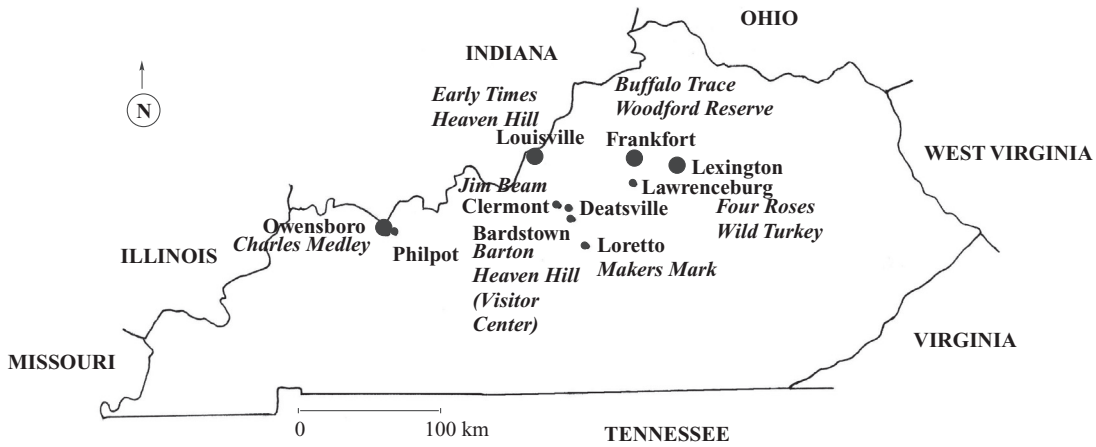

Figure 3.3.2 *Kentucky Bourbon distilleries*

to aid the colonialists in the Revolutionary War. Amongst the first settlers were (largely Protestant) Scots and Scots-Irish immigrants, who established small distilleries in the early 1800s, during the first colonization pushes into native American territories west of the original 13 states. Elijah Craig, a Baptist minister, set up one of the first stills in Georgetown, near Frankfort (Figure 3.3.2), in what was then Bourbon County, Kentucky. There were several small distilleries in Kentucky and surrounding states in the early 1800s, to which many farmers sent their corn crops for turning into whiskey at each harvest time. The whiskeys from this area soon acquired a good reputation and became known as Bourbon County Whiskey, but sometime before the Civil War, two processes that led to Bourbon whiskey having special distinction were developed and adopted: these were sour mash fermentation and the use of charred oak casks for maturation. In time, these two procedures were adopted by some distillers located outside Bourbon County and even in neighboring states, and so the general style eventually became known as Bourbon. Also in the then Lincoln County in the neighboring state of Tennessee, a process of trickling the new make whiskey through charcoal made from sugar maple wood was developed in the 1820s. This process became known as the Lincoln County process.

The origins of these special processes are uncertain, but it is known that the sour mash process was developed by James Crow, probably from the experience of producing a distinctive whiskey by using a portion of beer lees, rather than fresh yeast, from a previous fermentation to start a new fermentation. The starter contained lactic acid bacteria as well as yeast, thus giving a beer, and hence whiskey, with a special aroma/flavor profile.

The charring of oak casks was probably taken from the experience of importers of West Indian rum in the eighteenth century (or possibly from the rum distillers themselves); they found that rum matured in some accidentally charred casks tasted better than that aged in ordinary or toasted casks. Nowadays, once used charred Bourbon casks or their staves are used to mature many Scotch malt whiskies (Section 3.2.5) and rums, such as Puerto Rican rums (Section 3.5.4).

The use of charcoal filtration was probably developed by a distiller called Alfred Eaton, at the Cave Spring Distillery (now the Jack Daniel Distillery) in Lynchburg, Tennessee; at least he put his name to the process and helped to publicize it, if not actually inventing it. The idea probably came from the well known ability of charcoal to remove impurities and off flavors from liquids such as drinking water.

Bourbon whiskey, after a long period of consolidation up to the late 1990s, has experienced a boom during the first decade of the twenty-first century; production has doubled since 1999 (Kosar, 2008). The boom is

fueled by increased sales, especially export sales, of high-end and super premium Bourbons; in 2006–2007, export sales increased by 14.4%, with one particular super premium product, Woodford Reserve (Labrot and Graham/Brown-Forman), increasing its sales by 24%. Export sales of Bourbon whiskey totaled $713,000,000 in 2007 (Kosar, 2008). Increased sales began in the 1990s, with increased production of super/ultra premium brands, the gross revenue growth of this type growing by 20.1%, as opposed to 0.1% for the ordinary Bourbon category in the period 1996–2000 (Lioutas, 2004). A similar boom has been enjoyed in recent years by premium Scotch whisky, Scotch malt whisky and premium rum producers.

Present day Bourbon can be made anywhere in the USA, provided specific regulations (discussed next) are followed. In reality, all but one of the 11 large Bourbon distilleries are located in Kentucky, the odd man out being the A. Smith Bowman Distillery at Fredericksburg, Virginia. These are nearly all owned by large international corporations, such as Brown-Forman, Constellation Brands and Pernod Ricard. Additionally, several small 'specialty' or craft Bourbon distilleries have sprung up in the past few years. The latest craft distillery (2009) is the Corsair Artisanal Distillery at Bowling Green, Kentucky; it plans to produce Bourbon and rye whiskeys, as well as other spirits (Dowd, 2009). Tennessee has just two large distilleries, Dickel (at Tullahoma) and Jack Daniel (at Lynchburg), the latter being the largest producer of American whiskey.

Bourbon whiskey must be produced and matured in the USA, according to the following restrictions (see also Table 3.3.1).

- The grain bill must contain at least 51% (but not more than 79%) corn (maize)
- Maximum distillation strength must be not more than 80% (ABV) (160 American proof)
- New make whiskey must enter the casks at no more than 62.5% ABV (125 American proof)
- Maturation must be carried out in newly charred American white oak casks for a minimum of two years.

In practice, corn usually makes up well over 51% of the grain bill, with barley in second place, followed by rye, and much Bourbon is distilled at 70% ABV and is casked at below 60% ABV, for a maturation period of more than two years – often 10 years or more.

The corn is milled to a specified particle size, mixed with local limestone water and then steamed in a large cereal cooker. After cooling to ~65 °C, milled malted barley and rye are added to provide enzymes for hydrolysis of the starch to fermentable sugars. Most of the larger distilleries use an amylase enzyme mixture in place of some malt. Spent mash from a previous distillation (like the backset used in Scotch grain whisky production, but including the grains – see Section 3.2.3) is added at this stage or just before the pitching of yeast. The spent mash is of high acidity and hence lowers the pH of the new mash when mixed; this is now 'sour mash,' a medium that allows lactic acid bacteria as well as yeasts to flourish during fermentation. The sour mash method is used by the majority of American whiskey distillers. The remainder of the spent mash, a protein rich slurry, provides a valuable food for cattle and pigs.

The mash is then cooled to ~21 °C, and either a fresh yeast slurry or a slurry of yeast deposit (draff) from a previous fermentation is added. Fermentation proceeds for several days, after which the beer (grains and all) is transferred to the still, usually a pair of continuous columns, where distillation occurs and the new make whiskey is collected at 70% ABV, diluted to 60% ABV and is eventually transferred to new charred American white oak (*Quercus alba*) casks for maturation. Super premium Bourbons, such as Woodford Reserve are triple distilled in copper pot stills, just like the original Bourbon whiskeys in the early 1800s, and like Irish whiskey (Section 3.3.2) and a few Scotch malt whiskies (most are double distilled – see Section 3.2.4). In this case, distillation heads and tails management must be conducted, via a spirit safe (Section 3.2.4), during the second (high wines) and third (spirit) distillation, as for Scotch malt whisky distillation (Section 3.2.4) (Lioutas, 2004). The heart fraction is collected at ~80% ABV, vatted with fractions from previous distillations, diluted to ~60% ABV and eventually casked.

Compared with Scotch malt whisky wash, Bourbon beer derived largely from maize and having undergone a sour mash fermentation, has much higher concentrations of fusel alcohols and various carbonyl compounds, as a result of extensive lactic acid bacteria activity. Also, because of the much higher solids, protein and lipid content of maize, care needs to be taken during pot still distillation to ensure that no extraneous lipid oxidation products (such as unsaturated aldehydes) occur in the heart fraction going to the spirits receiver. This can be done by heating the still with steam, so that distillation occurs around 90 °C, and also by careful heads and tails management.

It is interesting to note that an 8750 l (2500 US gallons) charge of mash in a pot still will leave about 2000 kg of proteins, lipids and solids behind in the still after distillation, some of which forms a black deposit in the still and lyne arm. The black color is derived from copper (II) sulfide, which is formed by reaction of copper metal on the surface of the still with organosulfur compounds derived from both the cereals and the fermentation process. This has the important effect of removing the more objectionable aromas due to high levels of certain highly odor active organosulfur compounds, such as ethanethiol, dimethyl sulfide, dimethyl disulfide and others. After distillation, some of these organosulfur compounds will either be present below their odor threshold values or will be present at levels that make positive contributions to the whiskey aroma. See Section 3.2.5 for more detail about distillation and organosulfur compounds. This, of course is common to distilled spirits in general; distillates from purely stainless steel stills can have cooked vegetable and cabbage notes that are too pronounced. The column stills used to distil most Bourbon and Tennessee whiskeys are either hybrid stills (with stainless steel exterior shells, but the with copper internal structures, such as boiler plates and condenser tubes) or are made of stainless steel, in which case, it is common practice to include pieces of cut up copper tubing into the still.

The congener profile for new make Bourbon is quite different for pot still and column still processes. Pot still whiskey has higher levels of certain of the more volatile (lower boiling point) constituents, such as acetal, acetaldehyde, ethyl acetate and 1-hexanol, as well as higher ethyl esters such as ethyl butanoate, caprate, lactate, laurate, myristate and palmitate. Column still whiskey, on the other hand has higher concentrations of some fusel alcohols, *iso*amyl acetate, 2-phenylethanol and 2-phenylethyl acetate. Apart from these differences, pot still and column still Bourbons have many common congeners, with over 300 compounds being identified, if other whisky types are included. Of these, only a few (known as odor active compounds) possess odor activity values (OAV $>$ 1) (Section 4.7.3) that allow them an individual contribution the overall spirit aroma, with the most active of these being known as key odorants. However, many of the other congeners with lower OAVs ($<$1; background volatiles) are now thought to modify the aroma of key odorants and consequently it is believed that they make a valuable joint contribution.

Techniques such as GC-olfactometry ('sniff GC') (Section 4.3.2) and dilution to odor theshold methods such as aroma extract dilution analysis (AEDA) have been used to distinguish odor active volatile compounds from the background volatiles in the matrix (Connor *et al.*, 2001; Poisson and Schieberle, 2008a; 2008b). Connor *et al.* (2001) used GC-O on SPME extracts of Scotch whisky and found *cis*-whisky lactone and vanillin to be important contributors to whisky aroma. Previous to that, it had been proposed, on the basis of odor activity values (OAV = concentration/odor threshold value (OTV) in aqueous-ethanol; see Section 4.7.3) on components of model whisky, that butanal, 2,3-butanedione, 3-methylbutanal, methylpropanal and certain esters, such as ethyl acetate and ethyl hexanoate were important whisky aroma compounds (Salo *et al.*, 1972).

More recently, the application of aroma extract dilution analysis (AEDA) has been applied to the identification of key aroma compounds from fractionated solvent extracts of Bourbon (Poisson and Schieberle, 2008a). On the basis of the highest flavor dilution factors (FD), the key odorants (from 45 odor active compounds) were found to be (in order of decreasing FD values and not including an unknown flowery compound) (*E*)-β-damascenone; γ-nonalactone; 4-allyl-2-methoxyphenol (eugenol), γ-decalactone, 4-hydroxy-3-methoxybenzaldehyde (vanillin) and *cis*-whiskylactone (Figure 3.3.3). Overall, these compounds make an

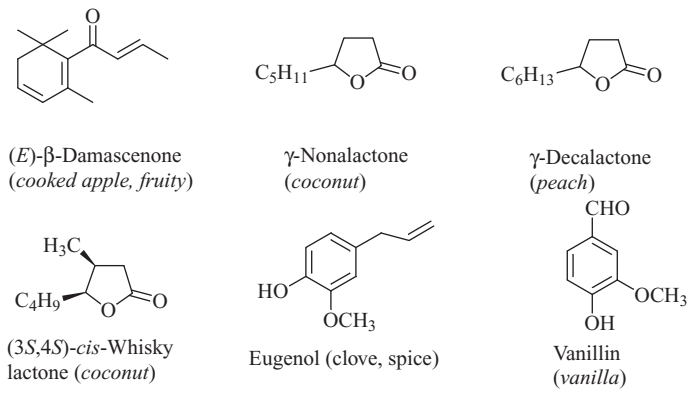

Figure 3.3.3 *The six most aroma active compounds of Bourbon whiskey. From Poisson and Schieberle (2008a)*

important contribution to the perceived overall fruity, smoky vanilla note of the Bourbon sample. Additionally, 3-methylbutan-1-ol (malty note), β-ionone (violet note), 2-phenylethanol (flowery), *trans*-whiskylactone (coconut note) and ethyl (*S*)-2-methylbutanoate (fruity) make important contributions. Furthermore, several unsaturated aldehydes ((*E*)-2-decenal, (*E*)-2-heptenal and (*E*,*E*)-2,4-nonadienal) contributing green or fatty notes were identified for the first time in Bourbon.

Thirty-one of the 45 odor active components of Bourbon whiskey were quantified by stable isotope dilution assays, their odor threshold values (Section 4.7.3) were determined in ethanol:water (4:6) and hence their odor activity values (Section 4.7.3) could be calculated (Poisson and Schieberle, 2008b). Twenty-six of these components had OAVs >1 and hence can be considered to make a direct contribution to Bourbon aroma. The compounds with the highest OAVs included (in order of decreasing values) ethanol, (*S*)-ethyl (2)-methyl butanoate, 3-methylbutanal, vanillin, (*E*)-β-damascenone, ethyl hexanoate, ethyl butanoate, ethyl octanoate, 2-methylpropanal, (3*S*,4*S*)-*cis*-whiskylactone, (*E*,*E*)-2,4-decadienal, 4-allyl-2-methoxyphenol (eugenol), ethyl 3-methylbutanoate and ethyl 2-methylpropanoate. Aroma recombination experiments using the 26 key aroma compounds dissolved in ethanol:water (4:6) allowed the aroma of Bourbon whiskey to be mimicked and omission studies supported the importance of all the esters, ethanol, *cis*-whiskylactone and vanillin for the overall aroma of Bourbon.

GC-O analysis of the headspace volume above the whisky indicated 23 compounds with the greatest odor activity, of which 3-methylbutanal (malty) had the highest RDF factor (smallest headspace volume in which the aroma compound is detectable), followed by ethanol, ethyl methylpropanoate (fruity), 2-methylbutanal (malty), 1,1-diethoxyethane (fruity), 3-methylbutanol (malty) and an unknown sulfury compound (Poisson and Schieberle, 2008a).

The norisoprenoids (*E*)-β-damascenone, α-damascenone and β-ionone are believed to be derived from carotenoids and have been found in a range of distilled spirits and wines. The immediate precursor of (*E*)-β-damascenone is thought to be 3-hydroxy-7,8-dihydro-β-ionol (Roberts and Acree, 1995).

The whisky lactones and the other odorous lactones are derived from the wood of American white oak during maturation; they are generally found at higher levels in whiskies aged for long periods in heat treated oak casks (Connor *et al.*, 2001). The unsaturated aldehydes (*E*)-2-nonenal and (*E*,*Z*)-2,6-nonadienal (already found in other whisky samples) and the new ones described above are probably formed by degradation of cereal lipids, which are abundant in maize. However, linoleic acid is also formed during fermentation and lipids can be released from oak wood during aging, so there are three possible sources for these aldehydes. See Figure 3.3.3 for the six most odor-active components of Bourbon whiskey.

A key feature of American whiskeys in general (and of course many Scotch whiskies and premium rums) is the use of charred casks made from the American white oak. Some companies, such as Brown-Forman have their own cooperages, but each year over half a million casks are supplied to the Bourbon industry by the Independent Stave Company of Lebanon, Missouri. Casks are made preferably from 80 year old trees, since at that age there is much tylose present to block the vascular channels in the heartwood, thus increasing the waterproofing ability and decreasing the permeability. American white oak casks are known to cause slower maturation than French oak casks of similar size, because of the coarser grain of French oak wood. Also, American white oak wood contains less tannin than French oak, so it is considered by many to be more suitable for the maturation of whiskies, where excessive tannin is not required.

Oak wood for making casks is treated in different ways according to the cooperage and according to the type or brand of whiskey it is destined to contain, but a general scheme is outlined next. Wood for casks is usually left in the open for four weeks or so, during which time its water content falls from ~40% to ~30%. It is then taken to a pre-dry shed for about four weeks where it is subjected to steam derived humidity at ~30 °C, starting at 60% humidity and dropping to 40% at the end. Following this, the wood, now containing ~20% water is moved to the kiln where it rests for about a week at ~60 °C in the presence of mildly turbulent hot air. After this, the water content is down to 10–14% and the wood is ready for fashioning into casks.

The wood for cask heads is planed and cut into circles. On the other hand to make staves, logs must be hand split to preserve the wood grain and endow. The logs are quartered and then cut to the appropriate length, tapered at each end, beveled, planed on the outside and given a second planing to induce a slight cupping on the inside. Sufficient staves for a cask are assembled together and a temporary steel hoop is placed over them. The whole is steamed for ~15 minutes to make the staves more pliable. Next, a cone device bends the staves into shape and a hoop is placed near the top. This is followed by dry heating to maintain pliability of the wooden staves, allowing them to be pressed together and leveled, so that later the cask heads can be fixed on securely. Next, two temporary bilge hoops are added to the middle, the staves are pressed tightly into place by a 'roll out' machine and a 'trusser' machine fixes the hoops tightly. The trussed staves are now ready for charring.

Charring of trussed staves is carried out using rotating blow flames so that an even char is obtained. There are five levels of charring, on a scale of 1–5, depending on the time of exposure to the flame. Each distillery has its own requirements for charring, but the most common is grade 4, which requires 1 min exposure to the flames. The heads are treated in a similar way, moving on a conveyer belt over the flame.

The whole cask is then assembled by fitting the heads onto the staves. The ends of the joined staves are trimmed and a 'croze' or groove is cut into the staves, near to each end, to receive the barrel heads (Figure 3.3.4). Barrel heads are custom cut to fit the croze, and are assembled using dowels and reed to make a perfect seal. The heads are fitted into the croze and the head hoops are place in position. The cask is tested for leakage and if passed, it receives a final planing and some sandpapering to give a pleasing finish.

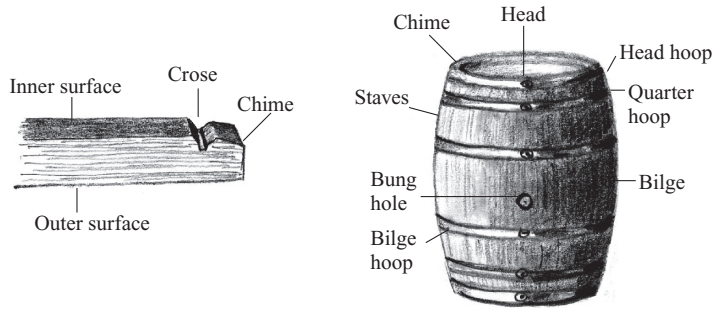

Figure 3.3.4 *Anatomy of a whiskey cask and close-up sketch of a stave top, showing where the cask head fits*

Some cooperages, such as the Bluegrass cooperage of Brown-Forman subject their cask interiors to toasting by a small flame as well as charring. Toasting results in:

- Decomposition of some of the wood lignins to vanillin and phenols (such as eugenol)
- Decomposition and oxidation of lipids to give lactones, such as *cis*- and *trans*-whiskylactones
- Caramelization of wood carbohydrates to furan and other derivatives
- Formation of brown coloring compounds via caramelization and Maillard reactions
- Decomposition of some polyphenolic compounds into simpler units, such as gallic acid.

The charring process results in a layer of black material over a thin layer of red toasted wood, rich in the products of toasting mentioned above. Processes occurring during maturation in oak casks are discussed for wine in Section 2.9.5 and for Scotch whisky in Section 3.2.5), so the discussion here will be limited to the influence of charring and toasting. During the aging of whiskey in charred oak casks, the charred layer, which contains many tiny fissures, acts as a filter, allowing some evaporation and some ingress of oxygen. It also acts as an adsorbent material for some unwanted compounds that when present in whiskey at higher concentrations give it an immature or rough character. At the same time, compounds from the red toasted layer are slowly leached into the whiskey, where they contribute certain aroma/flavor characteristics: vanillin and phenols give vanilla and spicy notes, lactones give coconut notes and furans give caramel notes. Additionally Maillard compounds contribute to the golden color of Bourbon and the phenolic compounds contribute to 'mouthfeel.'

Much Bourbon is matured in thermostatted warehouses, so that at higher temperatures whiskey expands into the wood material, but on cooling it contracts, bringing into solution flavoring and coloring compounds out of the red toasted layer.

A process that has some affinity with the charred cask method is the Lincoln County process of subjecting new make Tennessee whiskey to contact with charcoal immediately before transferring the whiskey to casks for aging. The charcoal is produced by burning sugar maple wood; it has a low surface area ($\sim$300 m^2/g) and so adsorbs a certain amount, but by no means all of the congeners that give new make whiskey its harshness. The purpose of charcoal treatment is to mellow the spirit before its long sojourn in cask. Hence the charcoal vats are sometimes known as mellowing vats. Treating spirits with charcoal is not unique to American whiskey, as it is used in much vodka production (Section 3.4.3), and sochu and soju production (Section 3.4.4). The main difference is that the use of activated charcoal with a surface area up to $\sim$1500 m^2/g to treat vodka strips away all flavor compounds and leaves an almost odorless and tasteless spirit.

The wood for charcoal is cut from shaded sugar maple trees in the autumn. After drying in air, the wood is cut into $\sim$10 cm $\times$ 10 cm $\times$ 1.5 m billets, which are stacked on open ground (not in a pit like charcoal is often made) six at a time, criss-crossed in layers forming 'ricks' about 2–2.5 m high. The ricks are set in squares of four, with a definite lean toward the center, so that the structures collapse inward when burning. The ricks are doused in alcohol and set alight, the fire being controlled by spraying with water. This prevents too much oxygen access to the burning wood, keeps down the temperature and hence prevents the formation of ash; too much water, however, leaves unburned wood with the charcoal. Burning takes about three hours, after which time the charcoal is allowed to cool before being broken up into pea sized pieces.

The vats at the Jack Daniel distillery are about 1.5 m in diameter and 3 m deep. They are filled to a depth of 2.5 m with charcoal pieces, with a white wool blanket at the bottom and new make whisky is sprinkled on to the surface from two crossed perforated tubes at a rate sufficient to give a flow of 30 l/min at the bottom of the vat. The charcoal bed is replaced every six months or so and in total there is about a 1% loss of spirit penalty to pay for the mellowing process.

At the Dickel distillery, the mellowing process is performed in refrigerated vats. A wool blanket is placed at the bottom of the closed off vat, followed by the charcoal pieces and another wool blanket at the top. New make whiskey (or whisky as it is called at Dickel) is trickled onto the top blanket whence it fills the vat,

permeating the whole of the charcoal bed. When filled, the valves at the bottom of the vat are opened and the mellowed whisky is transferred to a spirit vat for casking. The refrigeration is used because it was noticed many years ago at Dickel Distillery that the whisky mellowed in the cold winter months was superior. Soaking the charcoal bed with whisky is reckoned to give more efficient contact and to prevent inefficiencies caused by channeling. In this way, a charcoal bed at Dickel lasts about a year, but at either distillery, the time to replace a charcoal bed is judged by nosing and tasting the outcoming whiskey. A new charcoal bed takes out rather too much of the whiskey flavor components as well as less desirable elements, whereas toward the end of its life it is perhaps just beginning to lose its effectiveness at adsorbing all the less desirable components. Once again, taking into consideration the variations mentioned above as well as other variations (e.g. due to different cask characteristics), the blenders play their important role by assembling which casks are to be vatted (blended) before bottling, thus providing a uniform product.

3.3.5 American Rye and Corn Whiskies

Rye is an important cereal for the production of Korn (sometimes more generally termed Schnaps) and Vodka in northern Europe and so it was natural for the German immigrants of Maryland and western Pennsylvania to grow rye and produce rye whiskey from it.

American rye whiskey must be made from a minimum of 51% rye, distilled at less than 80% ABV and aged for a minimum of two years in new charred casks. Unless the distillation process includes extensive rectification, rye distillate has a characteristically pungent aroma and hard edged spicy, grain-like flavor. According to American law, a highly rectified spirit (say, distilled at 95% ABV) produced from rye beer could not be called rye whiskey. It is probably this rather uncompromising nature of rye whiskey that led to its decline during the second half of the twentieth century. National Prohibition played even greater havoc with rye whiskey than other with American whiskey types, since during that time and during World War II, peoples' palates became better attuned to more neutral and more delicate spirits; bootleg and smuggled liquor at first, imported and legally home produced liquor later. The market for rye whiskey diminished so considerably that nowadays the major brands of straight rye are produced by the Bourbon distillers of Kentucky and Indiana, as well by Jack Daniel's Distillery in Tennessee.

More recently, there has been a surge of interest in American rye whiskey, with several Bourbon distilleries producing premium straight rye whiskeys, often with old Pennsylvania or Maryland brand names or original distillery names (Regan, 2007). These include Rittenhouse Rye 100 Proof, Pikesville (both Heaven Hill Distillery), Sazerac 18 year old, Van Winkle Family Reserve Rye 13 year old (both Buffalo Trace Distillery), Red Hook Rye 23 year old and Mitcher's 10 year old Straight Rye Whiskey (Kentucky Bourbon Distillers), Old Overholt four year old (Jim Beam Distillery) and Russell's Reserve Rye (Wild Turkey Distillery). Other straight rye whiskeys produced at Bourbon or Tennessee distilleries include Jim Beam Rye, Wild Turkey Straight Rye Whiskey and Jack Daniel's Rye.

Apart from the products of the Bourbon distilleries, two premium rye whiskeys have recently emerged from new distilleries. The first is Old Potrero distilled at the Anchor Distillery (under the same ownership as Anchor Brewery) in San Fransisco. It is a single malt distilled in pot stills from beer made with 100% malted rye and is matured in toasted (rather than charred) oak casks. There are currently three versions: Eighteenth Century Style and Nineteenth Century Style (neither with an age statement) and Hotaling's, a 13 year old whiskey. The second is from the Mount Vernon Distillery in Washington, close to George Washington's home; the whiskey is allegedly based on the rye whiskey produced by George Washington. Although the number of straight rye whiskeys is increasing steadily and even includes a number of premium and single malt brands, the majority is used for blending with grain neutral spirit and other straight whiskeys (to produce blended whiskeys) or with other straight whiskeys (to produce blended straight whiskeys).

Canadian whisky was once referred to as 'rye whisky,' because at one time rye was high in the grain bill of such whiskies. However, for many years now rye has been a minor component of the grain bill of most Canadian whiskies (the major cereals being maize and wheat, but see Section 3.3.6 for some exceptions), which are characterized by sweeter, less spicy, perfumed and pungent characters than American rye whiskeys. Furthermore, Canadian law makes no distinction between (Canadian) whisky or rye whisky, provided it possesses the aroma, taste and character associated with Canadian whisky (Section 3.3.6). This has inevitably led to some confusion and there are still those who believe that Canadian whisky is essentially rye whisky. All this is especially ironic considering that American rye whiskey was displaced largely by Canadian whisky when prohibition ended in 1933.

According to US law, corn whiskey must be distilled from beer made from a mash containing at least 80% corn (maize); Bourbon must contain less than 79% corn. Like other American whiskeys, it must be distilled at less than 80% ABV, but unlike the others, there is no obligation to age it in oak casks. However, if it is matured in oak, then the law specifies two years in new or used uncharred casks. This type of whiskey, when made in pot stills, is probably the closest to the first grain whiskeys produced by the early colonial settlers in Virginia and neighboring states. It was produced openly by most farmers in these areas for home consumption and for trading until permanent excise taxes were imposed after the Civil War. Production of corn whiskey by small farmer-distillers then went 'undergound' and became known as 'moonshine.'

Today, most corn whiskey is produced using column stills and is used for blending after storage in cask for at least two years, but at Belmont Farm Distillery in Virginia a fresh (unaged) clear spirit 'Virginia Lightning' is made from almost 100% corn mash using pot stills. The home grown corn is ground and cooked in a stainless steel cooker. On cooling, crushed barley malt is added to achieve saccharification and the mash is fermented with added yeast. After fermentation (about four days), the mash is transferred to the 2000 US gallon (7000 l) copper pot still, where the first distillation takes place. A second distillation in a doubler is carried out to purify the spirit and to increase its alcoholic strength.

3.3.6 Whiskies from Other Countries

Canada

Canada, India and Japan figure prominently amongst a large number of other countries that produce grain spirits called whisky. Canadian whisky is consumed worldwide and constitutes a significant proportion of distilled spirits consumption in many countries, particularly the USA (Corrigan, 2004). Domestic Canadian whisky is often described as 'rye whisky,' despite the fact that rye malt is only a minor part of the grain bill for the big majority of products. According to Canadian law, Canadian whisky or Canadian rye whisky or rye whisky must be made according to the following criteria (CRC, 2009):

- It must be a potable alcoholic distillate, or a mixture of potable alcoholic distillates, obtained from a mash of cereal grain or cereal grain products saccharified by the diastase of malt or other enzymes and fermented by the action of yeast or a mixture of yeast and other organisms
- It must be aged in small wood for not less than three years
- It should possess the aroma, taste and character generally attributed to Canadian whisky
- It must be manufactured in accordance with the requirements of the Excise act and the regulations made thereunder
- It must be mashed, distilled and aged in Canada
- It should contain not less than 40% alcohol by volume
- It may contain caramel and flavoring.

In practice, the big majority of Canadian whisky is produced mainly from maize and wheat, with malted barley and rye generally playing a minor role from a quantitative sense, but the last two are important for supply of diastase enzymes for saccharification of gelatinized starch derived from maize (mostly) or wheat. Although barley and wheat are grown in nearly all the provinces, maize is grown extensively only in southern Ontario and Quebec, and indeed, Canada is a net importer of corn, most of the imports coming from the USA.

The production of most Canadian whisky in some ways resembles that of Scotch grain whisky (Section 3.2.3), based on maize or wheat, it is distilled in continuous Coffey stills and matured in oak casks for 4–12 years (although the law requires a minimum of three years). The main difference is in the blending: Canadian blends are composed of different grain whiskies, Scotch blends contain both grain and malt whiskies. The multiple continuous stills allow fine temperature control and, by increasing or decreasing the number of plates, at one extreme, grain neutral spirit (∼96% ABV) can be produced, or at the other extreme, more flavorsome distillates (at ∼60–70% ABV) can be made. The blander and more strongly flavored distillates are blended to give a product that is representative of the particular brand, according to the style of the company (nearly all Canadian whisky distilleries are owned by international companies). In general though, the emphasis is on lightness and delicacy, features that render Canadian whisky good for making mixes and cocktails.

Growth in Canadian whisky consumption was just 1.6% in the USA between 2002 and 2006. Value brand consumption dropped, but as in other distilled drinks sectors, sales of premium and super premium brands increased (Peck, 2008). Crown Royal (Diageo) was the sales leader in the USA (∼25% of Canadian whisky sales) in 2008, kept there by growth of two ultrapremium whiskies: Canadian Royal XR and Canadian Royal Cask No. 16. Wiser's was the leading Canadian whisky in Canada in 2008 (Peck, 2008), but Canadian Club (Beam Global/Fortune Brands) and Canadian Mist are better known outside Canada.

At the time of writing (2009), there is only one single malt whiskey produced in Canada, the 10 year old Glen Breton Rare Single Malt Whiskey, distilled and matured at the Glenora Inn and Distillery on Cape Breton, Nova Scotia. This whiskey, produced in the Scottish style was threatened a few years ago by an objection from the Scotch Whisky Association (Olver, 2007). The objection was related to the use of the word 'Glen' in the name – the product could be mistaken for being of Scottish origin. Canada's Trade-Marks Board over-ruled the objection, so the production of Glen Breton continues. Also at the time of writing, the construction of Pemberton Distillery (British Columbia) is underway. It is intended to produce a malt whisky, as well as vodka, using organic produce.

The Canadian distiller is probably subjected to more government control than any other distiller. Excise officers are allocated specific distilleries, where they exert supervision of the whole process, from arrival of the grains through fermentation, distillation, maturation to filtration and bottling. However, the excise office does not interfere with actual techniques; each distillery has complete control over the quality and character of its products.

Japan

Japan is the world's fourth largest producer of malt whisky (after the USA, Scotland and Canada) and the second producer of single malt whisky (after Scotland). The first Japanese distillery (Yamazaki) was set up by Shinjiro Torii (founder of Suntory) in 1924, in collaboration with Masataka Taketsuru, who in 1919–1920 had undergone some training in Scotland (Bunting, 2009). In 1934, the latter went on to found Nikka, Suntory's main modern rival in Japanese malt whisky. The military were the first major customers, whisky being an important beverage of the Imperial Japanese Navy. After World War II, the industry expanded enormously, under a protectionist regime that imposed high taxes on imported whisky, but the main product was blended whisky, sometimes made using neutral spirit in place of grain whisky in those days. In the 1990s the protectionist rules were removed, young upwardly mobile Japanese shifted their interests to the indigenous

Figure 3.3.5 *Sketch map of Japan, showing whisky distillery locations*

shochu (Sections 1.3.5 and 3.4.4), rich, more elderly men looked for imported whiskies, so the domestic market for mass whisky more or less collapsed. The surviving whisky distillers were forced to concentrate on premium products, particularly single malt whiskies for both domestic consumption and export. Blends are still produced, and have won international prizes (Bunting 2009; MacDonald and Ushio, 2008), but it is in the single malt category that Japan has excelled in the first decade of the twenty-first century. Several prizes have been won by Japanese malts from 2001, culminating in the capture of the gold award at the International Spirits Challenge by a Yoichi (1987) 20 year old malt whisky (Nikka) in 2008).

There are currently eight malt whisky distilleries in operation (Figure 3.3.5) and, like Scotland, several have been demolished or mothballed or diverted to other uses in recent years (Bunting, 2009). All but two of the working distilleries now producing malt whisky belong to the large conglomerates Nikka, Kirin and Suntory (Figure 3.3.5). The last mentioned company is the original and biggest whisky distiller in Japan. Its Yamazaki Distillery, built in 1923 was Japan's first, producing the Suntory Shirafuda blended whisky (released in 1929) initially. The distillery has 12 copper pot stills: six low wine and six spirit stills, most of the former being direct flame-heated (gas fired), whilst all the latter are steam heated. Good, iron free water is obtained from wells. This distillery produces single malts of various ages, from 10 to 50 year old, as well as Sherry wood aged malts, mostly at 43% ABV.

Suntory's Hakushu Distillery site has two buildings, the original one was built in 1973 and was for a few years the world's largest distillery. It housed no less than 24 copper pot stills (Jackson, 1989), producing malt whiskies for blends, but appears to be silent nowadays. The second building, known as Hakushu Higashi was built in 1981 and houses 12 pot stills with rectifying columns that produce 10, 12 and 18 year old malt whiskies at 40 or 43% (ABV). Suntory also has a grain distillery, known as Sungrain.

Japan's second largest whisky distiller is Nikka, with one distillery at Yoichi (Hokkaido) and the other near Sendai in northern Honshu (the Miyagikyo Distillery). The former was the original distillery, built in 1934 and housing at that time just one pot still that doubled as a low wine and spirit still. Now there are six coal fired stills. Hokkaido's climate is much more temperate than that of the Japanese islands further south, and although it is warmer than Scotland, it does have peat moors – peat was once used by the distillery. The

Yoichi Distillery is close to the sea and its water comes from underground aquifers. The single malts from Yoichi are some of Japan's most distinctive, ranging from cask strength versions to 10, 12 and 15 year old products of 43% or 45% ABV. The Miyagikyo Distillery was built in 1969 with eight stills. It produces a similar range of malt whiskies.

The Scots tradition has been largely upheld over the years and most of the distilleries are in maritime locations, so that Japanese and Scotch malt whiskies tend to have similar characteristics, with a similar range of congeners. Many of the production aspects of Japanese whisky have been studied over the years, especially during the present decade. This includes the influence of brewers' and distillers' yeasts (Wanikawa *et al.*, 2004) and the influence of lactic acid bacteria on spirit quality (Takatani and Ikemoto, 2004). Brewers' yeast was found to give higher concentrations of γ-lactones, unsaturated fatty acid ethyl esters (especially ethyl 9-decenoate) and other esters (such as ethyl 2-methylbutanoate) in the wash and also in the distillate (Wanikawa *et al.*, 2004). These components were shown by sensory analysis to be responsible for sweet, fatty notes and fruity notes (respectively) in whisky, especially new make spirit. It was shown that they are produced in greater quantities by lactic acid bacteria, which thrive in the wash fermented by brewers' yeast (but not distillers' yeast) as a result of the early death of the yeast cells. *Lactobacillus casei*, *L. plantarum* and *L. brevis* were found to thrive at lower temperatures, early in the fermentation, whereas *L. acidophilus* and *L. fermentum* were dominant at the higher temperatures of the mid to late fermentation period (Takatani and Ikemoto, 2004). The individual species of *Lactobacillus* gave specific estery, fatty, grassy sweet and other characteristics to the new make spirit, with *L. casei* giving, citrus, creamy and spicy notes of some complexity. If thermophilic bacteria such as *Streptococcus thermophilus* act upon the wort during mashing to reduce its pH from ∼5.9 to ∼5.5, both fermentation and the lactic acid bacteria activity were enhanced, leading to greater alcohol content of the wash and greater complexity of the resulting spirit, especially with regard to estery and fruity notes. Thus, although the basic flavor framework of the whisky is derived from alcoholic fermentation by *Saccharomyces cerevisiae*, beneficial supplementary flavors can be supplied by controlled (i.e. not dominant) bacterial activity. Lessons learnt from studies of these kinds by the Japanese and other whisky industries may well contribute to improved malt whisky quality.

At the other end of the production process, longer aging gives a higher concentration of phenols in the finished whisky, especially ellagic and tannic acids (derived from tannins) and lyoniresinol (derived from lignins) (Koga *et al*., 2007). It is thought that these compounds in the whisky contribute to the mature, smooth mouthfeel in two ways: firstly by enhancing the clustering effect of ethanol (Tanaka *et al.*, 2002; Nose *et al.*, 2004) and water molecules, and secondly by acting as reactive oxygen scavengers. Nonhydrated ethanol molecules are thought to give a rough mouthfeel through generating reactive oxygen species. Since many studies of the production methods and sensory characteristics have included Scotch and Japanese malt whiskies together, these have been discussed in greater detail in Sections 3.2.4 and 3.2.5.

India

Alcoholic beverages of various kinds were produced in India as far back as Vedic times and it is possible that the first distillations of fermented drinks were carried out in India or China. Nowadays, many traditional fermented beverages are still produced and large volumes of molasses based spirit are consumed by the countryside population. These are known as arrack and tharra (Section 3.5.2) and it is estimated that 250 million cases of arrack are consumed each year in India (Maitin and Stephen, 2004). At present, brandy, gin, rum, vodka and whisky produced in India are known as 'Indian-Made Foreign Liquors' (IMFL), of which *ca.* 80 million cases are produced, with *ca.* 60% of that being labeled whisky. Unlike the whisky of other countries, most Indian whiskies are based on grain neutral spirits distilled from fermented molasses. This is largely because molasses is a by-product of sugar refining (and cane sugar grows and yields well in much of India), so there is little competition for it as a food or other source, whereas the competition for barley, maize, rye and wheat as food means that very little of these crops are used to produce distilled spirits.

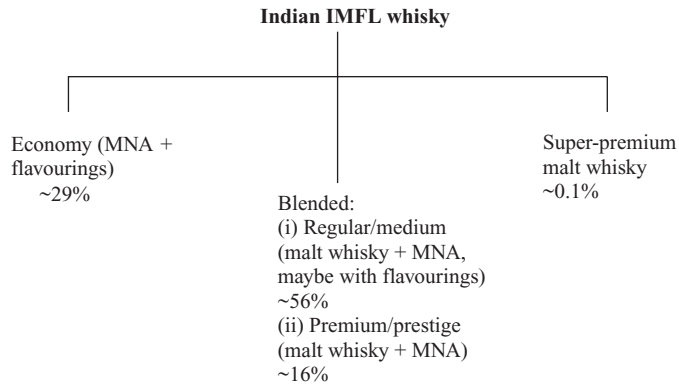

Figure 3.3.6 *Classification of Indian-made whisky. Approximate market share for early 2000s shown. Data from Maitin and Stephen (2004)*

The classification of present day Indian IMFL whisky is shown in Figure 3.3.6, where it can be seen that blends of malt whisky and molasses neutral alcohol (MNA) and flavored MNA type whiskies together form the overwhelming bulk of indigenous Indian whisky. Neutral spirits are produced from molasses in continuous column stills, as in other countries, and likewise the potable portion is only a fraction (in India about one third) of this, the rest being used for industrial and other purposes. Malt whiskies are produced in pot stills and matured in small oak casks, like their Scots counterparts.

A comparison of the flavor profiles of super premium Indian whiskies and the blended categories generally reflects the different materials and methods. Single malt or super premium whiskies have greater malt, oak and peat (smoke) character, along with a better mouthfeel, but some economy whiskies, whilst being low in these flavor attributes, can match the single malt whiskies on fruity, spicy and sweet characteristics, as a result of the flavorings used. The other blended whiskies tend to have intermediate flavor profiles, although some premium blends can match the single malts on floral and peat character (Maitin and Stephen, 2004). Correspondingly, Indian single malt whiskies are higher in congeners associated with pot still malt whisky: ethyl acetate, ethyl caprylate, ethyl laurate, *iso*amyl acetate, *iso*amyl alcohol, 2-methylpropan-1-ol and 1-propanol, for example.

Naturally, the blended whiskies and economy style are the lightest and were preferred by the majority of Indian whisky drinkers in 2004 (Maitin and Stephen, 2004). However, as more Indian people travel and taste foreign liquor, tastes may change and a growing middle class is already acquiring a taste for more flavorsome whisky of the premium or super premium type. Moreover, taxes on spirits imported into India have dropped, leading to greater competition from imports, and reciprocally, India is producing more malt whisky, including single malts, to improve its international standing and to challenge internationally renowned spirits such as Scotch whisky and rum (Battacharjee, 2004).

Europe

Whisky is produced in several European countries, apart from Ireland and Scotland. In the British Isles, there is a distillery in England (St. George's Distillery at Roudham in Norfolk), the Isle of Man (ManX) and Wales (Penderyn Distillery). The oldest of these distilleries is ManX, originally producing a whisky known as Glen Kella. Nowadays, the Manx distillery makes a unique spirit by taking a previously matured Scotch malt whisky and redistilling it in such a way as to preserve the major aroma and flavor profile. The result is a colorless, but flavorsome spirit that the company claims is ideally suited to making cocktails, but is also good to sip neat.

Much more recent is Penderyn Distillery, which opened in 2004 in the village of that name, near the Brecon Beacons. It produces a malt whisky using a hybrid pot still with a rectifying column above the pot, rather like stills that are used for some forms of pomace spirit (Section 3.7.2) and fruit spirit (Chapter 3.8). The column has seven perforated plates and so in theory can be used to produce a range of distillates, from light to heavy, by adjusting the amount of reflux. In practice, a highly rectified distillate (~92% ABV) is collected. It is diluted to 63.4% ABV before maturation in ex-Bourbon casks (many coming from the Buffalo Trace Distillery, see Section 3.3.4). Before bottling, it spends some time in ex-Madeira casks and it is probably this finishing touch that gives the whisky its distinction (it has been rated very highly by professional tasters). The Welsh Whisky Company, who owns the distillery, has succeeded in making the first Welsh whisky for about 100 years. The company also makes gin, vodka and a cream liqueur and is located in an area famed for the quality of its mineral water. Penderyn Distillery water flows through limestone into underground aquifers where it is retrieved and used for all distillery operations requiring water (except cleaning).

St. George's, which started distilling in 2006, is the youngest of these new distilleries. It produces unpeated and peated single malt whiskies that, at the time of writing (2009) are lying in casks in a bonded warehouse, with some of 2006 distillations being offered for sale.

There are several whisky distilleries in continental Europe, the best known being the Glann ar Mor Distillery on the northern coast of Brittany (Côtes d'Armor). This distillery, owned by the Celtic Whisky Compagnie, uses small flame heated copper pot stills with worm tub condensers, like a few Scotch malt whisky distilleries. Unpeated and peated malt whiskies are produced, all of which are aged in ex-Bourbon casks and some of which subsequently spend time in ex-Sauternes casks. The first distillation was in 1999, with the first bottles (Taol Esa) being offered for sale in 2005. The company also produces an impressive range of whiskies known as 'Celtic Connection,' which are matured Scotch malt whiskies rematured in ex-brandy or more exotic ex-wine casks, such as Jurançon, Monbazillac and Quartes de Chaume. Additionally, the company provides a good range of independently bottled Scotch malt whiskies (Section 3.2.6).

Sweden now has five malt whisky distillery companies (Box Destilleri, Gotland Whisky, Grythyttan Whisky, Mackmyra Svensk Whisky and Spirit of Hven), but only Mackmyra Svensk Whisky is distilling on a larger scale. This distillery started small-scale production in 1999, which was upgraded in size in 2002. So far, official bottlings are 'Den första utgåvan,' 'Preludium: 01 to 06' and 'Privus: 01 to 06.'

Korn is a German spirit that can be made from barley, buckwheat, rye and/or wheat. In practice, the most prestigious versions are distilled from wheat wash using column stills to give a highly rectified spirit, but one that retains some flavor of the original ingredients. It is lightly filtered and often spends a year in oak casks before dilution with deionized water and bottling, so it often has a pale golden color and has much more flavor than the more highly rectified and heavily filtered vodka, with which it is often compared. Korn is usually sold at ~32% ABV, versions with at least 38% ABV being known as 'Doppelkorn.' Korn is made in many areas of Germany, but is more popular in the north. One of the biggest producers is the Berentzen Gruppe; its output includes Berentzen Traditionskorn (from wheat), Dornkaat Korn and Strothmann Weizen (wheat) Korn and Doppelkorn. Other Korn manufacturers include Dieckmann (Helmstedt), Hegemann (Sprockhövel), Hoffe Alt (Rüssath-Hoffnungstaal), Kilian (Hünfelder-Dauborn), Mackenstedt (Bremen), Schmittmann (Düsseldorf) and Wöltingrode Kloster Kornbrennerei (Vienenburg, Braunschweig). The latter produces St. George Edelkorn, which is aged in ex-whisky casks. Many Korn distilleries produce other spirits, such as fruit spirits or aquavit; for example, the Berentzen Gruppe makes Bommerlunder Aquavit (Section 3.4.3).

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3.4
Other Cereal Based Spirits

When the clergyman's daughter
Drinks nothing but water
She's likely to finish on gin.
—Rudyard Kipling

3.4.1 Introduction and Scope

This section discusses a number of distilled spirits, other than whiskies, derived from cereals. Two of these, gin and vodka, have large world markets and together account for well over 30% of the distilled spirits consumed in the USA, presently the world's biggest consumer of spirits (Corrigan, 2004) and around 33% of the UK spirits sales by volume (Atkinson, 2004). The remainder, Akvavit (or Aquavit) and rice wine spirits, such as soju (Korea) and shochu (Japan) all have big markets in more restricted areas: northern Europe for the first two and Asia for the last named.

In many cases, versions of these spirits may be of noncereal origin. For example, some vodka is made from potatoes and either gin or vodka may be produced from molasses. Highly rectified spirit produced in three column continuous stills from any of the above sources has minimal flavor and so is ideal for use as a base spirit for cocktails and other mixes or for combining with natural or nature identical flavorings to give flavored spirits. The European Union merely stipulates that juniper flavored spirits (which includes gin) may be produced from 'ethyl alcohol of agricultural origin and/or grain spirit and/or grain distillate' (EEC, 1989). However, it is widely held that gin and vodka in particular are superior if originating from cereals (Lichine, 1982). That is why the following discussions on gin (Section 3.4.2) and vodka (Section 3.4.3) will be limited to their production from cereal grains; by the distillation of fermented cereal mash.

3.4.2 Gin and Similar Juniper Flavored Spirits

Gin is essentially a juniper flavored spirit, although several other flavoring agents (called 'botanicals') are also used in the many versions produced all over the world (Aylott, 2003). The name gin, now in universal use, is a corruption of the French word genièvre, meaning juniper. Other corruptions of genièvre, including geneva,

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

genever, genebra, ginebra and jenever, are used for specific kinds of gin. In the EU, all the above names may be used, depending on the circumstances, for what are classed as 'juniper flavored spirit drinks'; other, more local names such as peket and Wacholder may also be used where appropriate. Gin is normally produced from grain spirit to which juniper distillate and/or raw distillate of fermented juniper has been added. In many cases, this mixture is redistilled.

Although many botanical ingredients are used to flavor most gin, the major contribution comes from juniper berries. The juniper plant *Juniperus communis* grows in many areas of the world, where it produces an abundance of strongly flavored blueish berries. Juniper spirit per se is made in several central European countries by distilling a macerate of juniper berries in raw juniper distillate, itself produced by the distillation of a wine made by fermenting a juniper berry–water infusion. In Germany and Switzerland this 'juniper brandy' is known as Steinhäger. Juniper spirit is really a fruit spirit (Chapter 3.8), but is mentioned here as it is a close relative of gin distillates that are used to flavor many kinds of gin.

The first gin was produced in Holland during the seventeenth century at a time when spirits were still partly regarded as medicines. Fransiscus de la Boe, a physician and professor at the University of Leiden, while seeking a medicine for treating kidney disorders, found that the diuretic juniper oil could be introduced into the body more effectively by mixing it with either fruit or grain spirit. In the latter case, the juniper oil was found to greatly improve the taste, and hence his medicine, 'Genever' became popular in Holland and surrounding countries.

Its fame soon spread to England, where in the early eighteenth century (the early years of the Industrial Revolution) it was made essentially by distilling a juniper berry infused, hopped beer in small pot stills by a large number of artisanal distillers, particularly in London. Generally, it was not matured and the spirit probably contained high levels of undesirable and/or unhealthy components, such as methanol and fusel alcohols. Its rough, raw character was partially masked by the juniper and hop flavors and so its popularity grew alarmingly rapidly, causing great concern amongst governments, brewers and abstentionists alike. In an attempt to control the level of gin drinking, especially by the working classes, the government introduced the second Gin Act (1736), which required distillers to acquire an expensive licence and which imposed a duty on all the gin sold by the distillers, and further duties were payable by retailers, such as the owners of inns and taverns. Even though there was a heavy penalty for any person found selling gin without a licence, some 12 000 persons were prosecuted during the first two years following the passing of the Act (Young, 1979). Evasion was rife and so the Gin Act was repealed in 1743, the duty was removed and the distilling licence vastly reduced.

The discovery of redistillation and later of rectification allowed the production of a much more pure spirit that did not need aging in wooden casks.

Nowadays, gin can be found in three basic styles, as outlined in Figures 3.4.1–3.4.3. The Hollands, Geneva or Jenever style can be considered the original style, although there are several variations on this, as can be seen from Figure 3.4.1. Firstly, a beer (or wash) is made by fermenting the mash (Section 2.6.2) made from malted barley, rye and sometimes maize. Some distillers add juniper berries to the mash. After two or three days, the wash is distilled in pot stills and the middle distillate of which is redistilled with the added botanicals. The botanicals can be in the form of raw materials, but are more likely to be a distilled maceration of raw materials known as a gin distillate. The resulting spirit is usually aged in bulk, chill filtered and diluted with pure water to ~40% ABV before bottling (37.5% ABV is the minimum allowed by EU law). The process of a further redistillation in the presence of flavoring agents gives rise to double gin. Some Hollands gin is aged for several years in oak casks to give a more mellow character and golden color; this is virtually the only gin to be aged in this way. All Hollands gin has some grain flavor resulting from the congeners in the double or triple distilled spirit.

Nowadays, the London dry style of gin is the most prominent type, being produced in the USA and many countries other than the UK. An outline of its manufacture is shown in Figure 3.4.2, where it can be seen that

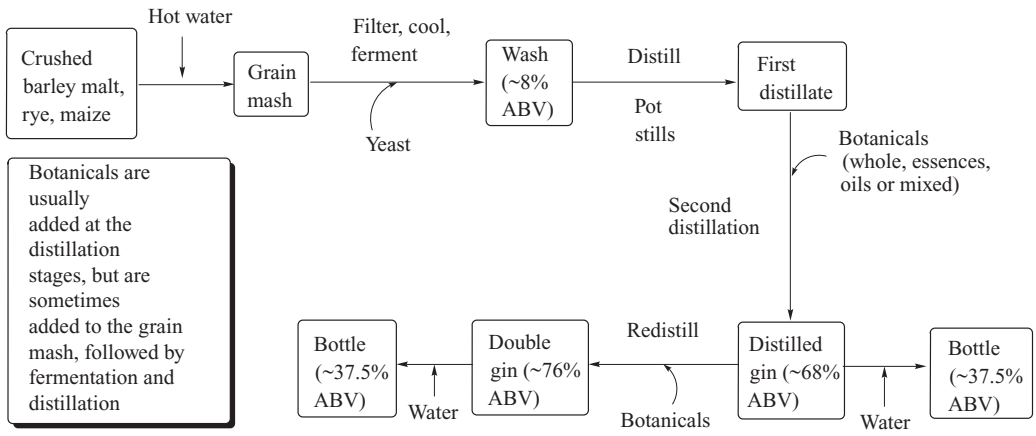

Figure 3.4.1 *Simplified scheme for production of distilled gin, such as Hollands or Geneva*

rectified or neutral grain spirit (usually produced from barley, maize and wheat) is the alcohol base, rather than the (essentially) more flavorful whiskey base used for Hollands gin. The neutral spirit will contain close to 96% (v:v) of ethanol concentration (EU law forbids use of spirit of greater strength than this), it will be low in congeners, including fusel alcohols and according to EU requirements (EEC, 1989) it must contain less than 50 mg/l of 100% ethanol by volume of methanol and less than 2 mg/l of 100% ethanol by volume of aldehydes (expressed as acetaldehyde). The botanicals are added and the mixture is redistilled. If this process is repeated, a London dry gin is the product. It contains ethanol, water and many flavor compounds from the botanicals, but very few of the compounds that render a new make double distilled spirit such as Scotch whisky harsh and raw. Hence, London dry gin needs no aging and can be bottled soon after distillation.

At the time of writing (2009), Plymouth gin is the only UK gin to have geographical protection; it can be produced only in Plymouth. Traditionally, it has been intermediate in style between London dry gin and Hollands gin, but nowadays it is probably rather closer to the former. Plymouth gin is made from crude spirit that has been distilled from wheat liquor at distilleries in London. The spirit is redistilled over botanicals 11 times in gas fired copper pot stills. It is diluted to ~41.2% ABV with deionized water before bottling.

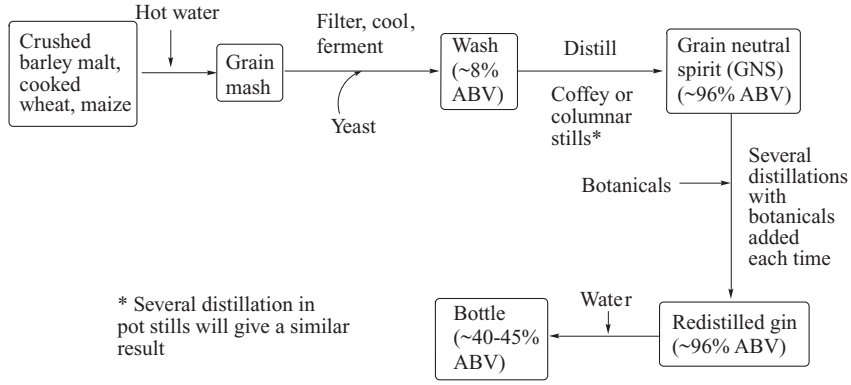

Figure 3.4.2 *Simplified scheme for production of redistilled gin, such as London Dry or Plymouth gin*

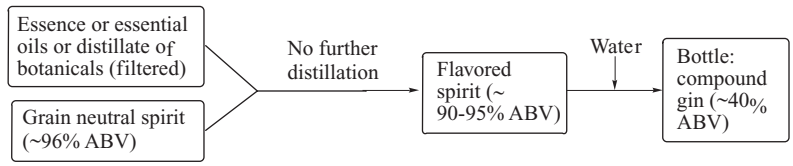

Figure 3.4.3 *Simplified diagram showing the production of compound gin*

In the EU, gin of the London dry or Plymouth type is known as 'distilled gin.'

The final type is an undistilled gin, whose typical manufacture is outlined in Figure 3.4.3. It is sometimes known as compound gin, although the EU allows the simple name of gin, but not distilled gin. It is simply made by mixing gin distillate with highly rectified neutral spirit of agricultural origin and diluting with deionized water to ≥35% ABV before bottling. This kind of gin can be made by amateurs and some less expensive commercial gin is produced this way; it is generally considered to be of inferior quality, but nonetheless is useful in the preparation of cocktails, mixes and 'ready to drink' beverages (alcopops).

The flavoring ingredients or botanicals are a crucial part of gin manufacture. Up to 100 ingredients can be used (Coates, 2000) to give additional complexity and each company has its own closely guarded secret recipe. Juniper berries, of course, are prominent, but many other ingredients are used, including allspice seeds, angelica root, cardamom pods, cinnamon bark, citrus peel, coriander seeds, ginger root, hop flowers, lavender flowers, orange flowers and orris root. Although mixtures of these raw materials can be used directly in the production of gin, most gin is made using mixtures of extracts and oils or most of all by using a distillate of a mixture of botanicals macerated in grain neutral spirit. The latter is known as a gin distillate or essence and an example of a method of preparing such a distillate, based on an example recipe given by Zach (2007), is shown in Figure 3.4.4. After steeping for two days the mixture is distilled slowly under vacuum, presumably not a high vacuum, and with the need to heat up to ~60 °C or so, as in the distillation of some commercial gin (Greer *et al.*, 2008).

The botanicals used to make gin, particularly juniper berries, are rich in monoterpenoids and other terpenoids, especially linalool from coriander seeds (see Table 2.12.1, Section 2.12.2). Although the monoterpenoids are important contributors to gin flavor, if present in high concentrations they can give the gin an overtly pungent aroma and flavor (Greer *et al.*, 2008). Furthermore, it appears that the application of heat during conventional distillation (~80 °C, no vacuum) of a maceration of juniper berries, coriander, dried

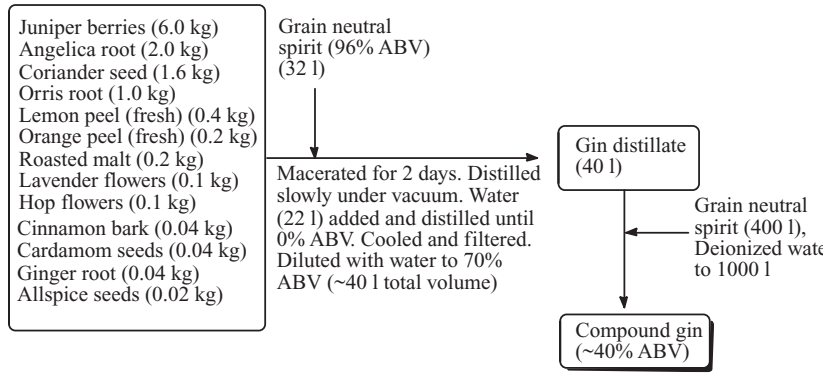

Figure 3.4.4 *Example recipe for compound gin. Based on Zach (2007)*

lemon peel and angelica in grain neutral spirit to make a model gin alters the flavor profile by causing the formation of extraneous monoterpenes (including α-pinene, (*E*)-caryophyllene, α-phellandrene and β-myrcene) compared with the predistilled botanicals (Greer *et al.*, 2008). Distillation of the same maceration at 0 °C under reduced pressure (<0.1 mm Hg) gave a distillate with much lower (fivefold in some cases) levels of monoterpenoids than the conventional distillate. The gin made with vacuum still distillate was judged by a tasting panel to be more flowery and pleasant compared with conventionally produced gin, which had greater pungency and spiciness (Greer *et al.*, 2008). The stainless steel and glass pilot plant consisted of a 20 l still attached via a lyne arm to a glass collecting reservoir containing a stainless steel liquid nitrogen coldfinger and connected to a positive displacement pump at the bottom and a vacuum pump (with 10^{-6} mm Hg capability) at the side. This vacuum distillation system has been scaled up to commercial size to produce trial lots of 'super premium' gin that has a flavor profile similar to the botanicals from which it was made.

Apart from the two mentioned above, there are several recent literature reports on the analysis of distilled gin or gin essential oil mixtures (gin distillates) (Vichi e*t al.*, 2005; MacNamara *et al.*, 2007; El-Ghorab *et al.*, 2008; Vichi *et al.*, 2008). Analysis of essential oils were carried out using gas chromatography, either by direct injection using two dimensional GC with spectral deconvolution (MacNamara *et al.*, 2007) (Section 4.3.2) or by solvent extraction (Greer *et al.*, 2008) or solvent extraction/column chromatography (Section 4.2.2) followed by GC analysis (El-Ghorab *et al.*, 2008).

Dichloromethane extraction of a steam distillate of *Juniperus drupacaea* L. berries, followed by fractionation using column chromatography and analysis by GC/MS/FID, El-Ghorab *et al.* (2008) were able to identify a large number of less volatile, as well as volatile, constituents. Although *J. drupacaea* is a native juniper shrub of the eastern Mediterranean countries, it is a relative of *J. communis* of northern and central Europe, the berries of which are used to make gin. Of the less volatile compounds, 4-*epi*-abietal, β-farnesol, laurenene, (*Z*)-nerolidyl acetate and rimuene (all terpenoids) were present in the highest concentrations. Otherwise *exo*-fenchyl acetate, (*Z*)-β-ocimene, methyl butanoate, α-pinene and thymol methyl ether were the most abundant volatile compounds. The solvent extracts of *J. drupacaea* berries showed high antioxidant activity according to 1,2-diphenyl picrylhydrazyl (Section 4.4.3) and β-carotene assays and were also active against various microbes, such as *A. parasiticus*, *C. albicans* and *S. aureus*. Although the flavor profile of *J. drupacaea* berries is different to that of *J. communis* berries, many compounds are common to both and it is reasonable to suppose that gin inherits some the antioxidant and antimicrobial character of juniper extracts (see also Chapter 5.8 and Section 5.11.2).

The composition of botanicals that are used in the formulation of gin is very complex and hence it is not usually possible to achieve a complete separation of volatile compounds by standard capillary gas chromatography. Even after a lengthy sample preparation (Section 4.2.1), this may still be the case. Hence a minor constituent that makes an important contribution to gin flavor can be masked by coelution with a major component, so that there is some difficulty in identifying all the key flavor constituents. Two dimensional GC (automated sequential heart cutting) has allowed the identification of 101 components of a test sample of a mixture of 10 different proprietary botanical and citrus oils used in making gin (MacNamara *et al.*, 2007). The deconvoluted data from the 2D process can then be routinely used to identify components in the same matrix by conventional (1D) GC/MS.

In this way, low level components are readily identified in the presence of major components and it is possible to include such minor components in routine quality control, aging or shelf life studies and consumer sensory perception studies.

Recent GC/MS analyses of the volatile constituents of commercial gin samples have used solid phase micoextraction (SPME) (Section 4.2.4) in either headspace mode (HS-SPME) (Vichi *et al.*, 2005) or contact mode (Vichi *et al.*, 2008) as an extraction/focusing device. The HS-SPME method allowed the identification of a large number of sequiterpenes and some of their oxygenated derivatives as well as monoterpenoids in a number of different representative gin samples. The sesquiterpenoids are in general less volatile than

monoterpenoids, but nonetheless may make important contributions to gin flavor, either individually or collectively, as background flavor. Gamma- and δ-cadinene, caryophyllene, β-elemene, γ-elemene, α-humulene and germacrene D were the most abundant sequiterpenes in the gin samples, whereas caryophyllene oxide, toreyol and α-cadinol were the most abundant oxygenated sequiterpenes, although the total concentration of former was much greater than the latter.

Gin with geographical denomination (Plymouth gin, UK and Mahon gin, Spain) had higher levels of the main sesquiterpenes than London dry gin. Discriminant analysis of the GC/MS data was able to differentiate between the geographically denominated gin samples and the London dry gin samples on the basis of both sesquiterpene and oxygenated sesquiterpene contents, as well as monoterpenoid and oxygenated monoterpenoid concentrations, but clear differentiation between all the London dry gin samples was not possible (Vichi *et al.*, 2005; 2008). However, on the basis of oxygenated monoterpenoids, it was possible to partially differentiate between London dry gin samples. The main monoterpenes in the gin samples were α-pinene, β-myrcene and limonene, followed by γ-terpinene, *p*-cymene, sabinene and β-pinene. Mahon gin had higher levels of α-pinene, β-myrcene, *p*-cymene, sabinene and β-pinene, suggesting a higher proportion of juniper berries were used during aromatization, whereas Plymouth gin had the highest concentrations of limonene and γ-terpinene, suggesting greater use of citrus peel in the manufacture of this gin. The oxygenated monoterpenoid linalool is a minor component of juniper berry, but a major one of coriander seed. Mahon gin had easily the lowest mean level of linalool (1.93 mg/l), suggesting coriander may be absent from or was a very minor component of the aromatization essence used to make this gin. At the other extreme, two samples of London dry gin had very high concentrations of linalool (23.18 and 36.99 mg/l), suggesting coriander is a major flavoring ingredient for these two. Interestingly, the Mahon gin had high (but not the highest) total contents of monoterpenes and oxygenated monoterpenes, and the highest total contents of sesquiterpenoids and particularly oxygenated sesquiterpenes, with a very high caryophellene oxide mean concentration and also high mean levels of torreyol, α-cadinol, spathulenol and *t*-muurulol. Plymouth gin had the highest mean totals of monoterpenoids (37.14 mg/l), with over half of that coming from limonene and γ-terpinene. One of the samples of London dry gin had the highest mean total of oxygenated monoterpenoids, with a high level of geranyl acetate; Mahon gin had the next highest mean total of oxygenated monoterpenoids, with particularly high mean level of α-terpineol, again indicative of a high juniper content. Thus by analyzing the terpene composition it was only possible to partially distinguish samples from different brands of London dry gin, but it was possible to fully distinguish Plymouth from Mahon gin and the different brands of London dry gin. It was also possible to obtain a qualitative estimate of the proportion of certain botanical ingredients used to aromatize the spirit, particularly, juniper berries, coriander seeds and citrus peel.

There is only one recent report on the analysis of diterpenoids in commercial gin (Vichi *et al.*, 2008), although the diterpenoid content of the bark, berries and leaves of *Juniper communis* have been investigated and reported in several studies (see references in Vichi *et al.*, 2008). The diterpenoids of juniper are of interest because of their known beneficial physiological properties (Barjaktarović *et al.*, 2005); after all gin was first formulated as a medicine for kidney problems. Using contact SPME followed by GC/MS for the analysis of the less volatile diterpenoids in eight commercial brands of gin, Vichi *et al.* (2008) showed, by comparison with a hexane extract of dried juniper berries, that the levels of diterpenoids were dependent upon the brand, which differed according to the proportion of juniper berries used in the aromatization mixture. Statistical analysis of the gin diterpenoid contents allowed differentiation of two geographically denominated gins (Mahon and Plymouth gin) and six London dry gins. The diterpenoids were all oxygenated – the most abundant four are shown in Figure 3.4.5. Interestingly, Mahon gin had easily the highest total diterpenoid content (190 μg/l compared with 10 μg/l for one of the London dry gins). Since all the samples of gin, by definition, had juniper berries as a component of their aromatizing essence (or gin distillate), it is unlikely that this big difference arises solely from the differences in proportion of juniper berries to the other aromatic ingredients (such as coriander and citrus peel). It seems more probable that production methods (e.g. the type

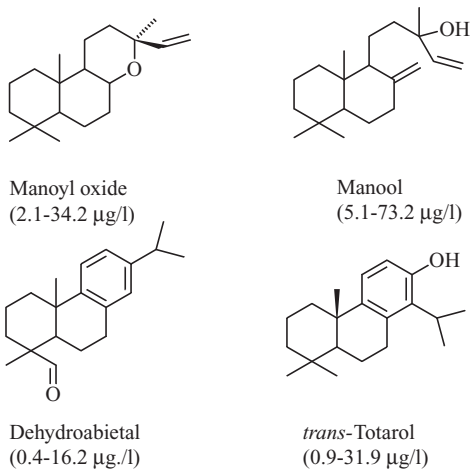

Figure 3.4.5 *Diterpenoids found in commercial gin. Vichi et al. (2008)*

of essence used, the mode of distillation and the degree of rectification) play a big role in determining the diterpenoid content of gin. In any case, this method was able to characterize different types and brands of commercial gin and hence could be used as a criterion of authenticity or quality.

A sensory vocabulary (Section 4.7.4) used to describe gin and distillates of individual botanical gin distillates has been developed along the lines of vocabularies used to describe Scotch whiskies (Phelan *et al.*, 2004). The sensory characteristics of gin could be described by 16 attributes: aniseed, buttery, citrus, floral, fruity, herbal, juniper, oily, pungent, soapy, solventy, sour, spicy, stale, sulfury and sweet. Variation in gin flavor could be quantified using this vocabulary and the contribution of individual botanicals to the overall flavor could be assessed. The major differences among commercial gins could be described in terms of aroma intensity and complexity, probably relating to both the total amount and number of botanicals used and their relative proportions. Juniper berry distillate was characterized by the terms juniper, pungent, but with citrus and floral components. On the other hand coriander seed distillate, usually the next major gin botanical, was described as pungent, citrus, floral and herbal, but with soapy, fruity and sweet components.

3.4.3 Akvavit (Aquavit) and Vodka

These are spirits that originate in northern Europe (especially Scandinavia and the Baltic countries) and Russia. They are treated here together because although their methods of production are quite variable and differ in a number of respects, their overall roles in the indigenous drinking culture of these areas are similar. In this respect, vodka differs from akvavit in being universally popular, as well as enjoying popularity in its regions of origin. Indeed, vodka is the most widely consumed distilled spirit worldwide. In the USA its share of total distilled spirits consumption is more than 25% (Corrigan, 2004) and in the UK, white spirits account for ~40% of spirits consumption, the major share being taken by vodka (Atkinson, 2004). Outside its countries of origin, vodka is the colorless, almost flavorless spirit that is widely used in cocktails (especially of the Bloody Mary, Martini and Screwdriver type) and as a base for the more recently popular 'ready to drink' (RTD) beverages, often known as 'alcopops.' Flavored vodka, for decades popular in northern Europe and Russia, is gradually becoming more popular worldwide.

Akvavit and vodka are distilled from the fermented mash of cereal grains, principally barley and wheat, but sometimes including rye and in a few cases other cereals, such as buckwheat, maize or millet. In principle, they can also be produced from any fermentable sugar source, such as molasses or hydrolyzed potato starch. The common feature of both spirits is their high rectification, so that almost all the flavor of the base materials has disappeared. This is normally accomplished using continuous column stills, but can also be achieved by multiple distillations in pot stills. Additionally, as well as high rectification, a series of filtrations (often triple filtration) through charcoal beds is used in the production of most vodka, thus ensuring that total extractives (components other than water and ethanol) are below 3 g/l (Zach, 2007).

Flavor compounds (congeners) in vodka are of course present at very low levels, often well below 1 mg/l (Simpkins, 1985) although some can be detected by tasters and the majority can be detected (and many quantified) by sorptive preconcentration/focusing techniques, such as solid phase microextraction (SPME) (Section 4.2.4), followed by GC/MS analysis (Ng *et al.*, 1996). The major congeners detected in vodka are ethyl esters, but terpenoids, 5-hydroxymethyl-2- furaldehyde (5-HMF) and triethyl citrate (TEC) have been found in some vodkas and as well as contaminants such as 2,6-di-*tert*-butyl-4-methylphenol (BHT) and bis(2-ethylhexyl)adipate (DEHP) (Ng *et al.*, 1996). Despite the generally low levels of congeners, Ng *et al.* (1996) were able to rationalize differences amongst 18 brands of vodka from different countries according to the base materials and method of production, on the basis of different ethyl ester profiles and the presence of markers, such as 5-HMF and TEC. For example, a Japanese vodka derived from barley and maize was the only one with ethyl decanoate as the most abundant ethyl ester and a German vodka from potatoes was unique regarding the presence of $C_{16:1}$ (ethyl palmitolate) and a high ratio ($\sim$2) C_{18}: $C_{18:1}$ (ethyl stearate to ethyl oleate). On the other hand, the American vodka GC profiles had relative intensities of $C_{18:1}$ and C_{18} to C_{16} ethyl esters of comparable values ($\sim$0.5). Also, the American vodkas were distinguishable by the presence of both 5-HMF and TEC. These were considered to exist as a result of addition of sugar syrup and citric acid, respectively, such additives being allowed by US Federal regulations. The presence of sugar and citric acid was confirmed by HPLC, where sugar concentration ranged from 40 to 2200 mg/l and citric acid was present in the range $<$0.1–357 mg/l. They are added after distillation/rectification/charcoal filtration and prior to bottling.

Akvavit (Akevitt in Norway), unlike most vodka is flavored, the usual dominant flavoring being from caraway seed, but many other botanicals (but not usually including juniper berries) can be used to give some complexity of aroma and flavor. The botanicals are macerated in alcohol/water mixture for several days, after which the mixture is distilled, filtered and diluted to the required alcoholic strength (Zach, 2007) (Figure 3.4.6(a)). Most examples of akvavit are pale gold, reflecting aging in old oak casks or the addition of a little caramel, which is allowed (EEC, 1989). Darker examples most likely indicate considerable aging in new or young oak casks (especially if Norwegian), but could instead be the result of the use of a greater quantity of caramel. Most Akvavit has a pronounced flavor of caraway (not unlike aniseed), but some, especially aged Norwegian versions have strong oak character superimposed on this and others, such as Aalborg (Denmark) have rather more subtle flavors due to the use of a special ingredient, such as amber.

Aquavit is sometimes marketed in clear glass bottles, in which case they should be stored out of sunlight or fluorescent light since, over a period of months, light catalyzes many reactions that lead to undesirable sensory changes (Refsgaard *et al.*, 1995). For example, photo-oxidation of limonene gives hydroperoxides (turpentine-like notes), carvone gives carvonecamphor (camphor notes), and possibly1,5-(*Z*)-octadien-3-one (metallic, geranium notes) is produced, although this was not confirmed. It is, however, responsible for metallic off flavor in caramel.

Denmark and Sweden make the greatest quantities of Akvavit, but Norway produces some of the most distinctive examples; in particular, Norwegian Akevitt is aged in oak casks for at least a year (up to 12 years), where it acquires some color and extra complexity of flavor if the casks are new or young. Well known producers include Aalborg, Den Bornhomske Spritfabrik (Denmark), Symposion International AB, Vin and

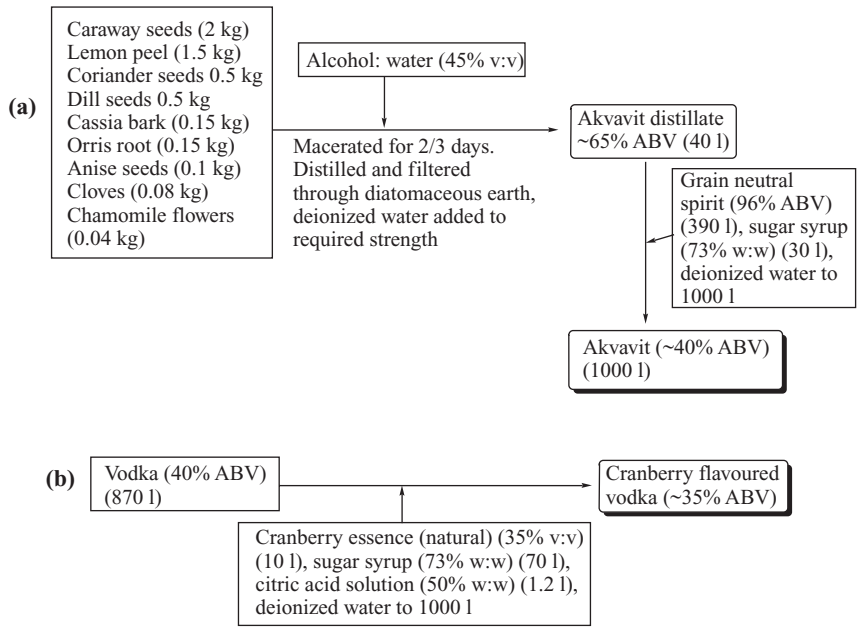

Figure 3.4.6 *Formulations for (a) Akvavit and (b) flavoured vodka. Based on Zach (2007)*

Spirit AB (Sweden) and Arcus (Norway). The Norwegian firm of Arcus make a brand of Akvavit known as Linie, which refers to the tradition of aging the spirit by shipping it around the world for 19 weeks or so in wooden casks, rather like the original Madeira process of estufagem (Section 2.10.6). As for Madeira, the rolling action of the ship during the voyage, coupled with the daily variation in temperature has a beneficial effect upon the maturation, but unlike Madeira, attempts on land to simulate the conditions of the sea voyage failed to give the same maturation benefits. Maybe this is because of the differences in alcoholic strength (~20% ABV for Madeira and ~60% ABV for Akevitt) and differences in humidity (Madeira is much more humid than Norway and hence has conditions closer to those of a sea journey). Traditionally, the ships should cross the equator twice before the casks are brought back to Norway to be sold.

Akvavit is also produced in Finland (akvaviitti), Iceland (ákavíti), Poland (okowita), the Baltic countries of Estonia, Latvia and Lithuania, as well as Northern Germany, (where it is called Aquavit), particularly Schleswig Holstein, which still has a sizeable Danish minority. German Aquavit producers include August Ernst (Sankt Petrus), Alte Mackenstedter (Lloyd Finest), Bartels-Langness (Kieler Sprotte Aquavit), Berentzen Brennereien (Bommerlunder Aquavit), Wilhelm Büchter (Nordkap), Dwersteg (Friedensreiter), A.H. Johannsen (Flensburg) (Aquavit No. 6), Kreuzritter (Dreiling), Schilking (Wikinger Feuer) and V&S Deutschland (Malteserkreuz).

3.4.4 Asian Grain Spirits

Grain spirits are produced all over Asia, but particularly in the far eastern countries of China, Japan, Korea, Thailand and Vietnam. In total, they are probably the most popular distilled beverages in the world, although they are derived from wines or mashes (porridges) made from a very wide range of saccharified cereals,

Table 3.4.1 *Distilled Spirits of China, Korea and Japan.*

Country	Beverage
Korea	Soju – traditionally distilled from rice wine, but commercially most commonly made in combination with other ingredients such as wheat, barley, or sweet potatoes to give a grain neutral spirit, which is then diluted to 20–30% ABV and sweetened. Traditional spirits are stronger, more flavoursome and unsweetened.
Japan	Shōchū – a distilled alcoholic beverage that can be made from rice wine, although it is more commonly made from barley, sweet potato, or sugar cane. Often of ~30% ABV, but traditional versions are stronger. Awamori – a distilled rice spirit from Okinawa.
China	Many liquors distilled from fermented mashes of sorghum or a mixture of grains, often maize, rice, sorghum and wheat (e.g. jiannanchun and yanghe daqu). Chinese liquors often have stronger flavours than their Japanese and Korean equivalents and are sold at 40–55% ABV. Rice baijiu – a potent spirit distilled from mijiu

including barley, buckwheat, maize, millet, rice, sorghum and wheat. Potatoes and sweet potatoes are also used as a carbohydrate source to produce some of the cheaper mass produced spirits. It is possible that the world's first distilled beverages were produced in China and it is possible that the Chinese passed on their knowledge of alcoholic distillation to other Asian nations at all points of the compass via normal trade. However, it is also possible that knowledge of distillation was passed from the Persians (who themselves learnt it from the Arabs further west) via Mongol invasions and wars, as well as by normal trade routes. Even today, traditional methods of producing distilled drinks in Asia tend to be broadly similar, from country to country, but with differences in fermentation and distillation techniques. A summary of the major distilled beverages of China Japan and Korea is given in Table 3.4.1.

Chinese Liquors

Today, Chinese distilled beverages are often known as liquors. Their annual consumption in China exceeds 4 billion (4×10^9) liters and they are produced from sorghum or more likely from whatever is available, but typically a mixture of maize, rice, glutinous rice, sorghum and wheat, with rice hulls being used as a fermentation aid (Fan and Qian, 2006a). Chinese liquors are often classified according to their aroma (Fan and Qian, 2006b):

- Light (milder fruity notes)
- Strong (more pronounced fruity, estery notes)
- Soy sauce (piquant, roasted notes)
- Sweet, honey (honey notes)
- Miscellaneous.

The basic process for the production of Chinese liquor is outlined in Figure 3.4.7, although in practice there are many variations. The fermentation starter is made from fermented/malted wheat or a mixture of barley, pea and wheat, and is available in solid (powder) form (daqu) or semi-liquid form (xiaodaqu). The starters contain a range of bacteria, fungi and yeasts, as well as amylolytic enzymes; they are similar to koji and nuruk used to make Japanese and Korean rice wines (Section 2.7.1). Daqu is added to cooked grains for fermentation in ratios of around 1:4 by weight. One of the most popular liquors, known as yanghe daqu is of the strong aroma type and is produced from barley, pea and wheat daqu, whilst two other popular liquors,

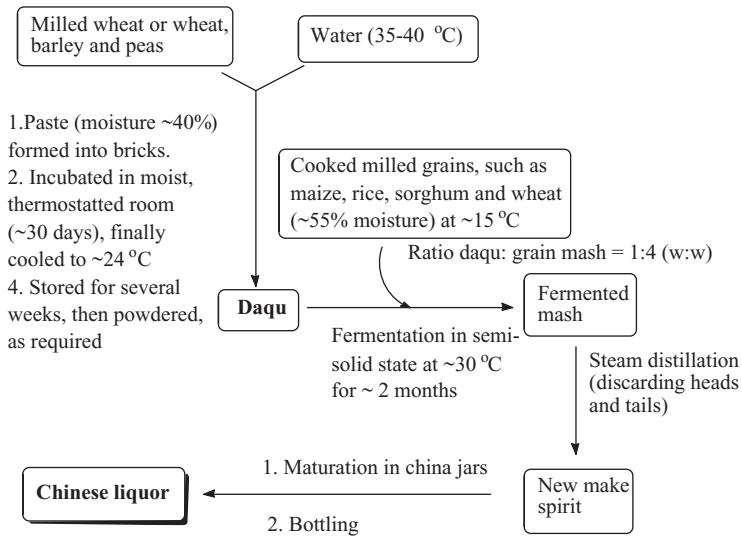

Figure 3.4.7 *Basic outline of Chinese liquor manufacture*

jiannanchun and wuliangye (of the soy sauce, roasted type), are made using wheat only daqu. A list of well known Chinese liquors is given in Table 3.4.2.

Not only does daqu vary according to its ingredient composition, but also according to the incubation temperature: < 45 °C (low temperature daqu), 45–60 °C (moderate temperature daqu) or > 60 °C (high temperature daqu). The spectrum of microorganisms likely depends on the incubation temperature, giving different aroma profiles in the different fermented mashes and hence in the corresponding distilled beverages. Additionally, higher temperatures are likely to produce greater amounts of Maillard products, which include furans with biscuit-like aroma and pyrazines with roasted aromas (Fan and Qian, 2007).

Yanghe daqu liquor is made in the following manner (Fan and Qian, 2006b). Firstly, to prepare the daqu, wheat, barley and peas (ratio 5:4:1 by weight, often to give a total weight of at least 1000 kg) are milled, mixed well and water (35–40 °C) is added until the moisture content is about 40% by weight. The resulting paste is pressed, formed into bricks (about 30 cm × 18.5 cm × 6 cm) and then kept in a fermentation room under controlled temperature and humidity. The temperature of the daqu climbs from ~25 °C to around 58 °C after eight days or so as fermentation gathers momentum, after which a careful ventilation and/or spraying of

Table 3.4.2 *Some well-known Chinese liquors*

Spirit	Typical ethanol content (%ABV)	Spirit	Typical ethanol content (%ABV)
Dongjiu	54	Moutai	53
Fenjiu	45	Shite	52
Gujinggong	55	Wuliangye	52
Jiannanchuan	52	Yanghe daqu	70
Jinshiyun	42	Yanghelansejindian	46
Langjiu	53	Xifengjiu	45

Source: Data from Fan *et al*. (2007); Fan and Qian (2006b).

cool water is used to keep the daqu temperature around 55 °C. It remains at this temperature for about 10 days – the daqu for other Chinese liquors can be held at lower or higher temperatures than this, according to the extent of ventilation/spraying. A further 12 days are required to cool the daqu gradually down to about 24 °C, after which the moisture content of the bricks should be about 17% (w:w). The bricks are finally transferred to another (dry) room where they are stored for three months or so before use.

Grains (maize, rice, sorghum, sticky rice and wheat total weight, say 800 kg) are milled, mixed with rice hulls (∼140 kg) and pressure cooked so that their moisture content is ∼55% by weight. After cooling to 15 °C, the mash is mixed with daqu powder (200 kg) and the fermentation of the semi-solid mixture occurs anaerobically at 28–32 °C for two months. The inner surface of the fermenting vessel is sometimes lined with a slurry of clay, bean cake powder and spent grains, containing populations of bacteria, such as *Clostridium* spp.

At the end of fermentation, the thick liquor is distilled a single time with steam, the head and tail fractions being discarded and the middle fraction being collected as the new make spirit. Chinese liquors are usually aged in sealed 1000 l porcelain jars for a minimum of three years, after which they are bottled in a variety of containers, including finely decorated porcelain bottles. Many distilleries make more then one brand of liquor, and, as with many other spirits, blending is often used to give consistently acceptable products.

The aroma and flavor compounds of Chinese liquors have been investigated by a variety of techniques, including HS-SPME or liquid–liquid extraction/GC-O AEDA (Section 4.7.3) (Fan and Qian, 2005; Fan and Qian 2006a), normal phase chromatographic fractionation/GC (Fan and Qian, 2006b) and liquid–liquid extraction and HS-SPME/GC (Fan and Qian, 2007). Many Chinese liquors possess distinct fruity/estery aromas, so it has been suggested, on the basis of GC-O analysis of column chromatographic fractions, that aliphatic esters, particularly ethyl hexanoate (fruity, floral), ethyl butanoate (pineapple) and possibly ethyl octanoate (fruity), 3-methylbutyl hexanoate (apple) and ethyl pentanoate (apple), make important contributions to the aroma of yanghe daqu liquor (Fan and Qian, 2006b). Acetals (fruity notes), some acids (cheesy, sweaty) some alcohols (fruity, pungent, solvent) and phenols (mostly spicy/smoky notes) were considered to make significant contributions. These findings were in broad agreement with earlier results using HS-SPME/GC-O AEDA on the same liquor (Fan and Qian, 2005), where aged samples had similar aroma profiles to young samples, but with aroma compounds in the former having higher flavor dilution (FD – see Section 4.7.3) factors.

Jiannanchun and wuliangye liquors, belonging to the strong aroma family of Chinese liquors (like yanghe daqu), also have strong fruity aromas and similar compounds (but in different ratios) were found in fractionated liquid–liquid extractions, analysed by GC-O AEDA (Fan and Qian, 2006a). However, these two liquors, especially wuliangye, have more roasted, soy sauce type aroma character than yanghe daqu, which is attributed, in part, to the presence of alkylated pyrazines with nutty, baked and roasted notes, especially in wuliangye liquor. These differences arise from different methods of manufacture, particularly with regard to the preparation of the daqu: jiannanchun and wuliangye liquors are made using wheat daqu, which is incubated at a higher temperature than that of yanghe daqu liquor.

Liquors of the roasted, soy sauce aroma family, jinshiyun, langjiu, moutai and moutaiyingbin were found to have the highest total pyrazine contents, corresponding to the highest temperatures for the incubation of daqu (60–65 °C for 10–12 days) (Fan and Qian, 2007). Jiannanchun and wuliangye had intermediate levels of total pyrazines, corresponding to daqu incubation at 58–60 °C for 10–12 days, whereas fenjiu had the lowest level, corresponding to incubation of daqu at the lowest temperatures (40–45 °C for 10 days). The major pyrazine components were the 2-ethyl-5-methyl-, 2,3,5,6-tetramethyl-, 2,6-dimethyl- and 2-acetyl-6-methyl-derivatives (in moutaiyingbin and moutai), but a total of 27 pyrazines were found amongst 12 different Chinese liquors (Fan and Qian 2007).

The alkylpyrazines in Chinese liquors are thought to arise from nonenzymic Maillard reactions (Section 2.6.2) between acetoin, 1-hydroxypropanone and similar compounds with ammonia, (all known to

be fermentation metabolites in Chinese liquors) although L-threonine was found to be the precursor of 2,5-dimethylpyrazine for *Bacillus subtilis* in the solid state fermentation of soybean (Larroche *et al.*, 1999). Similarly, acetylpyrazines are believed to be formed by the same route as alkylpyrazines, but with the initial pyrazine reacting further with C-methyl triose reductone (another Maillard intermediate) to produce a compound that dehydrates by the loss of two water molecules.

Pyrazines are important aroma/flavor components of many baked or fermented foodstuffs and they have also been found in several alcoholic beverages, including Chinese liquors, tequila, whisky and wine. In red wines, 3-alkyl-2-methoxypyrazines are of special importance, but have not yet been found in Chinese liquors.

Japanese Shochu and Related Spirits

Shochu (sometimes written shōchū) is the generic name for a large group of distilled drinks that are indigenous to and highly popular in Japan, although some Japanese people refer to these beverages, as to all domestic alcoholic drinks, as sake. Shochu is made from a wide range of ingredients by a variety of fermentation and distillation techniques and hence varies considerably in organoleptic character. The different kinds of shochu are broadly outlined in Figure 3.4.8. As in Korea, there are two basic types of spirit that depend mainly on the nature of the ingredients, the fermentation method and the mode of distillation. These factors also decide whether maturation is necessary before bottling and sale.

Traditional sochu (otsu-rui or honkaku), known as Type B Shochu, is generally produced from one ingredient, it is fermented with yeast and koji (Section 2.7.1), which is usually made from the same ingredient, distilled just once in a pot still (with appropriate head and tail management) and matured for about three years. The wine for distilling is usually of at least 15% ABV, because of the high levels of sugars in the mash. Hence the distillate is often in excess of 45% ABV, although it is often reduced to 25% or 30% ABV by the addition of deionized water before filtration and bottling. The original ingredient was probably rice (see awamori, below), but nowadays barley, buckwheat (soba in Japanese), rice, sweet potatoes or other sources of starch, such as chestnuts or sugar cane, are used. The most delicately flavored shochu is probably that made from rice, whereas the most strongly flavored is probably sweet potato or sugar cane shochu. Traditional shochu is usually enjoyed straight, on ice, or with a dash of water. In winter, it can be enjoyed with hot water (‘oyu-wari’).

There are various versions of koji whose use depends on the base material and which differ in the active species of *Aspergillus* present. For example, sake-koji (containing *Aspergillus oryzae*) (Section 2.7.1) is

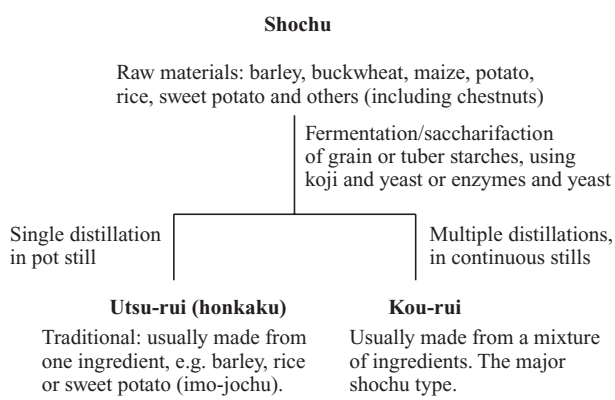

Figure 3.4.8 *Types of shochu*

produced from rice and is used with rice mashes, whereas barley-koji (containing *Aspergillus kawachii*) is made with barley and is used in the production of barley shochu. Barley-koji is made by taking pearl barley (70% pearling rate) steeping it in water and then steaming it. The cooled, cooked barley is then inoculated with *Aspergillus kawachii* and incubated at 30–40 °C for about 48 h (Iwami *et al.*, 2005). Fermentation is carried out in a similar manner to that described for sake (Section 2.7.1), but the flavor of the resulting wine, and hence the final spirit, depends on the concentration of precursors in the koji and therefore in the prefermentation mash. In particular, concentrations of aroma and flavor compounds (such as higher alcohols) in the spirit will be relatively high if high levels of precursors (such as amino acids) exist in the mash (Iwami *et al.*, 2005). Additionally, higher levels amino acids, such as alanine, glycine and phenylalanine will produce higher levels of Maillard products by reaction with sugars at higher temperatures (e.g. during nonvacuum distillation).

The levels of a number of amino acids in barley koji depended largely on the barley variety and the pearling rate (Equation 3.4.1), especially with regard to lysine, proline, histidine, threonine and lysine, phosphoserine (respectively).

$$\text{Pearling rate} = \frac{\text{Weight of 1000 pearled barley kernels}}{\text{Weight of 1000 barley grains}} \times 100 \tag{3.4.1}$$

In particular, the total concentration of amino acids is generally higher at higher pearling rates (say 80%), irrespective of barley variety, which means the resulting shochu is likely to be rather more highly flavored, with higher levels of congeners (all other things being equal). Besides the above considerations, the presence of high levels of amino acids is conducive to a good, trouble free fermentation. In the above respects, koji from the Japanese barley variety Nishinohoshi and the Australian malting variety Schooner have been judged to be suitable varieties for barley shochu produced in the traditional way (Iwami *et al.*, 2005). Additionally, these two varieties gave koji with higher acidities, higher levels of α-amylase and glucoamylase and higher levels of glucose and maltose than several other varieties; factors that give wines of high ethanol content.

The current trend (2009) in Japan is toward milder, less flavorful, lower strength spirits, but many of the older generation still prefer all round more powerful shochu. Competition has become fierce as the big breweries have diversified their interests to include shochu, as beer and sake sales continue to fall. Distillers of traditional shochu are striving to enhance the brand value of their products by modifying the production process in order to eliminate undesirable flavors and to moderate the intensity of desirable flavors (Minabe, 2004). Modifications have included use of high grade starting materials, reduced pressure distillation, filtration and maturation in wooden casks. Much shochu was once made from low quality ingredients (not fit for human consumption), which led to undesirable flavors. Reduced pressure distillation leads to a spirit that is lighter in body, having fewer congeners than shochu made by normal pressure distillation. Likewise, various filtration processes (activated carbon, ion exchange or chill filtration) that can be carried out before bottling generally lower the levels of congeners, as well as removing undesirable aroma components, such as certain organosulfur compounds (Minabe, 2004).

However, although much modern traditional shochu is lighter in body and flavor than in former times, it is still much more flavorful than the type A (kuo-rui) shochu, which is still produced in greater quantity than the traditional spirit. It can also be made from a single ingredient (e.g. maize), but is more likely to come from several ingredients. The saccharification process is more likely to be carried out on the cooked grains by the addition of fungal α-amylase, glucoamylase and other enzymes, rather by the use of koji, and fermentation is promoted by added yeast (*Saccharomyces cerevisiae*). The wine is distilled in continuous column stills to give a high strength (95% ABV) spirit, which after a short storage in bulk tanks is diluted with deionized water to around 30% ABV (or lower) before filtration and bottling. Like its Western counterparts, grain neutral spirits, vodka and others, type A shochu is clear and clean on the palate and is good for premixes ('ready to drink' beverages), but can also be enjoyed in cocktails or supped cooled or iced, with food.

Although Ohita prefecture is famous for barley shochu and Kagoshima prefecture for sweet potato shochu, it is probable that the first shochu (known as awamori), from rice, was made in the Ryukyu group of islands (Okinawa prefecture) prior to the sixteenth century (the first written record of Kagoshima shochu dates from 1559). The production of awamori is similar to that of traditional shochu, except that the major microbiological constituent of the rice koji is *Aspergillus awamori*, which gives a high concentration of citric acid, thus offering considerable protection from bacterial infection during koji manufacture and fermentation. Also, sometimes long grain rice from Thailand and western Asia is used in the brew, rather than the indigenous short grain japonica rice, perhaps a legacy of the possible Thai origin of the awamori production methods, although this is uncertain, since China and Korea probably exerted considerable influence in the fifteenth century. Awamori is distilled once in pot stills and is collected at 45–50% ABV, although like shochu, it is often diluted to 25–30% ABV with pure water before bottling. Because of the use of koji and single distillation, new make awamori is high in congeners, which can give it an earthy, pungent, even medicinal character. Consequently, awamori has traditionally undergone lengthy aging periods, often 10 years and more.

Aged awamori, matured in earthenware containers, is known as kuusu. The aging process is usually conducted over three years, but can be extended up to 25 years, during which time many reactions occur to give a smoother, more harmonious palate, as with other spirits. By law, a three year old awamori need only contain 51% spirit of that age, the remainder possibly being made up of younger spirit (compare this with the law regarding Scotch whisky age statements – see Section 3.2.5). However, a bottle labeled kuusu, 100% 10 year aged (or similar statement) will be entirely of 10 year old spirit.

The traditional method of aging awamori was known as shitsugi, and was a kind of simplified solera system. The household or restaurant would have several large covered earthenware pots of awamori lined up outside, with the pot containing the oldest spirit closest to the door and pots containing progressively younger spirit at greater distances from the house. As a sample was taken from the nearest pot, it was replaced by a sample from the second pot. This in turn was refilled from the third pot and so on. New make spirit was placed in the last pot, when available.

Phenolic substances play an important role in both undesirable and desirable aroma characteristics of awamori and shochu, the undesirable aromas receding with aging. Studies on the fate of ferulic acid in model shochu suggested that conversion of ferulic acid to the less desirable 4-vinylguaiacol and the more desirable vanillin and vanillic acid could involve chemical processes during distillation and aging, as well as biochemical processes during fermentation (Koseki *et al.*, 1996). The suggested processes are outlined in Figure 3.4.9, where the ferulic acid is derived originally from hydrolysis of ferulate ester linkages to arabinose residues of the arabinoxylans in rice cell walls.

Korean Soju and Related Spirits

The most famous and most popular Korean spirit is soju, which is similar in many ways to Japanese shochu. Like shochu, it can be subdivided into two major types, according to the ingredients, the method of fermentation and the method of distillation: hiseoksik soju, the everyday drink made by diluting rectified spirit and jeungryusik soju, a more traditional drink made by single distillation of (usually) rice wine (Section 2.7.1). The development of soju manufacture into the present situation, is not unlike the development of shochu in Japan, except the economic and political backgrounds are rather different (see Sections 1.2.3 and 1.2.4).

In either case, however, economic and political circumstances led to the domination of the markets by inexpensive, mildly flavored drinks made by relatively modern methods from a variety of materials. Thus, both the younger generations and some of the older generation acquired a taste for the mild, relatively low alcohol (20–25% ABV) products. The traditional products survived, used mainly for special occasions, but

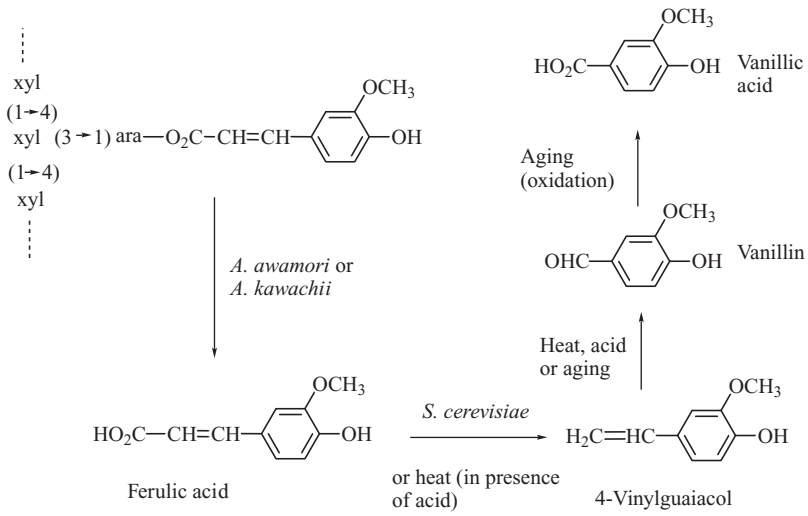

Figure 3.4.9 *Mechanism for the conversion of ferulic acid in awamori and shochu. Koseki* et al. *(1996)*

were seen by the younger generations as old fashioned, both in the their presentation (e.g. decorated porcelain bottles with Chinese inscriptions), flavor (very pronounced, often malty flavors) and alcoholic strengths (sometimes in excess of 45% ABV; double the strength of hiseoksik soju).

During the 1990s, distillers attempted to increase the market share of more traditional soju (premium soju), but these products were invariably more expensive than the standard drink, so that they were not a success. Additionally, their stronger flavors and higher ethanol contents were bucking the trend towards lighter drinks. However, during the past decade, Korean consumers have become more aware of the virtues of the traditional spirit and distillers are responding by producing milder premium versions by traditional methods and presenting them in modern, but attractive bottles (Cho, 2005).

The most popular type of soju is hiseoksik soju, produced by the fermentation of sugars from a wide variety of sources, including barley, maize, potato, rice, sweet potato, tapioca (dangmil in Korean) and wheat. The wine is distilled using continuous columns to give a highly ethanolic, mildly flavored spirit, which is then diluted to 20–25% ABV and sweetened with various ingredients before filtering and bottling, as described below. Because the undiluted spirit is highly rectified and has few congeners, like vodka and gin, aging is not required. A wide range of sweeteners is used, including natural ones such as honey and maple syrup to noncarbohydrate sweeteners such as aspartame, stevioside and xylitol. Stevioside is a carbohydrate free, natural sweetener derived from *Stevia Rebaudiana*, a South American plant, whereas xylitol, a sugar alcohol, is produced by the reduction of the carbohydrate xylose.

Traditional soju is basically yakju (makkoli) or takju (Section 2.7.1), made using the traditional nuruk starter and yeast, and singly distilled in pot stills, like the one shown in Figure 3.4.10. The most famous traditional soju is that of Andong, a city in North Gyeongsang. It is sold at 40% or more alcohol by volume, giving it a pungent introductory character, which broadens out into a fine malty palate that slowly disappears to leave a warm, clean finish.

In recent years, several distilleries (or brewery companies as they call themselves in Korea) have introduced lighter brands of traditional soju, including Andong Soju Ilpum and Sipguse, described below. Other companies have repackaged some of their traditional brands to attract the younger generation, an example being Cheongdam soju of the Sunyang Brewery Co.

Figure 3.4.10 *Traditional Korean soju still*

Jinro, based in Seoul, is the largest manufacturer of soju, controlling around 50% of the market and selling 72 million cases in 2007. The most popular variety of soju is currently Jinro's Chamisul (참 이슬 - 'real dew') brand, a quadruple filtered soju, but recently Cheoum Cheoreom (처음처럼 – 'like the first time') of Doosan (두산), based in Gangwon province, has become very popular in recent years. Because of government legislation and reorganization of the soju industry (Section 1.2.4) into one large distillery in each province, the market for a particular distillery company tends to be rather localized. That company can sell its products freely in its own province, but only in a few outlets in other provinces. The major distillery companies, apart from Jinro, are Bohae of South Jeolla province, Daesun of Busan, Doosan of Gangwon province, Hallasan of Jeju Island, Hite of North Gyeongsang province, Kumbokju of North Gyeongsang province, Muhak of South Gyeongsang province and Sunyang of Chungcheong province.

A brief description of some products of the major distillery companies is given next, based on Cho (2005).

Andong Soju

This is the 40–45% ABV jeungryusik soju from Andong, North Gyeongsang province, famous for its traditional soju. A powerful, pungent early palate, gives way to a long, malty flavor and finally a clean aftertaste.

Andong Soju Ilpum

This relatively new premium soju, made in North Gyeongsang province, is mildly malty, with a clean aftertaste. It is made using 51% rice and contains 21% alcohol (v:v).

C1 Soju

It has a pungent aroma and character, with a clean, but bitter aftertaste. This soju comes from the Daesan company in Pusan and can easily found only in southern Korea.

Chamsoon

This soju has a bland start, but with a sharp plate and slightly bitter aftertaste. It contains an extract from Japanese Raisins (*Hovenia dulcis* Thumb.), which is rich in both sugar and minerals.

Charm

From the Kumbokju company in North Gyeongsang province. It has a sweet, rounded palate, with a clean farewell.

Jinro Gold

This brand has a mild start, opening into a powerful, pungent palate (25% ABV).

Green

At 29% ABV, this is one of the stronger hiseoksik types of soju, leaving a heavy, sweet aftertaste.

Ipsaeju

From Bohae in South Jolla province, it is light and sweet, being sweetened by maple syrup (24% ABV).

Hallasanmul Sunhan Soju

This soju from Jeju island has a slightly pungent introduction, leading to a mild, sweet palate. It contains 21% alcohol (v:v) and is made with weakly alkaline water and aspartame as additive.

Hite Soju

From North Jeolla province, it has 21% ABV and added aspartame. It has a mild, sweet flavor.

Malgeul Linn

Made in the Chungcheong region by Sunyang Brewery Co., it has 23% ABV, is light and floral, leaving a slightly bitter, but clean aftertaste.

San

This new soju, introduced last year by the Doosan Company in Dongwang province, is made with mineral water from Daegwallyeong mountains, with green tea extract and aspartame additives.

Sipguse

This is new and with only 19% alcohol. It is a pure (jeungryusik) soju made in Andong, North Gyeongsang province. It has a mild, malty palate, creamy texture and soft, clean farewell.

White

This soju is made by Muhak in South Gyeongsang province. It contains 21% alcohol (v:v) and contains contains aspartame, honey and xylitol additives. It has a pungent introduction, with mild, sweet flavor and soft farewell.

Other Korean distilled liquors include goryangju made from sorghum and similar to Chinese gaoliang jiu and okroju. Another variety, called munbaeju (문 배 주), has the distinction of being South Korea's Important Intangible Cultural Property Number 86-1. Munbaeju is a traditional aged distilled liquor made of malted millet, sorghum, wheat, rice and nuruk (fermentation starter), with a strength of 40% alcohol by volume. It originates in the Pyongyang region of North Korea and is noted for its fragrance, which is said to resemble the flower of the munbae tree (similar to a pear).

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3.5

Cane Spirits, Vegetable Based Spirits and Aniseed Flavored Spirits

A Martinique rum is the perfect antidote for a rainy day.
—Ernest Hemingway

3.5.1 Overview and Scope

This section describes aspects of the production and character of a number of spirits that are distilled from various fermented vegetable and fruit juices. Principal amongst these spirits are sugar cane spirits rum and cachaça, produced mainly in the Americas. The corresponding spirits of Indonesia and southeast Asia are often known as arrack, rather than rum, giving rise to a certain amount of confusion of nomenclature, since arak is a word used to name often quite different spirits of western Asia.

Both arrack and arak are derived from the same Arabic word araq (عدق), whose meaning is sweat, referring to the beads of distillate in a still, but now is used for distilled alcoholic beverages. Arrack spelling tends to be reserved for spirits of southern and southeastern Asia; it is made by distillation of wine made from a range of vegetable or fruit sources, including, sugar cane, palm sap, dates, figs and plums. On the other hand, the word arak tends to be reserved for spirits of the Middle East derived from (grape) wine or pomace and anise – in essence, aniseed flavored brandies or pomace spirits. These beverages are made in countries in eastern Europe/western Asia: Bulgaria, Greece, Israel, Jordan, Lebanon, Palestine and Syria, although in the European countries non-Arabic names, such as ouzo and mastika are used. Further west, similar drinks are made in France (pastis), Italy (anesone) and Spain (ojén).

For the purposes of this book, arak is included in the number of aniseed flavored spirits, in Section 3.5.6, along with anesone, ouzo, pastis and others, despite the fact that some Egyptian and other Middle Eastern beverages named arak are produced from dates, figs or other sources. Likewise, arrack is the name used in Section 3.5.5 to describe the distilled wine of any vegetable or fruit origin (other than sugar cane) and is discussed alongside mezcal, tequila and related drinks. The sugar cane spirits of Indonesia (e.g. of Bali and Batavia) (often also known as arrack) are discussed as rum in Sections 3.5.2–3.5.4, even though some of them include rice in their production (Cooper, 1982).

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

3.5.2 History and Basic Description of Rum and Cachaça

Rum is the distillate of fermented sugar cane products, principally molasses, but some rum is made from sugar cane juice. It is produced wherever sugar cane is grown, in subtropical climates, like those of the numerous islands of and the many countries surrounding the Caribbean Sea. Figure 3.5.1 shows the main rum producing Caribbean countries, along with a selection of well known distilleries or distillery companies in certain countries. Although the best known rums are from the Caribbean countries, rum is also made in Australia, Fiji, India, Indonesia, Mauritius, Reunion, Sri Lanka and other countries. Cachaça is the Brazilian spirit made from fermented sugar cane juice. Similar drinks to rum and cachaça are made in the Central American countries of Mexico (aguadiente de caña and chasanda) and Panama (seco), as well as cane juice in Liberia and tafia in the Caribbean islands. Additionally, sugar beet spirit, known as tuzemák or tuzemský rum (domestic rum) is made in the Czech Republic.

The origin of the word rum is uncertain; the Spanish and French equivalents, ron and rhum respectively, appear to based on this word, although in Spanish and French, rum is often known as aguadiente (aguadente in Portuguese) and eau-de-vie de molasses, respectively. Two claims of origin (and there are others) are 'rumbullion' (a Cornish word meaning an uproar – no doubt a drunken one) and 'rummer,' from roemer, a drinking glass used by Dutch sailors (Blue, 2004). It is also possible, however, that the name rum is derived

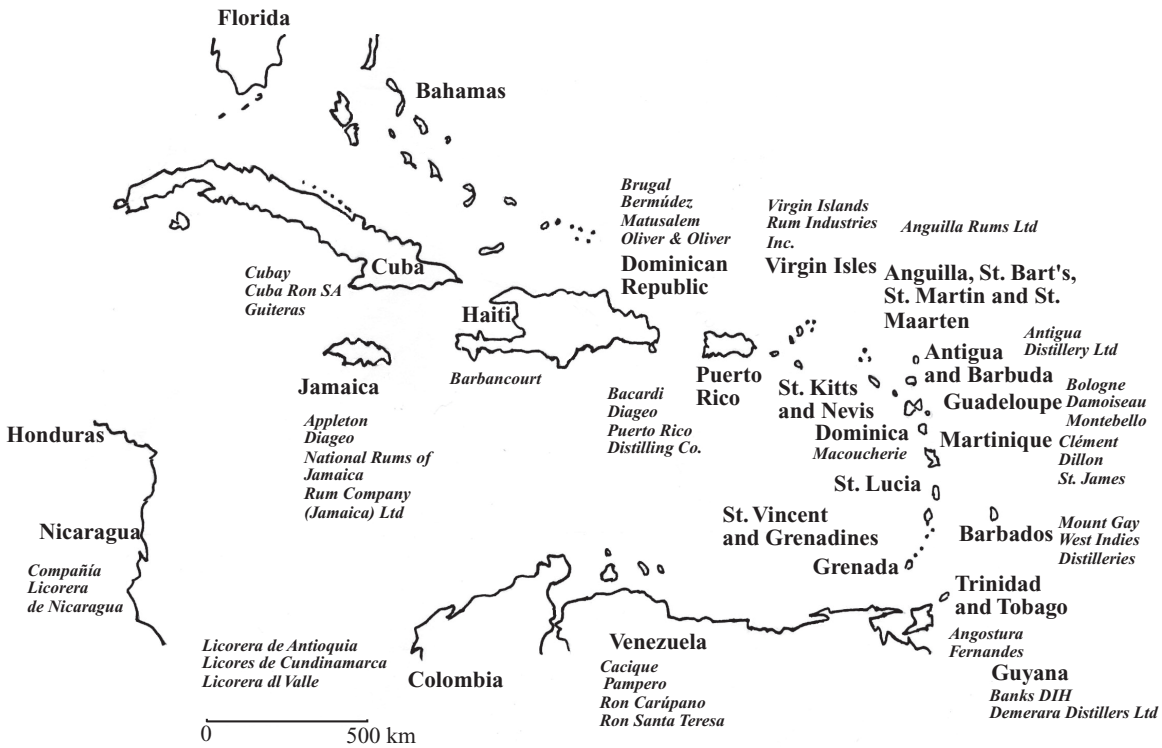


Figure 3.5.1 *The heart of rum production in the Caribbean area. Representative distilleries or companies only are indicated: the list is by no means exhaustive*

from 'brum', an ancient fermented drink made from sugar cane juice by Indonesian and Malay peoples (Blue, 2004).

The original home of sugar cane is probably Indonesia, where rum is still made. The Chinese introduced sugar cane to Arab merchants, who in turn traded plants with Europeans, where it was planted on subtropical islands such as the Azores and the Canary Islands. Sugar cane plants were taken by Christopher Columbus on his second trip to the Americas (1493), where he planted them on the island of Hispaniola (now Dominican Republic/Haïti). Later, Portuguese explorers started sugar cane plantations in northeastern Brazil, so that sugar production was well underway in the sixteenth century and it is probable that the first cachaça was distilled from fermented cane sugar waste ('borra') around the middle of the sixteenth century.

In the Caribbean area, sugar plantations sprung up on most of the islands and in most of the countries of the surrounding landmass, particularly those to the south and west. The first rums distilled from fermented molasses were probably made in Barbados around 1600, although there are reports of rum being produced in Puerto Rico as early as 1526. However, the Mount Gay Distillery on Barbados claims to be the oldest surviving Caribbean rum distillery (Figure 3.5.1) (Lichine, 1982).

The early Portuguese colonizers of Brazil soon began trading sugar and cachaça for west African slaves who were put to work in the sugar plantations, refineries and distilleries (Faria *et al.*, 2004a). It wasn't long before the colonizers of the Caribbean islands and countries (the British, Dutch, French and Spanish) involved sugar and rum in a similar trade, that later became known as the 'Triangle Trade.' It is not within the scope of this book to discuss the slave trade in detail; suffice it to say that rum and cachaça are unique among spirits in the roles that they played in the slave trade. They are also unique in that in the two modern centers of production, the Caribbean region and Brazil, cane sugar spirit is not indigenous; this claim most likely goes to Indonesia.

It was probable that the above situation, along with increasing colonization, accounted for the early popularity of rum and cachaça. Rum was, along with applejack (see Section 3.8.2 and also 3.1.1), a popular spirit of the early American colonists, whiskey not becoming popular until the influx of Irish and Scottish immigrants in the early to mid nineteenth century (Section 3.3.1). Indeed, New England rum was probably of the best quality available in the seventeenth and eighteenth centuries, because of superior technology. Rum was the basis of the early Colonial New England's largest industry and the Sugar Act of 1764 (imposing a sugar tax) did nothing to increase the Colonialists' love of the British (House of Hannover) monarchy.

Cachaça has increased its popularity in Brazil over the past few decades, so that today (2009) more than 1.5 billion litres are produced annually. Although only about 40 million liters per annum were exported in the last few years, this is growing steadily and there have been signs for many years that the drink is becoming more popular outside Brazil. The Brazilian government has been making concerted efforts for some time to regularize the quality of cachaça, in order to make it more attractive for export. See Faria *et al.* (2003) for an account of cachaça.

Rum, on the other hand, although popular where it is distilled, has always been exported, originally to the American colonies via the ports of New England (such as Boston) and to Britain via the western ports (such as Whitehaven – see Section 1.2.2) and to France via the Atlantic ports. When the United States obtained Puerto Rico from Spain (1898), that country became the major importer of Puerto Rican rum, so that today more than 70% of rum consumed in the USA today comes from Puerto Rico. Puerto Rico is presently the biggest producer of rum, exporting all over the world, but principally to the USA. Cuba, a large producer of light rum (like Puerto Rico) is the major exporter of rum to Russia.

The most popular rums of the seventeenth and eighteenth centuries were dark, full bodied types, derived from molasses and colored with caramel. In the twentieth century tastes began to change toward rums lighter in both body and color; these were (and still are) usually consumed in cocktails such as Daiquiri, rather than drunk neat. Initially, the lighter rums were produced largely by Cuba and Puerto Rico, with Bacardi, originally based in Cuba, becoming predominant. Nowadays, light rum is distilled from either cane juice or molasses in

most of the Caribbean area, although those of Puerto Rico and Cuba dominate. Likewise, darker or heavier rums are produced more or less all over the Caribbean area, but those of Barbados, Dominica, Guadeloupe, Guyana (Demerara), Haïti, Jamaica and Martinique are probably the best known. The major styles of rum and how they are made are discussed more fully in Section 3.5.3.

As with other alcoholic beverage industries, the rum industry has been influenced at various times by changing economic and political conditions, including wars and periods of prohibition. Most commercial rum nowadays is made in large distilleries owned by big international beverage companies, such as Bacardi, Banks DIH, Demerara Distillers, Diageo, Edrington Group and Pernod-Ricard. Many smaller distiller companies still exist in the Caribbean area, especially on the French West Indies islands, such as Guadeloup and Martinique, and on smaller, less well known islands such as Grenada, the Grenadines, St Kitts and Nevis, and St. Vincent. The larger companies have more than one rum distillery in the Caribbean region and some, like Diageo, have rum or other distilleries elsewhere. Rum producers often possess bottling, distribution and transport companies as subsidiaries. The larger producers also have diversified interests, owning (apart from sugar plantations and refineries), fruit juice companies (e.g. Demerara Distillers Ltd) and breweries (e.g. Banks DIH), for example.

Until the 1960s, production of rum was not particularly profitable and the industry was in a state of stability or slow growth. Interest at that time had shifted away from dark, full rum to lighter rum for use in cocktails and punch. The Cuban revolution in 1959 forced many rum distillers to other countries, notably Puerto Rico and the USA. Stimulated by a preferential status regarding rum imports into the USA (the 'Caribbean Basin Initiative,' which includes the US Virgin Islands as well as Puerto Rico, in which the US Treasury returned $10.50 excise tax for every proof gallon of imported rum from those two countries), there followed a period of rapid growth of Puerto Rican rum production, which fueled keener competition between distilleries all over the Caribbean area. Within 10 years or so, the industry had become dominated by a small number of relatively large distilleries. Demand for rum continued to grow into the 1970s and beyond, so that these days, rum, if cachaça and rum liqueurs are included, shows the greatest growth of all distilled spirits (Delevante, 2004).

Free trade agreements, or economic Partnership Agreements as they are known now (like that mentioned above for USA and Puerto Rico/Virgin Islands), also existed between the former European colonies in or around the Caribbean Sea (CARICOM) and the European Union, whereby selected imports from both partners were given preferential status, such as zero duty quotas. For the Caribbean countries, the duty free quotas included rum. Originally, the economic partnership between the EU and ACP (African, Caribbean and Pacific countries) was embodied in the Lome agreement, but pressure from the World Trade Organization has forced renegotiations of this agreement between the European Commission (EC) of the EU and CARIFORUM (= CARICOM + Dominican Republic), so that the new (2007) trade agreement (EPA) covers substantially all trade (involving goods, services, investment, government procurement and intellectual rights), and not just selected items, such as rum (Berridge, 2008). Rum from the CARIFORUM countries exported to Europe thus is free of duty and hence can be sold at more competitive prices in the EU market.

In Brazil, it was estimated that in 2004, there were more than 2500 small producers of cachaça (Faria *et al.*, 2004a). Although the larger distillers actually buy much of their spirit from small producers (in order to give consistent blended products), it is felt in government and trade circles that there are too many small producers and too great a range of products, particularly with regard to competing in international markets.

India is the biggest consumer of rum (followed by the USA) (2008). Distilled in the northern states of Bihar, Haryana and Uttar Pradesh, as well as in the Punjab, from fermented sugar cane molasses, Indian rum ranges from the rough and pungent tharra to sophisticated, aged dark beverages such as Old Monk. Tharra is distilled in countryside locations using small pot stills, often illegally. It is never aged and tends to be sold and consumed locally. The major distillery companies of Gilbey, Herbertson, McDowell, Simpson and others are now part of larger conglomerates, such as SOM and UB (Mumbai). The latter is India's largest

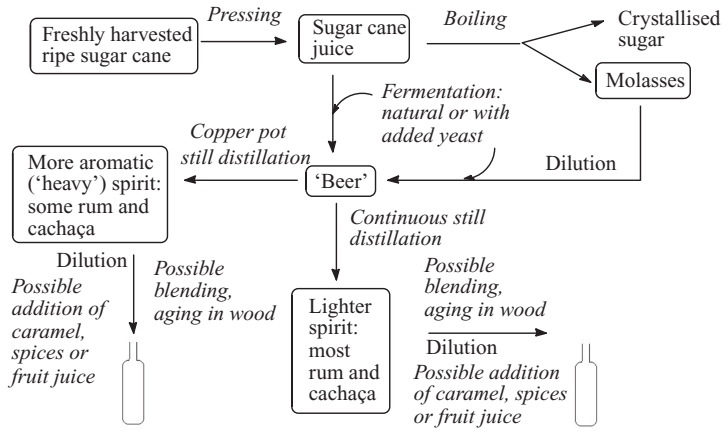


Figure 3.5.2 *General scheme for the production of rum and cachaça. Special distillation and other procedures are described in the text and in Figure 3.5.3*

distiller and probably amongst the world's top three distillers. India also produces whisky (including single malts) (Section 3.3.6) and other spirits, and at present (2009) India is the fourth largest producer of alcohol in the world. If one includes Mauritius and Sri Lanka, the countries of the Indian Ocean produce quite an impressive portfolio of distinctive rums: Old Monk, McDowell's No. 1 Celebration, Old Cascade (all India), Green Island (Mauritius), Rockland and White Diamond (both Sri Lanka).

Other rum producing countries include Fiji (e.g. Bounty rum), Hawaii (e.g. Maui dark rum) and the Philippines (Tanduay and Tondeña rum). Rum is also manufactured in Indonesia, the best known being Bali rum and Batavia rum. The latter is made by distillation of fermented molasses, although red rice is also used in the fermentation. Distillation is carried out in pot stills, originally of Chinese design, and the rums are aged in wood.

The basic processes (simplified) for the production of rum are shown in Figure 3.5.2. Ripe sugar cane is harvested, crushed and the resulting juice can be fermented directly (or somewhat diluted or even concentrated and then diluted) to give a liquor for distillation of *ca.* 7–8% ABV, called 'beer' in the rum industry. Much cachaça and some rum, especially from the French West Indies islands, are made in this way. However, most rum is made from molasses, the dark, viscous mass remaining after refinement of sugar cane juice. Refinement refers to the process of obtaining sugar crystals from cane juice. The juice is firstly clarified by the addition of slaked lime (calcium hydroxide), after which the clear juice is pumped off the deposit and boiled under pressure. Sugar crystals form on cooling and the molasses remaining after separation should contain around 50–60% by weight of total sugars. The reader is referred to Nicol (2003) for a detailed description of sugar cane processing. Dilution of the molasses with stream or rainwater (see Section 3.5.3) gives a substrate capable of producing a beer of around 7% ABV after fermentation, usually with specially cultured yeasts (Section 3.5.3). Fermentation can take from 24 h to 12 days, depending on the style of rum desired (Sections 3.5.3 and 3.5.4).

Distillation is carried out either in pot stills (Section 3.1.2), as with malt whisky (Section 3.2.4) and Cognac (Section 3.6.2) or in Coffey or continuous (columnar) stills (Section 3.1.3), as with many other spirits, including grain whisky (Section 3.2.4) and most Bourbon (Section 3.3.4). The former gives a spirit of around 65% ABV (for double distillation) with higher concentrations of congeners (flavor compounds), whereas the latter gives a more pure, lighter and less overtly flavored spirit containing close to 95% ethanol (v:v). Both types of distillation systems are open to modifications and a variety of operating options (Section 3.5.3).

Following distillation much cachaça and rum is not aged in oak casks, but usually spends some time ageing in stainless steel tanks. However, most cachaça and rum is blended, diluted and chill filtered before bottling. These spirits are naturally colorless (as are all freshly distilled or 'new make' spirits), but caramel can be used to give a golden or darker color. More usually, all traces of color may be removed by charcoal filtration prior to bottling, as with much cachaça, Cuban and Puerto Rican rum. Cachaça and rum can be aged in stainless steel tanks, wooden vats or smaller casks, such as ex-Bourbon casks, in which case color is acquired according to the length of ageing and the extent of charring of the cask interior (Section 3.3.4). This color can be further deepened or removed by the processes mentioned above. Charcoal filtration also removes certain aroma/flavor constituents, as observed in the depleted ester profile of white Brazilian rum, compared with the rich ester profiles of typical cachaca samples (Nascimento *et al.*, 2008).

Rum can be spiced or flavored with fruits or coconut before bottling (Section 3.5.4). Most cachaça and rums are diluted with rainwater, mountain stream water or distilled water (Section 3.5.3) to give beverages of around 40% ethanol (v:v), but 'overproof' rums can possess alcohol contents of 75% (v:v) and beyond (Section 3.5.4)

3.5.3 Fermentation and Distillation Practices in the Production of Cachaça and Rum

Most cachaça and rum distilleries are linked to or are situated close to sugar plantations and refineries, often owned by the same parent companies as the distilleries. Some distilleries (like some on the islands of the French West Indies and the Macoucherie distillery on Dominica, producing 60 000 bottles of rum per annum) are entirely self-sufficient in this respect, using only their own estate grown sugar cane. The majority, however, rely on imported sugar cane or molasses, as well as local products, so that import and export of molasses is a very important trade. Brazil is a major exporter of molasses to the Caribbean area, where the biggest producer, Puerto Rico, is a net molasses importer.

The quality of the molasses is dependent on the quality (ripeness) of the sugar cane and also on the quality of the slaked lime and water used in its preparation. Molasses should have close to 60% of its mass composed of total sugars, with a high sugar to ash ratio (>7), a low gum to sugar ration (<0.05) and a low acetic acid content (<5 g/l) (Delevante, 2004). If the last mentioned is too high, fermentation may not be satisfactory.

As is the case with most other spirits, the solution to be fermented (the beer, wine or wash, according to the industry) should contain enough sugar to produce 7–8% ethanol (v:v) when fermented right out; it should have an original gravity of around 50–60 °Oe ($\sim$12.5–15.0 °Brix). To achieve this, both sugar cane juice and molasses must be diluted with pure, clean water – rainwater, as in the case of Cruzan rum (US Virgin Islands), mountain spring water, as in the case of many Jamaican distilleries, or distilled water. Both cane juice and (diluted) molasses may be treated by boiling or pasteurization prior to further processing, in order to reduce populations of microorganisms.

Most cachaça is made from sugar cane juice, but most rum is made from molasses, although some rum made in Martinique ('rhum agricôle'), Haïti and a few others are derived from sugar cane juice. In many cases, distilleries that are located close to or are associated with sugar refineries use molasses, although imported molasses are also important.

Larger cachaça and rum distillers use special cultivated yeasts for fermentation, but smaller concerns may still use baker's yeast (e.g. in Brazil), wild yeasts or yeasts cultivated from generations of previous fermentations (e.g. Rhum Barbancourt, Haïti). Some fermentations are conducted with the lees (yeast residue) of previous fermentations (called 'dunder' or 'fermento-caipira'); certain aromatic rums from Jamaica and Martinique are typical of those using this type of fermentation.

The speed of fermentation has a considerable influence on the classification or style of the finished rum. Slow fermentations, taking up to 12 days, give beer that is rich in esters and higher alcohols (congeners). Such

beer, if double distilled in pot stills, results in the more 'aromatic' or 'heavy' rums of Demerara (Guyana), Jamaica, Martinique and certain other Caribbean islands. Rather more typical are rapid fermentations taking two days or less. These give a beer with fewer congeners, which if distilled in continuous (Coffey) stills, produces a more neutral or 'light' rum, spirit, typical of Brazil, Colombia, Cuba, the Dominican Republic, Puerto Rico and Venezuela. In general, wild yeasts and lees (dunder) conduct slower fermentations, while baker's yeasts or cultivated yeasts tend to ferment more rapidly.

Most fermentations are performed in temperature controlled carbon or stainless steel cylindrical vessels whose height is about twice the diameter, although other materials such as wood (as in former days), glass or plastic may be used, especially by small-scale distillers. Whatever the shape or material of the fermenter, its volume should be compatible with the distillation facilities.

The distillation process is also of high importance in deciding the style or character of the cachaça or rum, as with other spirits (see, for example, Scotch whisky, Chapter 3.2) The original method is double distillation using pot stills (Section 3.1.2). Rather in the style of Scotch malt whisky (Section 3.2.4), the first distillate (low wines) is mixed with the headings and tailings of a previous distillation and redistilled, whence the middle distillate, destined to become rum, comes over at about 65% alcohol (v:v). A variation on this general theme, originating in Jamaica, uses two retorts (called 'can pits') connected between the pot still and the condenser (Figure 3.5.3). This system removes one of the two distillation steps needed for double distillation and in effect is roughly equivalent to a triple distillation. The vapor leaving the still, containing around 30% alcohol (v:v), passes through the low wines in the first can pit where the aqueous part of the vapor condenses. This condensation releases heat into the low wine, which causes the vaporization, the vapors leaving the low wine retort now having around 60% alcohol (v:v). These vapors pass through the high wine in the second retort, where the above process is repeated, but this time the vapors leaving the high wine can pit contain close to 90% ABV. After collection of the headings the first fraction is collected as rum, then the following high wine (~75% ABV) and low wine (~30% ABV) fractions are collected (until the ethanol in the system is exhausted) and returned to the high wine and low wine can pits, respectively, ready for the next distillation batch (Figure 3.5.3).

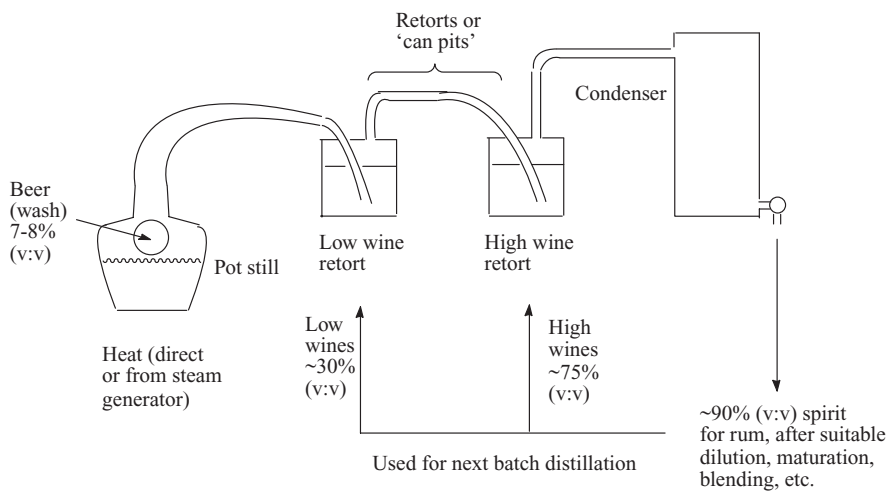
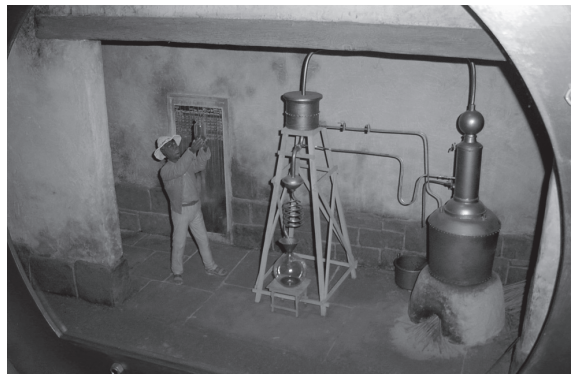


Figure 3.5.3 *Schematic representation of a twin-retort pot still distillation system. Used for certain heavy rums, originating in Jamaica. Not drawn to scale*

Pot stills, with or without double retorts, are used to make the more aromatic, heavier rums of Demerara, Jamaica and the French West Indies ('rhum arôme'). Similar rum is also made in Belize, Bermuda, Haïti, St. Kitts and other Caribbean islands. These rums all contain high ester and higher alcohol (fusel alcohol) contents. They are usually made from molasses, fermented slowly with dunder or special yeast, matured in oak casks and colored with caramel prior to bottling. These are the rums that go into the Navy and Demerara blends, although some are bottled 'straight' (e.g. 'Plummer' and 'Wedderburn' types from Jamaica and the 'Grand Arôme' types from Martinique). They are usually enjoyed neat, although they can be used to make cocktails, such as Planter's Punch. A model of a Caribbean pot still and part of a life size small Caribbean still from the eighteenth century can be seen in Figure 3.5.4.

Blending vat (1,720 gallon or 7,740 l). Bottom half of vat shown here, with two figures to give impression of size.



Model of West Indian rum still.

Life-size West Indian rum still

Figure 3.5.4 *Rum equipment at Jefferson's Rum Museum (Rum Story), Whitehaven, UK. This is effectively a museum based in Jefferson's former wine stores and warehouse. The emphasis is on rum, since the Jefferson family were involved in the import of rum into the UK via Whitehaven from the eighteenth century. (a) Vat (~1720 gallons; 7740 l) for blending or storing rum. Bottom part of vat is shown here, with two figures to give some idea of size. (b) Scale model of a small Caribbean rum pot still. (c) Life size model of small rum pot still*

Lighter rums (i.e. those that contain lower levels of congeners) and much cachaça are made using Coffey stills (Section 3.1.3), usually a two column system. The beer enters continuously at the top of the first column (the beer column), where steam entering the bottom vaporizes the ethanol (see Figure 3.1.6, Section 3.1.3). The vapors pass into the second column (the rectifier or analyzer column), where the heat of condensation of the aqueous component vaporizes more ethanol, but not the higher boiling point fusel alcohols. Thus, a relatively pure spirit containing ~95% ABV leaves the condenser (via the top of the rectifier column) to become rum, after appropriate dilution, maturation, etc. This type of rum has less volume of flavor than pot still rum and is a typical product of all the Portuguese or Spanish speaking countries of Central America, South America and the Caribbean region. These rums are often bottled after relatively short maturation times (in tanks or vats) and without coloration, although there are exceptions to this, and some is blended with darker more flavorsome rums to give medium bodied blends. The colorless versions (white rum) of Coffey still rum and cachaça are used in cocktails such as Daiquiri and long fruit drinks such as Caipirinha. In western Guyana, Coffey still spirit is matured over spices or fruit to give the popular spiced or fruit rums. A three column distillation system gives a highly rectified, almost flavorless spirit, like vodka, and indeed some molasses spirit distilled in this way can be called vodka in some countries (such as the UK) or can form base spirit of gin (Section 3.4.2).

The type of distillation system and the material from which it is made, not only has a bearing on the flavor of the resulting spirit (Section 3.1.1) but also on the levels of ethyl carbamate (urethane) (section 5.11.5) and dimethyl sulfide (DMS) and other sulfur compounds. In the latter case (a sensory defect), copper stills or aluminium stills with copper inserts were found to give cachaça with lower levels of DMS on an experimental/pilot scheme (Faria *et al.*, 2004b). Under distillation conditions, copper reacts with some of the DMS to form (as yet unknown) odorless substances.

The situation is more complex regarding ethyl carbamate (EC), a potentially carcinogenic compound found naturally in many foods and beverages. It appears that pot stills that are operated using low temperature/high reflux rate distillation (Section 3.1.2) produce cachaça with the lowest levels of urethane (Bruno *et al.*, 2007). This conclusion arises from a study of the spirits from 28 cachaça distilleries, who used a wide range of distillation systems, operations and materials. In general, pot stills produced spirit with lower EC levels than continuous stills, but the role of copper is not straightforward. The spirit with the lowest EC concentration came from a stainless steel pot still, but double distillation in a big pot still and distillation from stainless steel or copper alembics using a steam generator (rather than direct heating) and a copper serpentine (the 'worm' condenser used to make some Scotch malt whiskies – see Sections 3.2.4 and 3.2.6) gave spirits with low EC levels. This is important for cachaça producers, since the Brazilian government in 2005 put forward a five year limit for production to attain maximum EC levels of 150 μg/l, so that exports to countries such as Canada fulfill those countries' statutory limits for EC content.

Although the much rum and cachaça is not aged (much of it is consumed locally in long fruit drinks or cocktails, especially caiprinha), certain types for export are aged (in wood usually) because many countries have import regulations that stipulate a minimum ageing period for rum (usually one year). Ageing in wood is generally carried out in ex-Bourbon casks, their singed interiors imparting both flavor congeners and an amber or dark brown color, depending on the severity of singeing and on the resting time in cask. Most rum aged this way remains in the casks for no more than five years, but a few (particularly some Demerara, Jamaica and Martinique rums) are aged for longer periods – up to around 20 years. Rum aged in large wooden vats or stainless steel tanks remains almost colorless, but color can be added either by blending with dark rum or by adding caramel. On the other hand, any color acquired by a light rum during wood ageing can be removed by filtration through charcoal, a practice used extensively in Cuba. In general, casked spirits age more rapidly in subtropical climates than in cooler climates, so five years is normally sufficient to give well rounded mature rum. They also lose a greater volume of liquid (ethanol or water, depending on the cask strength of the spirit (see Sections 3.2.5 and 3.3.4): around 10%

per annum, as opposed to the 2% lost by Scotch whisky maturing in cask in the cool, damp climate of Scotland.

Most aged rum is destined for blends that are mostly medium bodied, consistent and whose bottles do not carry any indication of age. However, there is a growing market for premium rums, rather like the now well established market for malt whiskies. Premium rums are generally blends of spirits of different cask ages, whereupon the age of the youngest component is stated on the bottle label, as for Scotch whisky. Premium rums, like fine Cognacs and malt whiskies are meant to be sipped; they are usually medium to full bodied dark rums from Guyana, Haïti, Jamaica and the French West Indies, but premium rum is made elsewhere, notably Barbados, Bermuda and the US Virgin Islands. A few rums from the French West Indies are even vintage dated, like some Scotch malt whiskies (Section 3.2.6).

For rum and cachaça, like most distilled beverages, the art of blending plays an important role in maintaining consistency of product character from year to year. The blender's job is to ensure that consistency, which depends on the marriage of several distillates (or 'marks') to strike the best balance in the final product. This depends on the age and congener profile of the distillates available for blending. After ageing and blending, but before bottling, cask-aged rum is chilled and then filtered to remove flocs formed by the precipitation of fatty acids (Delevante, 2004).

The production of all alcoholic beverages involves material waste (see, for example, Sections 3.2.5 and 3.5.6). In other spirits industries the wastes can be disposed of in economically and environmentally acceptable ways. For example, spent grains from the mash used to produce whiskies make good animal feed and spent lees, pot ales, etc. from the stills are partially recycled in some distilleries. Distillation residues discharged into the environment require prior dilution because of the accumulated heavy metal salts (Section 3.2.5).

In the rum distillery, the most problematic waste is the still residue (mosto or 'rum slops'), which correspond to the residue of the rectifier Coffey still. This residue contains high levels of heavy metals and organic compounds making it both toxic and a heavy demander of biochemical oxygen, so untreated effluent is not permitted to be discharged into rivers or coastal seawater. Pretreatment (acidification) of the diluted molasses before fermentation, removal of yeast and fermenter sludge prior to distillation both help to minimize sludge build up in stills and to reduce the toxicity of distillery effluent. The sludges can be applied to the land and the still residue can be evaporated to give condensed molasses solubles (CMS), which can be used as animal feed or can be incinerated, the ashes being used as fertilizer.

3.5.4 Flavor Characteristics of Rum and Cachaça

Several studies have been performed on the flavor characteristics of rum and cachaça (e.g. Nykänen and Nykänen, 1983: Pino *et al.*, 1996; Ng, 1999; Faria *et al.*, 2004b; Faria *et al.*, 2004c; Pino, 2007; Nascimento *et al.*, 2008; Silva *et al.*, 2009). As with other spirits, the majority of volatiles fall into the categories esters, aromatic compounds, terpenoids, alcohols, acetals, aldehydes, phenols, ketones, furans, carboxylic acids and benzopyrans. In a recent detailed study (Pino, 2007), the number of compounds in each category is in the order listed above, although this may not necessarily reflect their relative contributions to the overall odor and flavor. Using a highly sensitive solid phase microextraction (SPME)/gas chromatography-mass spectrometry (GC-MS) technique (Sections 4.2.4 and 4.3.2), Pino identified 184 volatile components in unaged sugar cane spirit (aguadiente) and also in three and seven year old cask-aged rums.

In all the samples, the major volatile compounds were ethyl esters, 3-methyl-1-butanol and 2-methyl-1-butanol. The ethyl ester profiles for the samples were similar, suggesting that this particular profile may be influenced by fermentation and distillation methods, but not by maturation in oak casks. As expected, there were many differences in the concentrations of certain components in the unaged white spirit and the

cask-aged rums. In general, the aged rums had higher levels of ethyl acetate, ethyl butanoate, ethyl isobutanoate, a number of other esters, benzaldehyde and some terpenoids. On the other hand, the white sugar cane spirit had higher levels of certain esters, some hydrocarbons, (e.g. benzene and naphthalene derivatives), phenols and some edulans. Compounds normally associated with wood ageing (e.g. acetals, certain lactones, phenols and terpenoids) were present only in the aged rums. Principal component analysis based on 15 volatile components allowed discrimination between three and seven year old rums. The first principal component, contributing 69% to the total variance, was highly correlated with acetic acid, benzaldehyde, 3-*tert*-butyl-4-hydroxyanisole, (*E*)-β-damascenone, diethoxyethane, dodecanal, 2-dodecanol, ethyl butanoate, limonene and menthol.

Analysis of the ethyl esters (by direct injection GC) of 136 cachaça samples distilled in copper alembics, mixed copper/stainless steel stills or steel columnar stills revealed ethyl acetate and ethyl lactate to be the most abundant (Nascimento *et al.*, 2008). High levels of ethyl lactate in cachaça (compared with whiskeys) were suggested to be the result of greater *Lactobacillus* activity during fermentation; spontaneous fermentation at high pH and reaching temperatures of 45 °C, as opposed to the yeast culture, temperature controlled fermentations at lower pH used for whiskey production. Cachaça produced in alembics (the majority: 109/136) had higher mean levels of ethyl acetate and ethyl lactate than those produced in column stills, reflecting the greater rectifying power of the latter types of stills. Other ethyl esters (butanoate, hexanoate, octanoate, decanoate and laurate) of cachaça were present in much lower concentrations (as in whiskeys) and were slightly more abundant in column still samples.

Supercritical fluid extraction (SFE) (Section 4.2.2) using CO_2 has been applied to the assessment of aroma compounds in the intermediate streams in the Cuban rum production process (Gracia *et al.*, 2007). The head alcohol fraction contained the greatest number of higher alcohols, as determined by GC-MS analysis of the CO_2 extract. This corresponded to an organoleptic (sensory) evaluation of 'fruity.' The levels of higher alcohols diminished and concentrations of carboxylic acids, esters and other compounds increased from the head alcohol fraction through crude (unaged) spirit to aged rum. Sensory evaluation correspondingly changed from fruity, through sweaty to whiskey, the last named descriptor meaning aged or mature. The head alcohol fraction was suggested as the best source of natural flavoring compounds for the food industry, extracted by supercritical CO_2. This fraction is not only rich in flavor components, but is normally used as a fuel source (as with cachaça – see Section 3.5.2), thus isolation of flavor compounds could increase the value of this fraction and contribute to increased profits.

In Brazil, since the government is pushing to optimize cachaça quality, there is much interest in the isolation and selection of yeast strains that give cachaça with high sensory value and levels of EC below those specified by current legislation. Recent experiments have evaluated 233 *Saccharomyces cerevisiae* isolates from traditional cachaça fermentations for good flocculation characteristics, high attenuation capability and nonproduction of hydrogen sulfide (Silva *et al.*, 2009). It was found that although several strains possessed these desirable characteristics and gave cachaça with good sensory evaluations, one in particular (UFMGA-1031) was recommended for use as a starter for the production of high quality cachaça. It is expected that as the industrialization and rationalization of cachaça continues, more and more use will be made of cultured yeast starters.

Wood ageing naturally contributes to the flavor of rum and cachaça, as with other alcoholic beverages. Ex-whisky or Bourbon casks are often used to age Caribbean rums (Section 3.5.3), but this practice is a relatively newcomer in the production of premium cachaça in Brazil. That country grows a wide variety of trees whose wood is capable of being made into casks and it is possible to buy cachaça aged in wood other than oak. In an experiment, the sensory values of cachaça aged for six months in specially made 15 l casks made of various indigenous woods and oak were compared (Faria *et al.*, 2004c). Sensory analysis by a panel based mainly on overall aroma and flavor suggested that amendoin (*Pterogyne nitens*), pereiro (*Hymanaea stigobocarpa*) balsámo (*Myroxylon peruifum*) and pay d'arco (*Tabebuia impetiginosa*) may be suitable for

ageing cachaça, but the remainder, amarelo (*Plathymenia reticulta*), pan d'oleo (*Copaifera langsdorfii*) and louro *Aniba parviflora*) were considered unsuitable.

Although cachaça and rum have similar origins, experienced tasters usually have little difficulty in distinguishing the two spirit types, implying that they have significant differences in aroma profiles. Gas chromatography-olfactometry (GCO) (Section 4.3.2) was used to identify the most potent aroma compounds in extracts of new make (unaged) cachaça and Puerto Rican rum, and the results were compared with the results of descriptive sensory analysis (Section 4.7.4) (De Souza *et al.*, 2006). Cachaça was found to be more intense in the grassy, spicy, sulfury and vinegar descriptors, whereas apple and caramel were about the same in cachaça and rum. This corresponded to GCO observation of spicy eugenol, 4-ethylguaiacol and 2,4-nonadienal being much more potent in cachaça, but fruity β-damascenone, ethyl butanoate, ethyl isobutanoate and ethyl 2-methylbutanoate were at about the same potency in cachaça and rum.

Application of chemometric techniques to the chemical profiles (including not only certain volatile components, but also nonvolatile components such as minerals and phenols) of various types of rum allows classification or identification, as with other beverages (e.g. see Marsala, Section 2.10.8; pomace spirit, Section 3.7.2). Sampaio *et al.* (2008) used principal component analysis (PCA), partial least square-discriminate analysis (PLS-DA) and linear discriminate analysis (LDA) of chemical descriptors derived from GC analysis of aroma compounds, atomic absorption analysis of minerals and HPLC analysis of polyphenols to discriminate between Cuban and other light rum types. In total, 23 analytes were chosen as relevant descriptors, including the aroma components acetaldehyde, acetone, 1-butanol 2-butanol, 2-butenal, cyclopentanone, ethyl acetate, isoamyl alcohol, isobutyl alcohol and vanillin.

3.5.5 Arrack, Mezcal and Tequila

As explained in Section 3.5.1, arrack (and its variants alak or arak) refers to any distilled alcoholic beverage in Asia, irrespective of the source of fermentable sugar (fruit, grain, sugar cane or vegetable. For the purposes of this book, the term arrack is used to describe the spirits of southern and southeastern Asia, particularly those derived from coconut palm sap. Arrack derived from sugar cane is also called rum and is dealt with in Section 3.5.4. The term arak is reserved for spirits of western Asia, especially those of the Middle East derived from grapes and anis or aniseed and is dealt with in Section 3.5.6.

Coconut arrack is distilled from palm wine, a beverage made by fermenting the sap (neera) tapped from the preblossom flowers (i.e. the unopened spadix) of coconut palm trees (*Cocus nucifera* L.). It is a popular alcoholic beverage of Sri Lanka, where a variety of brands are available, having alcohol contents of from ∼33% to over ∼50% (v:v). The cheaper brands are blends of arrack and neutral spirit; these are drunk with cola, ginger beer or soda water. Better quality coconut arrack tends to be consumed neat, with water or in cocktails. Sri Lanken arrack is produced by companies such as Indian Distillers Ltd, Distillers Company of Sri Lanka (DCSL), Mendis and Rockland. The brands often have sophisticated names such as VSOA ('Very Special Old Arrack') and Old Reserve. Some of these companies also produce rum.

A comparable drink to arrack, known as lambanog, is produce in the Philippines. It too is distilled from palm wine (fermented coconut palm sap: called 'tuba' in Luzon), though it is often flavored with fruit (e.g. blueberry or mango) or spices (e.g. cinnamon). At the time of writing (2009) it is just emerging from its long existence as essentially a cottage industry. Its situation is rather like that of cachaça in Brazil a few years ago.

The palm sap (called neera in southern India) is usually clarified by filtration before fermentation and distillation. As in other cases, alcoholic, lactic and other fermentations cause changes in the aroma profile, so that fermented neera (called 'toddy' in southern India) contains a greater quantity of volatile compounds. However, several aroma compounds are retained from the fresh neera, including ethyl lactate, farnesol and

2-phenylethanol (Borse *et al.*, 2007). Ethyl lactate persists through the distillation process and is considered a characteristic volatile constituent of all commercial coconut arrack (Samarajeewa *et al.*, 1981).

Mezcal and tequila are distillates of wines made by the fermentation of syrup exudates from cooked pines of *Agave* (Maguey) plants. Plants of the *Agave* family (*Agavaceae* genus) are tall succulents, although there is considerable biodiversity within the genus: various species of *Agave* grow in Mexico and other Central and South American locations. *Agave tequilana* var. Azul ('blue agave') grown mostly in west-central Mexico (especially in the states of Jalisco and Guanajuata), originally around the city of Tequila, is used for the production of Tequila, by law. Other species of *Agave* can be used for mezcal: *A. salmania* in the Mexican altiplano and *A. angustifolia* with *A. potatorum* in southern Mexico (De Léon-Rodríguez *et al.*, 2006). *Agave* plants are cultivated on farms and are harvested when between seven and nine years old, whereupon the magueyeros (maguey farmers) cut the plants from the roots and strip off the leaves to reveal the pines (piña), which can weigh up to 50 kg each.

Tequila must be made from blue agave within certain designated locations in the states of Guabajuato, Jalisco and others. Mezcal is made from Agave plants other than blue agave in a much wider range of locations. Much mezcal is produced in the state of Oaxaca, but the drink may have local names such as bacanova (Sonora region) or sotol (Chihuahua region). Both mezcal and Tequila are protected by an international Denomination of Origin: the production of Tequila is subject to the Mexican regulation NOM-006-2005 (SECOFI, 2005), whereas mezcal is subject to the regulation NOM-070-1994 (SECOFI, 1994). The annual production of Tequila in 2006 was 242.6 million litres, whilst that of mezcal was 6 million liters (Lappe-Oliveras *et al.*, 2008). They are both are consumed straight, with water, or as components of long drinks or cocktails.

The production process for traditional mezcal and Tequila are summarized in Figure 3.5.5. In all cases, the *Agave* hearts or pines are heated to ~100 °C for at least 36 h. traditionally this performed in sunken ovens (hornos) using preheated stones, but many modern distilleries use pressure cookers or autoclave-like ovens. The mean reasons for the cooking process are to hydrolyze the polymeric fructans into fermentable sugars (Waleckx *et al.*, 2008), to soften the pines so that crushing and extraction of fermentable sugars is easier, and to create a number of flavor compounds via the Maillard reaction (Chapter 2.6). The baked pines are separated

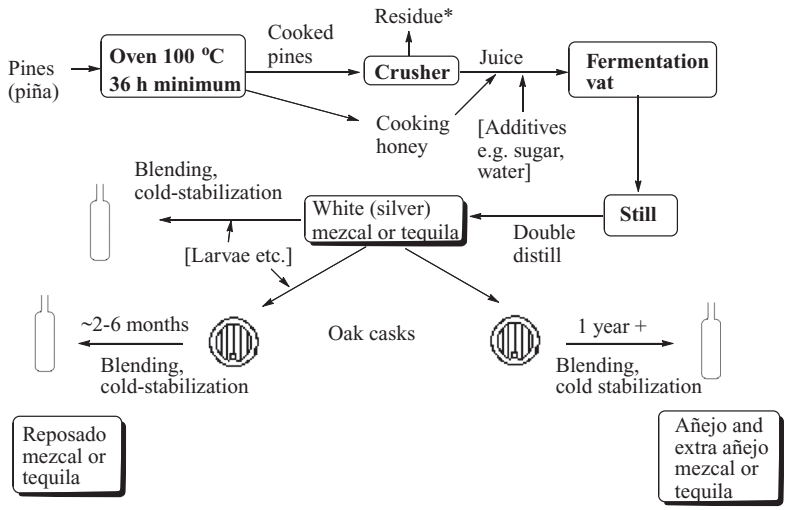


Figure 3.5.5 *Simplified scheme for the production of mezcal and tequila. *Some traditional distilleries ferment whole crushed, cooked pines and distil from this, in which case the fibers and debris have to be removed from the still before the first distillate is returned for redistillation*

from the viscous 'cooking honey' produced by the cooking process and are then crushed. This gives *Agave* juice, which is mixed with the cooking honey ready for fermentation. Additives, such as cane or corn sugar may be added at this stage. In some distilleries the whole crushed *Agave* pines (i.e. the juice plus pulp and fiber) is fermented, but in the majority of cases fermentation is carried out by microorganisms in the juice and on the distillery equipment. Fermentation takes place in vats or tanks of 500–10 000 l capacity, made of wood, stainless steel or stone, and lasts several days, depending mainly on the sugar extract of the must and local weather conditions (Lappe-Oliveras *et al.*, 2008). The resulting liquor is double distilled in metal or earthenware pot stills. As with other spirits (e.g. see Section 3.2.4), the first distillation yields a relatively crude spirit containing *ca.* 30% ethanol (v:v), which is returned to the still (after removal of residues) for a second distillation, producing a spirit of about 60–65% ABV.

Industrial mezcal and Tequila are often made by the stepwise process shown in Figure 3.5.5, but with modifications at various points (apart from scale of operation: 2000–120 000 l fermentation tanks) including the use of steam injection ovens or autoclaves for cooking, the use of a combined water extraction/milling process, the use of dried yeasts or inocula of yeast starters (∼5–10% of must volume) and the use of rectification columns on the stills. The last mentioned technique increases distillation efficiency and allows greater control of the organoleptic qualities of the final product (Prado-Ramírez *et al.*, 2005). In particular, monitoring ethanol content is a crucial factor determining how the slop cuts (the first distillate) and final distillate meet the Tequila norm for regulated compounds: ethanol, higher alcohols, methanol, acetaldehyde, ethyl acetate and furfural).

By law there are two categories of mezcal and Tequila, depending on how much of the fermentable sugars are derived from the maguey plant. For mezcal these are type I (100% Agave) and type II (80% Agave) and for Tequila, they are called Tequila 100% (100% Agave) and Tequila 51% (51% Agave). Each category is subdivided according the extent of ageing in small white oak casks: 'mezcal joven,' white or silver Tequila ('Tequila blanco') and 'Tequila joven u oro' (golden Tequila) are unaged, 'reposado' indicates 2–6 months cask ageing, 'añejo' indicates up to one year in cask and 'extra añejo' indicates at least three years of cask ageing. The most popular Tequila is the reposado type. Tequila 100% is required by law to be bottled in the region of production, but Tequila 51% can be exported in bulk and bottled outside Mexico.

Mezcal joven and reposada (but not añejo) are sometimes sold with various animal or mineral additions in the bottle. These include 'maguey worms,' scorpions and glass figurines wrapped in gold foil. The 'maguey worms' are in fact the larvae of certain insects found on *Agave* plants and up to four of these can be found in certain bottles of mezcal. For example, the well known mezcal brand 'Gusano Rojo' (Nacional Vinicola) makes use of the gusano rojo (red worm) – the larva of the *Hypopta agavis* moth.

Like other alcoholic beverages, general organoleptic characteristics, including flavor, depends on the number and type of constituents in the distillate. The presence of some Tequila and mezcal constituents (such as ethanol, acetaldehyde, higher alcohols, methanol and ethyl acetate) are regulated by Mexican law: their concentrations must fall within specified limits. However, the majority of flavor congeners, generally present in lower concentrations, are not regulated and it is these that generally contribute most to the overall character of the spirit.

As with other alcoholic beverages, the microbiota involved in fermentations play an important role in establishing the aroma/flavor profile of mezcal and Tequila. Utilization of 'spontaneous' fermentation or of starters originating from such fermentations is still widespread in the production of these spirits. These rely on the natural microorganisms already present in the *Agave* must and on distillery equipment and hence fermentation comprises of a number of phases in which successions of yeasts and bacteria dominate. In the early stages of fermentation of *Agave tequilana* to produce Tequila, *Dekkera bruxellensis*, *Hanseniaspora* spp., *K. marxianus*, *P. membranifaciens* and *T. delbrueckii* were active, but *S. cerevisiae* was the dominant yeast, whereas toward the end of fermentation the last mentioned was totally dominant (Lachance, 1995). Other workers have observed somewhat different microbiotic profiles isolated from different Tequila distilleries and

totally different ones (in which LAB and *Z. mobilis* dominate in some; *S. cerivisiae* dominating in others) from mezcal distilleries (Escalante-Minakata *et al.*, 2008; Lappe-Oliveras *et al.*, 2008).

It seems clear that different *Agave* species, locations and distillery conditions favor different microbiological populations and this will account for differences in flavor profiles amongst mezcal, Tequila and related spirits, such as raicilla. Also, if a dominant species in one must (say mezcal) is transplanted into an alien must (say, Tequila or raicilla) both biological activity and formation of flavor compounds often differ (Arrizón *et al.*, 2007). *S. cerevisiae* strains from fermenting Agave musts were found to produce β-glucosidase, whereas non-*Saccharomyces* secondary yeasts, such as *Candida*, *Kluyveromyces* and *Hanseniaspora* spp., produced both β-glucosidase and β-xylosidase enzymes, with *C. magnoliae* having the greatest β-glucosidase activity (Fiore *et al.*, 2005). These secondary yeasts could thus play an important part in the release of flavor active terpenes from their flavorless glycosides during fermentation, thereby making an important contribution to the flavor profile of the spirit. Similar relationships have been observed for natural fermentations versus inoculated yeast monoculture fermentations in the resulting flavor profiles of wine (Section 2.2.6), beer (Section 2.6.7) and cider (Section 2.8.4).

There are flavor differences between mezcal and Tequila that are made from highland or lowland plants. The highland plants give pines that are bigger and juicier, and produce liquor and spirit with more fruity notes, whereas lowland pines tend to be smaller, giving a spirit with more earthy notes. Characteristic flavor compounds of mezcal and Tequila include ethyl acetate, ethyl lactate, acetic acid, higher alcohols (major contributors), other alcohols, carbonyl compounds, carboxylic acids, ethyl esters, furans, terpenoids and hydrocarbons (De Léon-Rodríguez *et al.*, 2006; Cardeal and Marriott, 2009). In this respect, they resemble other distilled spirits. However, two dimensional GC/MS (Section 4.3.2) can distinguish Tequila from other distilled drinks (Cardeal and Marriott, 2009), and mezcal produced from different species of *Agave* can also be differentiated using SPME-GC/MS (Sections 4.2.4 and 4.3.2) by the presence of unique compounds. Mezcal from *A. salmania* contained limonene and pentyl butanoate, which could be used as markers of authenticity (De Léon-Rodríguez *et al.*, 2006). Similarly, mezcal obtained from *A. angustifolia* was distinguished from Tequila and sotol by the presence of ethyl nonanoate, 2-acetylfuran and 2-methylnaphthalene, respectively (López and Guevara-Yáñez, 2001). Furthermore, mezcal with larvae could be distinguished by the presence of 3-hexen-1-ol and 6,9-pentadecadien-1-ol (De Léon-Rodríguez *et al.*, 2006). Also, using principal component analysis of the fluorescent background of Raman spectra (Section 4.4.2) it has been possible to distinguish Tequila blanco (unaged) from aged Tequila (Frausto-Reyes *et al.*, 2005).

In recent years, mezcal and Tequila have received international recognition and worldwide consumption of tequila has increased enormously; for example, its sales in the USA are about the same as brandy, prepared cocktails and Scotch whisky (Corrigan, 2004). Frequent fraud, particularly aldulteration of Tequila with grain spirits or the labeling of 'mixed' category Tequila as '100% Agave' (the 100% *Agave* category is more expensive) has accompanied this growth and hence it is necessary to exert authenticity control of *Agave* spirits (Lachenmeier *et al.*, 2006). This is particularly, so since much of the lower grade 'mixed' Tequila is shipped in bulk and bottled outside Mexico. In a study of a wide range of Mexican *Agave* spirits, using GC and ion chromatography (Section 4.3.3), Lachenmeier *et al.* (2006) found that the two Tequila categories (100% *Agave* and mixed; made with added sugar) could be differentiated on the basis of methanol, 2- and 3-methylbutanol and 2-phenylethanol. The mixed category samples had lower levels of these constituents. Likewise, all *Agave* spirits had concentrations of oxalate (0.1–9.7 mg/l), derived from the *Agave* plant material, although there was considerable overlap of concentrations in samples of 100% *Agave* and mixed Tequila. Hence oxalate content was not recommended as a discriminator between the two Tequila categories. Additionally, higher total levels of anions were observed for artisanal *Agave* spirits, reflecting the lesser technological sophistication in the production of these spirits; less pure water used for dilution, for example.

Raman spectroscopy, although used primarily to determine the ethanol content of Tequila (using the OH region profile of water), was able to distinguish silver Tequila from aged tequilas, via application of a

chemometric (principal component analysis, PCA) to the fluorescence background of the Raman spectra (Frausto-Reyes *et al.*, 2005).

More recent work has shown that the four categories of Tequila and mezcal could be differentiated on the basis of chemometric analysis of the concentrations of 12 elements (Al, Ba, Ca, Cu, Fe, K, Mg, Mn, Na, Sr, S and Zn), determined by inductively coupled plasma optical emission spectrometry ICOES (Section 4.4.4) (Ceballos-Magaña *et al.*, 2009). Several chemometric treatments were tried, with the probabilistic neural networks method giving 100% success in the differentiation of extra aged, aged, gold and silver Tequila, and mezcal.

3.5.6 Aniseed Flavored Spirits

Aniseed flavored spirits are produced in many countries around the Mediterranean Sea: Egypt, France, Greece, Israel, Italy, Lebanon, Palestine, Spain, Syria and Turkey. They are also made in a number of Balkan countries, such as Bulgaria and Macedonia, as well as in some Middle Eastern countries, such as Iraq and Jordan. Most are based on brandy (distilled wine; Chapter 3.6) or grappa (distilled pomace; Chapter 3.7), although some are based on fruit brandies (Chapter 3.8) and others on cane sugar spirits or grain/vegetable spirits (Chapter 3.4). In the Middle East their generic name is arak. Here, they can be made from distilled date, fig or plum wine, rather than brandy or grappa, and in Israel, spirits are made from sugar cane 'beer' as well as wine. In the European Mediterranean countries, they are usually distilled from wine or pomace and are known by many different names, rather than arak. For example, there are ouzo, raki, tsikoudia and tsipouro in Greece; mastika in several Balkan countries; pastis in France; anesone and sambuca in Italy; ojén in Spain.

Methods of production of aniseed flavored beverages and related spirits vary widely, as does the spirit base and the flavorings, thus giving rise to a family of drinks with an obvious similarity, but at the same time with subtle differences. Middle Eastern arak (e.g. of Lebanon, Palestine and Syria), based on brandy, is usually made in the weeks following the grape harvest – for example in the mountainous areas of Lebanon, in October or November. The grapes are often fermented on the pulp for 2–3 weeks and the resulting vinous mixture is distilled in an alembic (pot still) to give a crude brandy. The alembic is emptied of the residue, washed and the crude brandy is then returned to it and mixed with aniseed, the amount of which depends on the distiller. A second (slow, low temperature) distillation yields arak as the middle distillate, usually of ~65% ethanol (v:v) content. In the Lebanon and some other countries, such as Greece and Macedonia, completion of the first distillation is accompanied by a ceremonial party at the distillery.

The major flavor component of aniseed, and hence of arak, is anethole. In the 1980s, the anethole content of 30 commercial Lebanese Arak samples was found to vary between 1.2 and 3.8 g/l, with an average value of 2.4 g/l. Arak from artisanal distillers had a much wider range of anethole content: 0.5–4.2 g/l, but with a similar average of 2.5 g/l (Geahchan *et al.*, 1991). Acetaldehyde, methanol and 1-propanol were found in all Lebanese arak based on brandy, where the ranges were 6–220 m/l, 9–540 mg/l and 17–760 mg/l, respectively. These components were missing from arak based on high proof alcohol. Other components were ethyl acetate (0–870 mg/l), 2-butanol (0–524 mg/l), 1-butanol (0–400 mg/l) and amyl alcohol/*iso*amyl alcohol (0–1692 mg/l) (Geahchan *et al.*, 1991).

Aniseed flavored spirits are popular in Greece, where again, they are usually based on brandy or grappa. These include tsipouro (τσίπουρο; called tsikoudia or raki on Crete), masticka (μαστίχα; similar to the мастика of Bulgaria and Macedonia) and the most popular and most heavily commercialized spirit ouzo. Tsipouro and tsikoudia are often unflavored pomace brandies, like aguadiente, grappa or marc (Chapter 3.7), but some versions of tsipouro are flavored with aniseed and some tsikoudia is flavored with herbs, such as thyme. Masticka (and sometimes other spirits) is flavored with gum mastic.

One the most famous aniseed flavored spirits is ouzo, whose name is probably derived from uzum, the mediaeval Turkish word for grapes. The drink probably evolved from tsipouro and rose to prominence

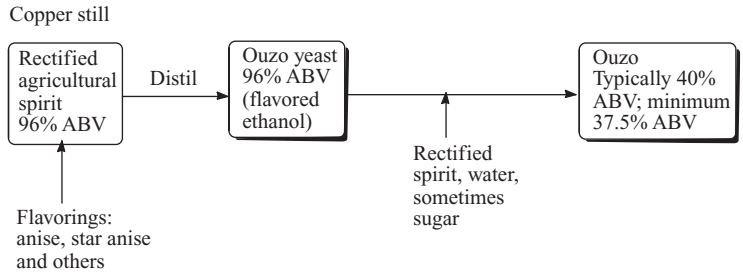


Figure 3.5.6 *Simplified diagram for the production of ouzo*

following Greek independence from the Ottoman Empire (1832). Its popularity increased when absinthe (see later) was banned by the French government in 1915. Nowadays, ouzo is distilled all over Greece, but the island of Lesbos remains a major producer and in fact claims to be the originator of the beverage. Most ouzo is now made like gin (Section 3.4.2) from rectified spirit, which in theory can be of any agricultural origin, in practice is most likely to be of wine or pomace origin.

Flavorings, which include natural anethole, anise (aniseed; *Pimpinella anisum*), cinnamon (*Cinnamomum verum*), coriander (*Coriandrum sativum*), cloves (*Syzygium aromaticum*) and star anise (*Illicium verum*), according to the style of the distiller, are added to the spirit in a copper still and then distilled (Figure 3.5.6). The highly flavored distillate (called μαγιά ούζου or 'ouzo yeast'), containing around 96% ABV is either diluted with distilled or mountain spring water to *ca.* 40% ABV or mixed with neutral spirit and then diluted. Ouzo based only on ouzo yeast is generally considered to be the finest. Greek law stipulates that at least 20% the total final alcohol all ouzo must be derived from ouzo yeast. Ouzo, tsipouro and tsikoudia were all accorded Protected Designation of Origin status by the European Commission (2006), so that according to EU law, these names may only be used for the spirits produced in Greece and certain of its island dependencies.

Further west, pastis (France) and sambuca (Italy) are the most common aniseed flavored spirits. Pastis came into being after 1915, when the French wartime government banned absinthe, a drink that had already received much criticism relating to its perceived unhealthy effects, including claimed hallucinogenic effects. The blame for these undesirable properties fell largely upon α-thujone, a major constituent of wormwood (*Artemesia pontica*), one of the major flavoring ingredients of absinthe. There is now considerable doubt regarding the focus of this blame – see Section 5.11.2. At the time it was banned, absinthe was a very popular beverage, so the two main producers, Pernod Fils and Ricard (now Pernod Ricard) created pastis: absinthe without the wormwood constituent, without the green coloring matter, but with more star anise component and less alcohol.

Aniseed flavored spirits are often consumed with cold spring water (about 1:3), to which ice is sometimes added. A characteristic common to all these beverages is their milky appearance when mixed with water. This occurs because some of the oily components of the aniseed flavoring (e.g. anethole) are soluble in 40–45% ethanol–water mixtures (as in the undiluted spirit), but not in the diluted drink. The micrometer sized oil droplets grow only very slowly, thus preserving the attractive milky appearance of the mixture. Slow growth has been attributed to very low interfacial tension of the droplets. Growth occurs by two processes:

- Ostwald ripening. This involves diffusive transport of dissolved oil through the medium, giving a linear droplet volume increase with time.
- Droplet coalescence (creaming). This involves the merging of two or more droplets, resulting in an exponential increase in droplet volume with time.

A study of the interfacial tensions of the three phase system anethole–ethanol–water (a pastis–water simulation), measurement of both types of growth rates and comparison of the results with predicted by Ostwald ripening and creaming models led to the conclusion that normal Ostwald ripening theory may not be applicable to this system because of the different gradients of concentration, density and pressure that exist in a critical system such as the three phase system studied here (Scholten *et al.*, 2008).

In the eastern Mediterranean and certain Balkan countries, these spirits are often consumed at ceremonies or with meals such as mezedes (small dishes made from fish, meat and vegetables). They are sometimes mixed with fruit juices or liqueurs, like the Bulgarian 'Cloud' (облак) – masticka and menta, a mint liqueur. Otherwise, aniseed flavored spirits, including pastis, are used as apéritifs. There are also many cocktails based upon pastis, like canditini, cornichon, mauresque, parroquet and tomate.

Anisette and sambuca are aniseed flavored liqueurs, that is to say they are rather sweeter than arak, ouzo, pastis and others, so they are dealt with in Chapter 3.9. The former was created to replace absinthe, but the latter dates back to the latter half of the nineteenth century.

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3.6
Brandy

3.6.1 Introduction and Brief History

The word 'brandy' originates from the Dutch word *brandewijn*, meaning 'burnt wine.' In South Africa (where brandy is the top selling spirit drink), the Afrikaans term for the beverage is also *brandewyn*. The German term *weinbrand* is similarly derived, but outside the German speaking countries it is usually only used to refer to brandy produced in Austria and Germany. The 'burnt wine' label is appropriate, as most brandies are made through heating wine (without burning it), distilling over the alcohol (ethanol) and volatiles, and ageing the distillate in small oak barrels.

Distillation as a process is so old that the origins are uncertain. It may have been carried out as early as *ca.* 3000 BC in China, and was certainly used in Egypt around 2000 BC to make wood extracts during charcoal processing. There is evidence that the making of rose water in Persia and India in ancient times involved distillation (Amerine and Singleton, 1977), possibly using wool to trap and condense vapors, and the technique spread through Europe in the sixth century AD with the influx of Arab traders and scholars.

According to Léauté (1990), the art of distilling *wine* was first 'perfected' by a returned crusader, Arnaud de Villeneuve in France, in around the middle of the thirteenth century. Villeneuve proclaimed that the distillates prolonged life. In the Cognac region around 1600, the Chevalier of Croix Marron mastered double distillation, which further concentrated the alcohol. Distillates were also mixed or infused with a variety of herbs, and the medicinal (and antiseptic) properties associated with these infusions were probably the reason that they were known as *aqua vitae* (water of life) in Latin, and are still known in France today as *eau de vie*. The tradition of herb infused distillates led to the development of many of the flavored liqueurs (for example, Benedictine, Chartreuse, Jagermeister – see Section 3.9.3) that are available today.

It is probable that wine was initially distilled in order to decrease the volume before transport on ships. Distillate may also have been added to wine to preserve it during transit. The containers in which the fortified wines and distillates were stored and transported were made from wood, and extraction of various aromatics and phenolics would intensify flavors further during lengthy voyages. When water was added back to the distilled liquor before consumption, the resulting product had greater complexity, and was probably a considerable improvement on the original harsh wine. Jackson (2000) noted that although there are records of distillation of alcoholic beverages being carried out in Europe as early as the twelfth century, distillation on a larger scale for beverage production really only took off in the sixteenth century, with the first Cognac being made in the seventeenth century. According to the South African Brandy Foundation, brandy was first

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

distilled in the Cape on 19 May 1672 by a chef on the Dutch ship *De Pijl* (South African Brandy Foundation, 2010).

Modern brandy is usually made from the fermented juice of *Vitis vinifera* grapes, but distillates can be made from any fermented beverage that originally contained carbohydrates (for example whisky is the distillate of fermented grains). According to modern EU regulations (EEC, 1989) brandy or *weinbrand* '. . .is a spirit drink, produced from wine spirit, matured for at least one year in oak receptacles or for at least six months in oak casks with a capacity of less than 1000 litres, containing a quantity of volatile substances equal to or exceeding 125 grams per hectolitre of 100% vol. alcohol, and derived exclusively from the distillation or redistillation of the raw materials used, having a maximum methanol content of 200 grams per hectolitre of 100% vol. alcohol. The minimum alcoholic strength by volume of brandy or *weinbrand* shall be 36%. No addition of alcohol as defined in Annex I (5), diluted or not, shall take place. Brandy or *weinbrand* shall not be flavored. This shall not exclude traditional production methods. Brandy or *weinbrand* may only contain added caramel as a means to adapt color.'

The EU definition excludes fruit spirits (Chapter 3.8), pomace spirits (Chapter 3.7), and even grape brandy that has not undergone ageing. The same European Union regulation defines the names of these excluded spirits as 'fruit spirit,' 'grape marc spirit,' and 'wine spirit,' i.e. in terms of the legislation, these beverages are not allowed to be known as 'brandy.'

Brandies are usually classified by region of production, even though the soil conditions, climate and grapes used to produce the base wine have less influence on the final product than the distillation process used and ageing practices (Amerine and Singleton, 1977). The best known brandies are Cognac and Armagnac, which are described in more detail in Sections 3.6.2 and 3.6.3. Cognac is produced in the Charente region north of Bordeaux, and is made from the wine of the St. Émillion, Colombard (or Folle Blanche) grapes. The soil of the district contains a high percentage of calcium carbonate and it is generally accepted (although the theory remains to be tested scientifically) that the best brandies come from grapes grown on these calcareous soils.

The Base (Rebate) Wine

It is incorrect to assume that all wine that is not seen as good enough to be bottled as wine for the table is simply carted off to distilleries for brandy production. Certainly, some wineries will try to recoup financial losses through selling off poor quality batches for distilling, but most good brandy production is dependent on a good quality base wine, albeit one that is often rather thin and acidic.

Wine that has oxidative or reductive taints, or is oversulfured, is infected with microorganisms or has produced peculiar characteristics during fermentation as a result of yeast stress, is unlikely to be accepted for distilling, except perhaps to make industrial alcohol. Good quality brandy is generally distilled from wine made for the purpose and should have a moderate alcohol content, crisp acidity and little or no sulfur dioxide added to it. The choice of grape variety, the *terroir*, the time of harvest and the wine making process are specifically defined to produce the best possible raw material to distil. Healthy grapes from white cultivars are usually used that are necessarily neutral in character, for example in South Africa, Chenin Blanc and Colombard account for 90% of production, but Cinsaut and Sultana may also be included. Grapes are harvested early so as to avoid high concentrations of primary aroma compounds, which would not suit the style of the final product. In France, Ugni Blanc (also known as Trebbiano) is often used for base wine production for Cognac, but other grapes such as Colombard and Folle Blanche may also be used. In the cool Charente region, these varieties yield grapes that naturally produce between 8 and 9% alcohol in most years (Amerine and Singleton, 1977).

In California, cultivars without distinctive varietal character such as Colombard and Thompson Seedless (a table grape) are commonly used (Jackson, 2000). Other pale colored varieties such as Tokay, Mission and Emperor may also be used. According to Léauté (1990), Professor James F. Guymon studied several

Table 3.6.1 *Requirements set for quality wine that is to be used as pot still brandy (rebate wine) as stipulated by the Wine and Spirits Control Act (Act 47 of 1979) in South Africa*

	%w/v Total solids ≤ 45	%w/v Total solids > 45:
Total polyphenol content (mg/l GAE)	<250	<1200
Residual sugar content (g/l)	<4	<4
Volatile acidity (g/l)	<0.7	<1.2
Total sulphur dioxide (mg/l)	<20	<70

grape varieties used for brandy production in the United States in the early 1970s and came to the conclusion that early harvested French Colombard wines produce a better brandy than Thompson Seedless. Generally, producers recommend that grapes are pressed at a sugar content of 17 to 18 °Balling and a total acid content of between 7 and 9 g/l. If possible, free run juice that has had little skin contact is used so that the wine will meet the requirements of the legislation in terms of the total polyphenols (see Table 3.6.1 for the South African example). It is generally recognized that the lower the total polyphenol and volatile acidity (VA) content of the base wine, the higher the resultant quality of the brandy. Brandy quality tends to increase as the total acidity increases or the pH of the wine decreases. Skin contact is undesirable, and usually only free run juice is used so that the concentrations of total phenols are kept as low as possible. A high polyphenol content can catalyze oxidation, resulting in a product with an aldehyde character. Such a character may be desirable in the grape spirits used for the production of fortified wines, such as Sherry (Section 2.10.2) and Port (Section 2.10.7), but is unwanted in brandy per se.

After pressing, the must is usually clarified by means of settling or centrifuging to remove any scraps of skin and unwanted microorganisms and enzymes. Centrifuging is often used for clarification as the must needs to be cooled as quickly as possible, and long settling times without the protection of sulfur dioxide are not advisable. As very little SO_2 is allowed, it is also of great importance that the wine is made under very hygienic conditions. If pectins are extracted, and pectolytic enzymes are used, methanol will be formed and will distill over into the brandy. The methanol content of distilled spirits is the subject of strict regulation.

A pure yeast culture will be inoculated into the must, in order to prevent any off odors associated with wild strains. The choice of yeast strain can have a marked effect on the quality of the final product. For example, a strain that produces elevated levels of 2-phenylethanol (a floral scent), and low levels of acetaldehyde ('Sherry odor') is advantageous, as these characteristics will influence the distillate positively. In a study conducted by Stegers and Lamprechts (2000), 107 yeast strains were screened for their ability to produce a brandy base wine of exceptional sensory quality. Volatile acids, esters and higher alcohols (or 'fuselols' containing more than two carbon atoms in their structure) were quantified and significant differences between yeast strains for higher alcohol, fatty acid ester and acetate concentrations were observed. On the basis of their chemical profiles, only 16 of the original 107 were re-evaluated in larger-scale fermentations and subsequent double distillations. Not surprisingly, sensory evaluation showed that representatives of the three largest brandy producers in South Africa preferred yeast strains that suited the style of brandy produced by their company, and strains B7, LL2 and 20-2 were therefore recommended for further evaluation on a semi-commercial scale at each of the respective companies.

The fermentation temperature for producing brandy base is generally between 15 and 18 °C, which is higher than normally employed in a white wine fermentation. The reason for a slightly elevated temperature is to guarantee the fermentation proceeds and does not lag or produce off odors, and because the preservation of primary aroma is not an issue. The level of accessible nitrogen (free amino nitrogen) is usually tested and, if necessary, increased to a level of between 600 and 700 mg/l by adding diammonium phosphate. Apart from

suppressing the formation of hydrogen sulfide by nitrogen deprived yeast, the diammonium phosphate helps to prevent a sluggish fermentation, increases ester formation and suppresses the formation of higher alcohols. Wine is fermented dry in order to maximize yield of alcohol and minimize sugars. The latter requirement is due to the fact that during heating, the pentoses give rise to the formation of furfural (burnt), and the hexoses give rise to the formation of hydroxymethylfurfural (scorched or smoky) character.

du Plessis *et al*. (2002) determined the extent to which lactic acid bacteria occurred in brandy base wines, and their effect on the quality of the base wine and the brandy distillate. Lactic acid bacteria were isolated from grape juice and brandy base wines in which spontaneous malolactic fermentation (MLF) had occurred in approximately half the wines. In samples where MLF occurred there was a loss of fruitiness and intensity of aroma. Volatile compounds like isoamyl acetate, ethyl acetate, ethyl caproate, 2-phenethyl acetate and hexyl acetate decreased in samples having undergone MLF, while ethyl lactate, acetic acid and diethyl succinate increased in the same samples. The authors concluded that malolactic fermentation occurred spontaneously in commercial brandy base wines in their study, and that it had a significant effect on brandy quality.

Wine is frequently distilled immediately after fermentation, after partial racking from heavy fermentation lees (Léauté, 1990). Studies have shown that yeast lees have an important effect on the final concentration of higher alcohols and esters in the distillate (Stegers and Lamprechts, 2000). Usually, only a partial racking from the heavy lees takes place after fermentation as yeast lees contain high concentrations of fatty acids and fatty acid esters, and impart important qualities to the product. Samples of the young rebate wine, including its lees, are submitted to the brandy house as soon as the fermentation has finished. No fining agents may be used in the making of the rebate wine so as not to remove or to reduce substances that occur naturally in it.

In South Africa, if the wine is approved for brandy production, it is delivered to the distiller as soon as possible, otherwise it is cooled to around 10 °C. If it has not been delivered within 14 days, a new submission is done. Rebate wine is delivered with all the lees (including the gross lees). The specific requirements for brandy base wine will vary a little according to production region. In South Africa, the Wine and Spirits Control Act (Act 47 of 1970) determines two categories: wines with a solids content of 45% per volume or less and wines with 45% or more solids. Within these categories, the total polyphenol content, residual sugar content, volatile acidity and sulfur dioxide concentration will be lower, or higher, respectively (See Table 3.6.1). In order to carry out the tests, a sample must be taken at a height that is no lower than 30% from the bottom of the tank.

Compounds That Influence the Quality of the Base Wine and Resulting Brandy

Elevated levels of esters and moderate concentrations of the higher alcohols are known to contribute positively to the overall quality of brandy base product, but highly elevated levels of ethyl acetate and *iso*amyl acetate are undesirable. A slight defect, which is below detection in wine may well be detectable in brandy because of the concentrating effect of distillation on volatiles. In a good quality distillate, as mentioned previously, levels of 2-phenylethanol may be higher and volatile acidity (acetic acid and ethyl acetate) and ethyl lactate will be reduced. Undesirable flavors such as rancidity, wet cardboard and cabbage-like aromas (reduction), and aldehydic character (oxidation) that are present in the brandy base wine will have a negative effect on the resultant brandy quality. The compounds responsible for the off characters such as SO_2, H_2S, mercaptans and aldehydes have high volatility and are therefore usually removed in the 'heads' (or early distillate) in column stills. A large portion of the SO_2 and CO_2 leaves the system through 'breathers,' and will not end up in the distillate at all. During batch distillation in copper pot stills, many volatile sulfur compounds, such as H_2S and SO_2, redissolve in the condensed alcohol and water in the coolers, react with the copper of the still and are removed completely in the form of copper sulfide deposits. The quantity of acetaldehyde present will depend on the sulfur dioxide content as well as the oxidative condition of the wine. Normally the aldehyde section of a wine column is effective in concentrating aldehydes and related products, so that they

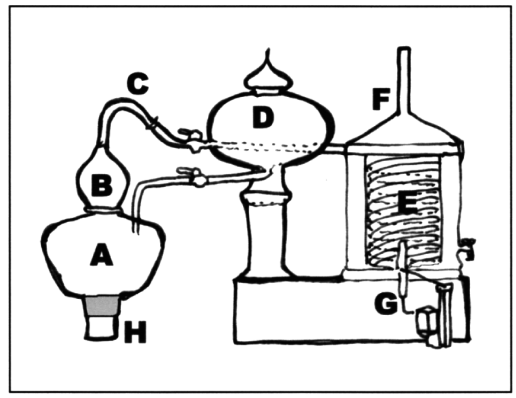
can be removed with the heads, but if acetaldehyde undergoes reactions to form α-hydroxy-ethane sulfonic acid and acetal, removal is difficult. If aldehydes occur in large concentrations, the distillation material may be treated with caustic soda. Another aldehyde that can cause problems is acrolein (the compound in tear gas), which causes phenolics to taste bitter at concentrations as low as 10 ppm, and has an acrid smell. It appears that acrolein originates from bacterial spoilage during the storage of wine for distilling (de Bod *et al*., 2008).

The Distillation

The objective in distilling wine for good quality brandy is to capture the alcohol and agreeable aromas of the underlying fruit and fermentation in the distillate, and leave unpleasant odors behind. Despite sounding relatively easy, this is in fact a complicated and challenging task, and there is surprisingly little published research available on the effects of various factors on the quality of distillates, considering its commercial importance.

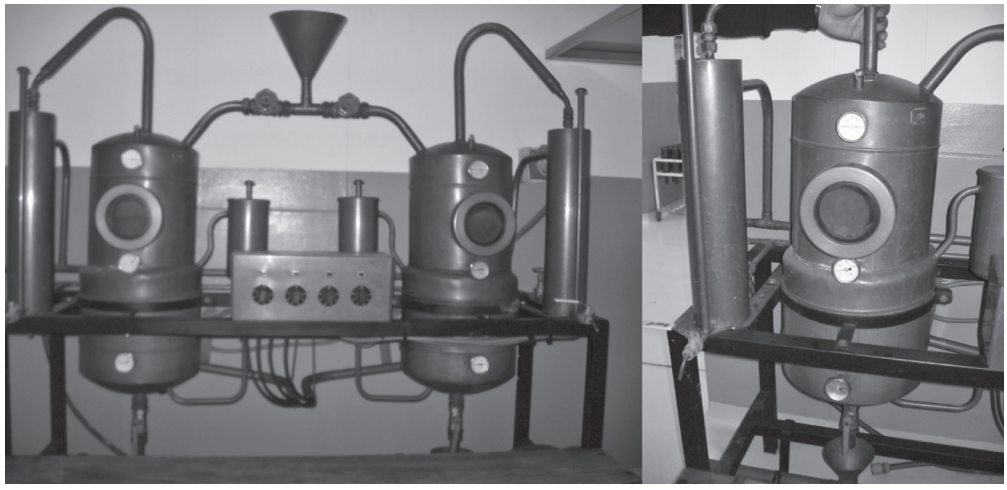
Two main methods are used to produce brandy: batch or pot still distillation, and column or continuous distillation. It is generally recognized that the latter method often produces a lower quality product – but this is not always the case, see Sections 3.6.3 (Armagnac) and 3.8.2 (apple spirit). Figure 3.6.1 shows a diagram of an alembic used to produce Cognac. Although it is similar to pot stills used to make other spirits, such as Scotch whisky (Section 3.2.4), there are several differences, which are described below. Figure 3.6.2 shows a small-scale double pot still apparatus, which can be used in either batch or continuous mode to produce a more highly rectified spirit (a more pure distillate, but one with fewer flavor compounds) than a single pot still.

The equipment used for distillation is simple in principle: it consists of a container (or series of vessels) where wine can be heated, an enclosed headspace (or series of headspaces) above the pot where vapor can collect, cooling conduits where volatiles condense and reception vessels for condensate. Volatile substances



***Alambic Charentais*:**
A: boiler (*chaudiere*) B: hat/cap (*chapeau*) C: swan's neck (*col de cygne*) D: preheater *(chauffe vin)* E: coil *(serpentin)* F: condenser *(condenseur)* G: hydrometer port *(porte alcoolmétre)* H: gas burner *(bruleur)*

Figure 3.6.1 *Pot still setup for batch distillation (adapted from R. Léauté, 1990)*



(a) (b)

Figure 3.6.2 *(a) Double pot still at the University of Stellenbosch, South Africa for small scale continuous or batch distillation. (b) Close up of boiler, showing oil heater unit below and condenser on the left*

evaporate and are collected in the reception vessels, nonvolatiles such as acids and sugars remain behind in the heated vessel, and are eventually discarded. Ethanol is soluble in, but less dense than, water and boils at a lower temperature (around 78–79 °C depending on the atmospheric pressure). The initial fractions of the condensate will therefore be enriched in ethanol and other lower boiling volatiles. As the distillation continues, water will also evaporate and condense, gradually diluting the condensate. As a result, a second distillation is frequently carried out to concentrate the alcohol. Methanol will boil off along with the ethanol and also collect in the distillate, but wine from *V. vinifera* does not normally contain a lot of methanol unless pectinases have caused pectin ester hydrolysis with methanol as a by-product.

For a more general account of batch distillation and continuous distillation, see Sections 3.1.2 and 3.1.3, respectively. For discussion of distillation of other beverages, see, for example, Sections 3.2.4 (Scotch whisky), 3.3.4 (Bourbon and Tennessee whiskey), 3.5.3 (rum and cachaca), 3.7.2 (pomace spirits) and 3.8.2 (apple and pear spirits).

Higher alcohols are also present in small amounts, but usually the sum of all the concentrations of these do not amount to more than 1% of the total alcohol concentration in the distillate (Amerine and Singleton, 1977). After one distillation, the distillate, called 'low wine,' will contain roughly 30% alcohol (ethanol) by volume. The low wine is then distilled a second time in a batch distillation, or is redistilled automatically in a continuous system.

Distillation does not simply enhance the alcohol content of wine. The heat under which the product is distilled and the material of the still causes chemical reactions to take place during distillation. This leads to the formation of numerous new volatile aroma components, changes in relative amounts of aroma components in the wine and the hydrolysis of components such as esters.

Traditionally, fine brandies are produced in small batches using copper pot stills, which are large pots, usually made out of copper and brass, with a bulbous top. Modern alembics will have stainless steel fittings (valves and the condenser tank) incorporated into their structures, but little else in the design has changed over the ages. The standard alembic copper boiler (see Figure 3.6.1) has a capacity of 660 gallons or 2500 l

(Léauté, 1990), and when filled has a headspace of around 130 gallons or 500 l. Pot stills can come in a variety of capacities, and may have volumes of anything up to 22 000 l, depending on the producer's requirements.

The *boiler* base is convex in relation to the inside of the still, which not only makes it easier to clean, but also means that the pot still can be emptied completely. The thickness of the copper base is an important function of the volume of the pot, as it has a direct influence on the rate of heating of the product that is being distilled. Copper is still the only metal that is used in the production of pot stills and the parts that are in direct contact with the wine. The malleability and ductility of copper, as well as the fact that it is an excellent heat conductor, contribute to the reasons for its use. In addition, copper is eroded very slowly by acids and it bonds undesirable volatile compounds, e.g. the mercaptans and sulfides. Copper pot stills need to be cleaned regularly in order to remove the fatty deposits on surfaces that are in regular contact with vapors. Parts that are not in direct contact with the vapors and/or solutions may be made of stainless steel. For further discussion of the influence of copper on distillates, see Sections 3.2.4, 3.3.4, 3.5.3 and 3.5.4.

Boilers are robustly built to withstand direct heat for sustained periods of time, and resist corrosion by the hot, acidic wine. They may be heated directly by gas burners, with propane, butane and natural gas used (around 200 kg of liquid propane is required to produce approximately 370 l of pure brandy). Most modern facilities use steam, which is less intense and likely to cause localized hotspots and burning of the wine. According to Léauté (1990), the temperature under the boiler may reach 760–870 °C, which is essential in creating the characteristic aromas in the distillate. The inside surface is smooth and well polished for easy cleaning between batches.

The still head (cap) of the pot is the section of the still that is responsible for the fortification (rectification) of the distillate. The total surface of the still head is important and is determined by the shape thereof. The quaintly named 'hat' or *chapeau* is located directly above the boiler and has a capacity of between 2 and 10% of that of the boiler. The volume and shape of the *chapeau*, according to Léauté (1990), directly influence the concentration, separation and selection of volatile components.

The characteristic 'swan's neck' type spirit pipe is a lengthening of the cap and its length is also a function of the capacity of the still. During the distillation process, volatile components may move through the *chapeau* and down into the swan's neck, or reflux back into the boiler. The height and orientation of the swan's neck are reputed to influence the quality of distillate, but there is not much evidence in the literature at this time to support this. It is sloped downwards in the direction of the preheater in order to prevent counter pressure and also to prevent the fluid from being sucked back. The main function of the wine prewarmer is to save energy, in that the heat of the alcohol vapors are used to partially heat the next load of wine and, in addition, it also condenses the rising alcohol vapors to a certain degree.

The pipe from the swan's neck runs through the preheater, and condensate collects and heats here for the second part of the distillation process. The *condenser* is a cylindrical tank made of copper or stainless steel, which is filled with cold water and which contains the coiled copper *serpentin* through which hot vapors pass and condense during distillation. The condenser capacity is high, usually between 4500 and 5000 l. The cold water enters at the bottom of the condenser, and warm water, heated by the condensing fumes, is drawn off at the top. The *heads tank* is a small stainless steel tank, which is used to collect the first part of the distillate. The *serpentin*, or coil pipe, has a number of functions: to react with sulfides and precipitate them from the distillate, to condense the vapors and to cool the distillate. The *serpentin* is contained within the condenser, which is filled with cold water during distillation. Fresh cold water enters at the bottom of the condenser, and warm water is removed from the top. The hydrometer port is also made of copper and has several functions: to monitor the alcohol concentration of the condensate, to filter the distillate and to monitor the temperature of the liquor. The heads tank near the hydrometer port is used to collect the first part of the distillate, which is generally returned to the boiler or discarded.

The First Distillation (*Brouillis* Production)

During the first batch distillation, the base wine, with an alcohol concentration of 8% to 12% v:v and high acidity, is boiled in the pot still. The first distillation is essentially a concentration process in which water is removed, and wine is distilled to raw brandy ('low wine' or *brouillis*). The first 1% or so of this distillate (from the first 3–5 min after the vapors begin to rectify) has an alcohol concentration of about 83% v:v and usually contains unpleasant and highly volatile compounds like ethyl acetate and acetaldehyde. It is known as brandy 'feints' and is usually discarded. The raw brandy therefore contains all the (desirable) volatile compounds that occurred in the wine, and the wine is concentrated threefold by the process. The initial distillation usually takes between six and nine hours. In his review of alembic distillation, Léauté (1990) stated that three separate distillations take place the production of Cognac (discussed later in this section in detail), the first one giving three separate fractions of which only the heart (*brouillis*) is used for the second distillation. This in turn gives four fractions, heads, heart 1 (Cognac), heart 2 (*secondes*) and tails. The *secondes* are redistilled with the next batch of *brouillis*.

After the first distillation, which takes about 8 h, over 13 000 l of wine will be converted to about 4500 l of concentrated liquid with around one third of the original volume of the wine. The volatile components are separated from the nonvolatile components and most of the water in this process, giving a distillate has an alcohol content of between 26 and 32% v:v. This raw brandy or low wine is hard and harsh, and is not protected against microbiological spoiling if it is not distilled again immediately.

The Second Distillation (*La Bonne Chauffe*)

The second stage entails the distillation of raw brandy to pot still brandy with an alcohol content that does not exceed 75% v:v. This phase is usually divided into 'fractions,' and the distiller will monitor alcohol concentrations and quality during the process. French producers limit the second distillation to batches of 2500 1, which makes it far easier to manage the various fractions, and the distiller plays a very important role in evaluating the quality of the distillates.

In a typical pot still distillation, the 'heads' will be collected within the first hour or two, the 'heart' from the 2 h mark to around 7 h, and the 'tails' thereafter. Of an original 2500 l of brouillis (of around 30% v:v), the 'heads' fraction will have a volume of about 25 l, and be approximately 75% v:v in concentration. The first 'heart' fraction distils until an alcohol concentration of 55% per volume registers on the hydrometer in the test glass, in a process lasting 6–7 h, and yielding a product that is approximately 70% v:v alcohol, on average. This distillate comes across until all the alcohol has been distilled, i.e. an alcohol concentration of 0% per volume registers**,** and the process lasts about 5 h. The portion of low wine that remains after distillation, called the 'tails,' will be mixed into another batch of low wine for future use. In total, the second distillation may last about 10–12 hours, depending on the type of pot and the starting temperature, as well as the duration of stoking.

Most pot still brandy is double distilled, so that the alcohol is concentrated twice. As a rough estimation, takes about 8.75 1 of wine to make three l of low wine, which then is distilled again to around 330 ml of brandy. The product of the second distillation usually has an alcohol content of around 72%. The higher the alcohol content the more neutral (cleaner) the distillate will be, with fewer of the underlying flavors remaining in the brandy. A lower alcohol distillate may be more flavorsome, but there is also a greater chance that off flavors will make their way into the final product.

Cognac is a well known example of brandy produced in batches using pot stills, and is discussed in detail later (Section 3.6.2).

Effects of Distillation

There are four broad groups under which most of the flavor compounds from the grape and fermentation can be classified: aldehydes, higher alcohols, esters and volatile acids.

Many different reactions occur between the compounds of mixtures, which are at high temperature, and in this regard the boiler acts as a large reaction vessel. The reactions may involve volatile components in the wine decreasing or increasing in concentration, and new volatiles can appear in the solution as a result of associations between the components. Reactions can involve copper, and include hydrolysis, esterification, acetalisation and occasionally pyrolysis. Monoterpenes (linalool and alpha terpineol, <1 mg/l), ketones (α-ionone and β-ionone, <0.01 mg/l) and vitispirane and trimethyl dihydronaphthalene <0.1 mg/l) may be generated through break down and rearrangement of various terpenoid components (de Bod *et al*., 2008). Most products are fairly easily detected by gas chromatography (Section 4.3.2). The reactions occurring during the first distillation are very important in determining the future characteristics of the brandy. These reactions will be affected by various factors, which include the chemical composition of the wine (i.e. sugar, pH and acidity), the size and condition of the still, the temperature within the boiler and the duration of the distillation. If lees are included during distillation, the distillate is more likely to contain high levels of fatty acids and their esters (e.g. ethyl caprylate, ethyl caprate, ethyl laurate), and nitrogen compounds (like amino acids). Fatty acid esters enhance fruity aromas in Cognac, and according to Léauté (1990), fatty acids give the brandy body and are like 'fixatives' for many other aromatic components. Amino acids and reducing sugars may be involved in Maillard reactions in the heated vessel, which are one source of a complex range of heterocyclic compounds that include volatile flavor components and brown melanoidins of higher molecular weight. The wood used for maturation of brandy would be another source of Maillard products due to the toasting of barrels.

Continuous Distillation

A continuous column still is in essence a series of integrated consecutive pot stills (see Section 3.1.3). The still is a cylindrical column, typically about 30 ft (9 m) high, which is divided into a series of plates or horizontal, hollow baffles that are interconnected. Columns differ in their engineering and orientation, but in principle, wine to be distilled is fed into the middle portion of the column, which is heated by steam from below. The alcohol and other low boiling point liquids in the wine evaporate. The vapors rise while the less volatile liquids drop down so that there is a liquid layer on each plate (known as the liquid phase). The space remaining above the liquid layer accommodates the vapor phase. When the liquid heats on a specific plate, vapors bubble through the liquid layer on the plate directly above it, and are partially condensed in it. The vapor from an alcohol–water mixture will be enriched in alcohol as it is more volatile, and the concentration of the alcohol in the column therefore increases on the upper plates. When the still is in equilibrium, the alcohol concentration remains constant, as the liquid phase is always replenished from the plate above. Continuous stills for brandy production usually consist of a 'stripping' column and a 'rectification' column. The basic function of the stripping (or 'wine-stripping' or just 'wine') column is to remove the nonvolatile compounds, organic and inorganic suspended material, and produce a partially fortified, impure vapor that can be concentrated and purified further in the rectification section.

Although continuous stills do not generally produce the same quality distillate as pot stills, neutral wine spirit, produced in an integrated five column continuous still, is an important building block in the production of various kinds of distilled beverages, including many liqueurs (Chapter 3.9). The basic design allows for side stream removal of the higher alcohols and high boiling point fatty acid esters, first flow removal of aldehydes, low boiling point fatty acid esters and methanol, hydro selection purification of raw spirit and the extraction of the final product, neutral wine spirit, from the base of the final column (De bod *et al.*, 2008). Wine that is not good enough quality to be used for pot still or continuous column still brandy is distilled in such a column to remove all suspended material and to remove all secondary volatile compounds, and the resulting product is a pure spirit, colorless, odorless and tasteless, with an alcohol content of about 96.5%.

Column still brandies are more likely to be made from table grape varieties like Thompson Seedless rather than from fine wine grapes. Column still brandies are also aged in oak casks and blended, diluted to around

40% alcohol, and bottled, although regulations for quality brandy production will usually stipulate a portion (around 30%) of pot still brandy in the blend.

Coopering and Maturation

The brandy is not ready to drink after the second distillation, as it is a harsh, fairly unpleasant product. It is first allowed to age in oak casks for a specified time period; this is viewed as an essential part of the production process and lends most brandies their warm flavorful characteristics. Grape brandies are among the very few foods and beverages that improve with ageing over many decades. Although most brandy consumed is less than six years old, the ageing process is integral to the quality of the product and as a general rule of thumb, the older a brandy is, the better its perceived quality. Fine brandy can be ready for bottling after two years, some after six years and some not for decades. Some fine French Cognacs are alleged to have been made during the time of Napoleon, but as a great number of Cognac makers continually remove 90% of the Cognac from an old barrel and then refill it with younger brandy, this is unlikely.

According to South African legislation, the pot still distillate must be aged in vats with a maximum size of 340 l for a minimum period of three years under the supervision of Customs and Excise officials. The vats are usually constructed from French Limousin oak, but reused bourbon or other barrels of indeterminate lifetimes may also be used (Singleton, 1995). Initially the aroma of the immature brandy is composed mainly of volatile compounds derived the grapes and those formed during fermentation, and it is therefore fairly fruity. Immature distillates also have varying intensities of herbaceous, smooth associated and other aromas, depending on their quality. Snyman (2004), in a study of young distillates, found that examples that were most intense in the 'fruity' aroma descriptor were perceived as good quality, whereas distillates in which the 'herbaceous' descriptor dominates, were perceived as poor quality.

The alcohol content of the distillate placed into the oak barrel may vary from 55 to 70 % (v:v) depending on the method of distillation employed. Once the distillate is transferred to the oak barrel, the extraction of the wood by the alcohol begins almost immediately. Investigators have found various alcohol strengths ranging between 40 and 60% v:v optimal for maximal lactone extraction, with optimal extraction levels of tannins and lignin in Armagnac at 55% v:v (Peuch, 1984). Above 60% v:v the extraction rate of color, solids, tannins and volatile acids tends to decrease (Nykänen, 1986). During barrel maturation, the brandy changes in flavor and color from colorless immature distillate to the golden brown aged version.

Changes in chemical composition as maturation progresses are brought about by extraction of aroma and flavor compounds from the oak (Table 3.6.2), or through reactions between wood components and/or compounds in the distillate. Nonvolatile, polyphenolic compounds such as tannins and lignins, may impact on brandy flavor by reducing the solubility of volatile compounds. Fatty acids and sterols, which may be derived in part from oak wood, can cause cloudiness or precipitation in distilled beverages. The gradually acquired rancid taste of Cognac is associated with the hydrolysis of fatty acid esters and their oxidation to ketones during maturation (Mosedale and Puech, 1998). This 'rancio taste,' also called '*rancio charentais*' is a highly desirable characteristic of aged Cognac and on of the ways in which the quality of a Cognac is measured (Macrae *et al.*, 1993).

Oxidation and evaporation of some volatile compounds through the cask also play a role in the formation of brandy flavor. Wood not only contains flavor and color compounds which may diffuse into the distillate, but as it is porous, it allows minute amounts of oxygen to slowly diffuse into the distillate where it may participate in maturation reactions between chemical components. As the brandy ages, it is known to become less astringent and aggressive. During the maturation period, volume and alcoholic content decline as the porous nature of the barrel also allows some of the volatile constituents like alcohol and water to diffuse from the ageing brandy, through the oak, into the air of the surrounding barrel storage area. Ethanol has a higher molecular weight than water and therefore diffuses more slowly through the wood, except under high humidity

Table 3.6.2 *Compounds that can be extracted from oak during the maturation of brandy*

Compound	Contribution to brandy flavour
Coumarins	Bitterness
Dimethylpyrazines	Chocolate, biscuit & fresh bread aromas
Ellagitannins	Mouth-feel/ astringency/ colour
Eugenol	Cloves aroma
β- Methyl-γ-lactone	Coconut, sweet, woody aroma
Furfural, 5-methylfurfural	Toasted almond aroma
Maltol, cyclotene	Sweet, caramel aroma
trans-2-Nonenal	Sawdust, raw wood aroma
Terpenes and terpenoids	Floral, fruity, fresh aromas
Vanillin, syringaldehyde	Vanilla, caramel aroma
Volatile phenols: guaiacol, phenol, cresol, dimethoxyphenol	Smoky, spicy, medicinal aromas

conditions. The relative humidity of a barrel storage area is therefore important and is usually kept around the 70% level. The temperature of the maturation process (i.e. the storage area) will influence both the extraction of compounds from oak as well as the formation of flavor congeners in the maturing brandy. However, it is not simple to specify an optimum maturation temperature, since for each compound or compound group this will be different. Generally speaking, barrel rooms are kept cool to discourage evaporation and the activity of molds and other microorganisms.

For discussions on wood maturation of wine and fortified wines, the reader is directed to Section 2.9.5 and Sections 2.10.2, 2.10.6 and 2.10.7, respectively, and for other distilled spirits to Section 3.2.5 (Scotch whisky).

A good quality final brandy product is characterized by a successful balance of the desirable fruit aroma of the original young spirit and the warm, rounded qualities imparted by the oak and maturation process (Table 3.6.2). Unfavorable characteristics such as 'hotness,' 'greenness and 'rawness/sharpness' are often found in young distillates, but are lowered by ageing in oak and the flavor is transformed to more rounded, soft, mellow flavors. The change in sensory character from the unaged distillate, with its prominent 'fruity' and other less desirable aroma descriptors, to that of a mature brandy with the above-mentioned aroma notes is brought about by a change in the chemical composition as maturation progresses. Panyosan *et al.* (2001) used headspace gas chromatography-mass spectrometry (Section 4.3.2) to identify various alcohols and carboxylic acid aldehydes, acetals and ethylates in Cognacs aged for three, 10 and 20 years and found significant differences in their concentrations. The concentration of Cognac flavored compounds, such as diethylacetal and carboxylic acid esters, significantly increased with age, whereas the concentration of alcohols (butanols, allyl alcohol, hexenol and toxic methanol) considerably decreased. The 'nutty,' 'sweet associated' and 'spicy' aroma notes are often associated with brandies aged for longer periods in oak, usually 15 years and older. It is not easy to attribute a specific flavor to a certain compound, since flavor nuances are often due to synergistic effects of several compounds (see Section 4.7.2), and differ depending on their concentrations. Not all compounds are found in brandy at levels above their sensory thresholds, which means they may only add to complexity, rather than contribute unique notes. Fine brandies are also usually blended from many different barrels over a number of vintages, which again increases complexity.

As part of a broader study into techniques for the rapid induction of ageing character in brandy products (van Jaarsveld *et al*., 2009a), the effect of oak type on quality and chemical composition of oak wood extracts,

and matured and unmatured pot still brandy was investigated. Extracts of American and French oak chips at different levels of toasting were added to 70% (v:v) unmatured pot still brandy and stored for eight months in glass containers at room temperature, and then analyzed by HPLC and GC. Although French oak initially yielded better quality products with higher concentrations of wood derived congeners (including eugenol, the furan derivatives and aromatic aldehydes), these effects lost prominence over time. American oak generally contained higher concentrations of oak lactones than their French counterparts, with higher proportions of the more sensorially potent *cis* form of lactone than its *trans* isomer. In an additional study, van Jaarsveld and his coworkers (2009b) found that the treatments that yielded the highest observed quality were those that made use of toasted oak as opposed to untoasted oak, and that the medium toast level yielded higher quality products overall. Higher toasting levels could also lead to charring, the formation of unwanted polycyclic hydrocarbons, and immediate degradation and loss of flavor compounds. The authors stated that there was a definite relationship between wood derived congener concentrations and pot still brandy quality.

Blending

When the distillate (heart fraction) is at least three years old, it is pumped from the barrels into tanks and cold stabilized (cooled to between –10 to –12 °C) in order to precipitate unstable dissolved substances such as calcium and iron containing compounds. After filtration, the taste of the aged product is still fairly harsh, but it is assessed and classified according to fullness, balance, softness of taste and intensity of flavor. Once all the components of a brandy have been combined according to its recipe, the blend is diluted with pure (distilled) water to around 43% v:v. During Cognac production, this process, known as 'reduction' is carried out very slowly during the barrel aging period. If color adjustments need to be made, caramel may be added, but not all brandies have caramel in them. Although brandy should have a maximum sugar content of 15 g/l, the 'degree of sweetness' is expressed as the 'obscuration' of a brandy. There is a direct relationship between obscuration and reducing sugar content or RS. An RS of 15 g/l is known to give an obscuration of 3% v:v. Fruit extracts, in particular prune extract, also provide smoothness and may also be added up to a maximum of 3% of the 430 ml of alcohol. The fruit extracts used are called '*bonificateurs*' and may consist of prune extract, almond etc. A small amount of caramel may be added to obtain consistency of color across the entire production run.

Like many other distilled alcoholic beverages (see, for example, Section 3.2.5), blending is an important part of the brandy production process. The true skill of the brandy master is demonstrated in the choice and proportion of components used in blending the final product. A variety of matured distillates from various grape varieties, wooden barrels of varying ages, oak cask toasting levels and maturation periods will be blended to create varying tastes and styles to create products for a specific market sector or with a particularly unique flavor and mixing qualities. The success and value of the brand is dependent on consistency and quality of the blend, which is often a carefully guarded secret.

Blended brandies are usually bottled at 43% v:v. This means that in every 1000 ml of brandy there must be at least 430 ml of absolute alcohol. Regulations vary according to region of production but in South Africa, at least 30% of this 430 ml of alcohol must come from the matured pot still distillate. A maximum of 70% of this 430 ml of alcohol may be neutral wine spirit.

3.6.2 Cognac

The European Union legally enforces 'Cognac' as the exclusive name for brandy produced and distilled in the Cognac area of France, and 'Armagnac' from the Gascony area of France, using specified traditional techniques. Brandy made, for example, in California in an identical manner to the method used to make

Cognac, and which tastes very similar to Cognac, cannot be so called in Europe as it is not from the Cognac region of France.

The most well known French Cognacs are produced in the Charente district north of Bordeaux. The Cognac district is one of only three officially designated brandy regions in Europe, along with Armagnac, France, and Jerez, Spain. The name comes from the city of Cognac in the central part of the area. Cognac has been made in these areas for centuries, with some of the well known houses like Martell and Remy Martin being established in the early eighteenth century. Today there are only about 200 producers of Cognac, and most of them use the same methods that they have been using for hundreds of years. Popular brands include Hine, Martell, Rémy Martin, Hennessy, Ragnaud-Sabourin, Delamain and Courvoisier.

The Charente area is demarcated by legislation – only brandy produced here and adhering to mandated guidelines may legally be designated Cognac, and all Cognac must be produced from grapes grown and harvested within these regions. Charente is divided into six vineyard districts, which are listed below in order of decreasing quality: the Grande Champagne (not to be confused with the Champagne region that gave its name to champagne), which comprises around 12% of the region, Petite Champagne (also around 12% of the area), Borderies (3.9%), Fins Bois (33%), Bons Bois (28%) and Bois Ordinaires (11%), which is westernmost of the regions. Cognacs from the Fin Bois are said to reach their peak quickly and are used as blending components to soften younger, hard brandies. Those from the Bons Bois are reputed to have the lowest quality, and yield 'coarser' liquors. Brandies from the Bois Ordinaires are said to have a distinct '*gout de terroir*,' or characteristic aroma and are usually drunk by the locals in the area, rather than being blended with other brandies for export.

Cultivars used in the production of base wine for Cognac include St Emillion, Ugni Blanc, Folle Blanche, Colombard, Jurançon Blanc, Montils, Sémillon, Sauvignon Blanc. The Ugni Blanc cultivar accounts for 94% of the total vineyards planted today. Grapes for Cognac base wine are grown in chalky soil (that is rich in lime, and it is recognized that a high percentage of chalk in soil yields the most fragrant and subtle brandies. A decrease in percentage chalk in the soil occurs across the appellations, with the Grand Champagne region having the highest chalk content and Bons Bois having a mixture of sand and chalk. Because of the climate and the latitude of Cognac area, Ugni Blanc (known as Trebbiano in Italy; see Robinson, 1986) has not yet reached maturity when grapes are harvested at the beginning of October, and so are reasonably unripe, containing sugar levels between 14 and 18 °B. If the grapes are left longer than this, rotting often creates problems as the weather grows cool and damp. Acidity is usually very high; pHs are generally low (2.5 to 2.8).

The grapes are directly crushed in hydraulic presses (pressed in bunches) at moderate pressures in order to avoid phenolic extraction. The juice is fermented to dryness with the preferred house strain of *Saccharomyces cerevisiae*, or less often with 'wild' strains (yeast that are present naturally on the grapes, almost always *S. cerevisiae*). Very little (less than 20 mg/l) of sulfur dioxide is added, or none at all, depending on the producer's preference and the condition of the grapes. The wine that is obtained has is neutral in flavor, light in alcohol, highly acidic and produced specifically for distillation (Léauté, 1990).

The yeast lees content of wine used for distillation is highly correlated with the even numbered fatty acid ester content of the brandy distillate, and this in turn plays a role in the concentrations of the corresponding methylketones, which are associated with quality of Cognac (Watts and Butzke, 2003). As lees content is a function of juice nitrogen status, grape composition could also have some impact on the availability of methylketone precursors in the distillate.

The characteristic Charente pot (*Alambique charentais*) gradually developed the specific flask shape it has today, and the distillation therein is a selection and a concentration of volatile compounds, which give the specific character to Cognac. In addition, during distillation in alembic, the wine and the *brouillis* are heated intensely, and reactions occur between the compounds generating the delicate aromas characteristic of the product. The first distillation gives three fractions: the heads (which distill over for around 15 min),

the heart (*brouillis)*, which distill over for around 6 h, and the tails (which distill over for another hour). The heads and tails are redistilled with the successive batches of wine. After the first distillation, the *brouillis* is cooled to form an opalescent liquid (the 'soul' out of the wine) with an alcoholic strength of 27–30 % v:v. This is then redistilled into four fractions: the heads (around 30 minutes of distillation time), the heart 1 (Cognac), which takes around 6 h to distill over, the heart 2 (*secondes*, which takes around $4\frac{1}{2}$ h to distil) followed by the tails – which distil for an hour. The first distillation therefore takes around 9 h, and the second around 14 h, giving a total of nearly a full day's distillation to go from base wine to immature brandy. The very young brandy has an alcohol content which may not, by law, exceed 72% v:v. During the long Cognac distillation process at approximately pH 3.0, volatile esters may form and distil over. Some of these can then hydrolyze in the slightly acidic distillate (pH 4–5), resulting in free fatty acids. Despite their low volatility, some fatty acids may also distil over directly during distillation through azeotrope formation. In some areas of the world, this liquor is marketed and consumed, but in Cognac, a strictly mandated ageing process in 350 l barrels is necessary before the product is considered ready for sale. The French '*appellation d'origine controlée*' (AOC) *eaux-de-vie*, are traditionally matured in barrels made from oak from nearby regions (Limousin, Allier, Troncais and Nevers), with Cognac almost exclusively matured in oak from the Limousin and Troncais regions. Cognac producers are careful to use barrels of exceptionally good quality, as their products are dependent on the wood for flavor and maturity. Many houses employ their own coopers to ensure that their barrels are made to a particular specification in terms of their conditioning, construction and toasting. The brandy is vatted at full strength of around 65 to 70% alc/vol. The barrels are not filled completely or topped up, and oxygen is constantly in contact with the distillate. Chemical reactions take place throughout the process, and depending on the age of the distillate, different aromas become apparent, ranging from exotic fruits, nuts and dried flowers, to port wine, balsamic and tropical fruit characters. The required minimum period of oak ageing is two and a half years, but the vast majority of Cognacs mature for a longer period than this. The maturation stores are cool in temperature, so maturation processes are slow and controlled.

Genuine Cognac should be 100% pot still brandy. However, due to the expense of long maturation periods and the need for rapid turnover, there is a tendency even in France to mix pot still brandy with column distilled spirit, which does have the same complexity or quality. The lack of esters and other components is supplemented with wood extract, lees oils and other *bonificateurs* (essences and aromas).

Cognacs are usually sold as blends made from distillates of different vintages. Some Cognacs can contain brandy from up to a 100 different barrels. The unaged brandies from the different farms/producers may be purchased by the large Cognac houses, and blended according to origin and character with older batches. Regulations specify that all Cognac be aged for at least two and a half years from 1 October of the year the grapes were harvested. Even though the ageing process is paramount to Cognac's quality, Cognac makers rarely use vintage years as identification, preferring instead to use a lettering system. Brandies are classified using a '*compte*' system to designate their age. For example, '*compte* 00' will be a young distillate that was produced between harvest and the following 1st of March. '*Compte* 1' are Cognacs that are more than a year old on the 1 April of a given year. The *compte* system now goes up to *compte* 10 (10 years old on 1 April of that year). This has relevance as Cognacs may not be sold in France until they are at least *compte* 2. Most other major markets insist that Cognacs must be at least *compte* 3. Reserve, VO, VSOP type Cognacs have the youngest component as a minimum *compte* 4 (around four and a half years old). Extra, Napoleon, Vieux and Vielle Reserve types all have their youngest components as *compte* 6, and XO indicates at least *compte* 10.

Although these regulations specify the minimum age of the youngest component in a blend, blends often contain much older distillates, as with Scotch whisky (Section 3.2.5). The blending process is complex and overseen by an experienced cellar master. Cognac is often aged for decades and sometimes over a century, possibly the longest of all consumables. Some finer Cognacs may be the result of blending as

many as 50 different Cognacs in effort to achieve a house's desired flavor and maintain a uniform standard of taste and quality. Since 1988, vintage Cognacs are also possible. Very old Cognacs are known to develop unique complex flavor characteristics collectively called *rancio charentais* or *Cognac rancio*. It is usually recognizable in the aroma and taste of Cognacs aged between 15 and 20 years and is therefore mostly characteristic of expensive Cognac blends. It has been described as an aroma of 'rancid butter and mushrooms, accented by notes of dried fruits and touches of raisins and nuts' (Watts and Butzke, 2003). Similar notes can be found in extensively aged (and oxidized) fortified wines, such as oloroso Sherry (Section 2.10.2), Madeira (Section 2.10.6), tawny Port (Section 2.10.7) and the *rancio* styles of vin doux naturel (Section 2.10.9). Despite the term 'rancid' usually being used in a negative sense, traces of a rancid character are very desirable in aged Cognacs. Methylketone concentrations were correlated with Cognac age classifications, despite a large variation due to the variable nature of the production practices. It has been determined that 2-heptanone and 2-nonanone are the most abundant methylketones in aged Cognac, but 2-undecanone and 2-tridecanone have also been shown to play an important role in the development of the *rancio* character (Watts and Butzke, 2003). Oxidation products of lignin, lignans and lactones in oak wood contribute a balsamic component of the *rancio*. The ketones are likely to form as a result of the β-oxidation and subsequent decarboxylation of even numbered long chain fatty acids (C8, C10, C12 and C14) during barrel ageing. Peroxidases from molds present on the barrel surfaces have also been implicated in the process. Higher fatty acid esters such as propyl octanoate and ethyl octanoate may add a certain dimension to the ketone aroma, modifying it to a more fruity character often associated with *rancio*.

3.6.3 Armagnac

Armagnac was the first distilled spirit in France, is the oldest French brandy and has been distilled since 1411. The area is in the southwest of France in the Gascony province and is divided into three demarcations, namely Bas Armagnac (or Armagnac noir), Haut Armagnac (or Armagnac blanc) and the Ténarèze. It comprises 66 000 ha in southwest of France framed by the Pyrenees, and is generally warmer than the Cognac region, with grapes ripening earlier and more completely. Sandy soils such as those found in the Bas Armagnac region on the west coast of France are reputed to produce Armagnacs with the greatest suppleness and finesse, while clay soils (found in the Ténarèze region further inland) produce lighter brandies, which mature more quickly. Chalk and limestone soils such as those found in Haut Armagnac are reputed to give spirit of a mediocre quality, according to some critics (compare this situation with that of Cognac). Cultivars used in the production of Armagnac include Folle Blanche (around 7% of total plantings), Baco blanc or 'Baco 22' (47% of total plantings), Ugni blanc or Trebbiano (40% of total plantings – mainly in St Emilion), Colombard (4% of total plantings) with Graisse, Jurançon and Clairette de Gascogne making up the balance.

Like Cognac, Armagnac is distilled in copper stills (known as '*alambique Armagnacais*' or the *Verdier systéme*), but in a column still rather than the pot still used in Cognac production. The wine flows into a feed tank situated above the still house containing the still, preheater, cooler and condenser. The Armagnac still is an arrangement of five to six plates situated in a column above the boiler. The wine is preheated by flowing over the condensing coils, and then is fed in to the top of the column, whereupon it flows down over the column plates, and alcohol and other volatiles evaporate as they are heated. The wine collects in the bottom of the column where it boils, generating vapors that bubble through the descending wine and eventually collect in the condenser. The condensate forms the immature brandy, and has an alcohol content of around 53% v:v. It is blended and diluted to 40% ABV.

Periodically, wine will be removed from the lower part of the column, but no heads or tails are collected, as it is a continuous process. The distillate is put into new casks sourced from the Monzelun forest in Gascony

or Limousin until it has enough woody flavor and is then further matured in old casks. The size of the casks varies from 400 to 420 l. Vintages are kept separate, but vintage Armagnac is becoming less popular. Strict control is carried out under AC laws. Until 1999, Armagnac followed roughly the same regulations as Cognac, but recently has adopted a more simple, sensible classification: 'Armagnac' is between two and six years old, 'Vieil Armagnac' is over six years old, and 'Millesimes' must be more than 10 years old. Well known Cognac houses with an interest in Armagnac include Martell in Janneau, Camus, Marquias de Puysegur, Prince de Chabot, Courvoisier and Maillac, and other popular brands of Armagnac include Darroze, Baron de Sigognac, Delord, Laubade, and Gélas.

3.6.4 Other Brandies

Spain and Other Southern European Countries

Brandy de Jerez is a product that originates from Jerez de la Frontera in southwest Spain. It is used to fortify some Sherries and is also available as a separate product. The vast majority of Spanish brandy is produced using wine made from the very widely planted Airen cultivar from various locations around Spain. Nowadays most of the distilling is done using column stills, and a continuous distillation process which has as its end product a distillate known as *hollandas* with an alcohol concentration of 65 to 70% v:v. Brandies are matured within certain areas of production (Jerez de la Frontera, El Puerto de Santa María, and Sanlúcar de Barrameda). They can be aged in American oak casks with a capacity of 500 l, which previously contained sherry, or produced through the use of a traditional ageing system of criaderas and soleras (see Section 2.10.2). A solera is a series of large casks or butts, each holding a sequentially older spirit than the previous one. Some soleras have over 30 stages. When brandy is racked from the last butt, no more than a third of the volume is removed and it is replenished with brandy drawn from the previous butt in the system. This process, which imitates that used in sherry production, is repeated all the way down the solera line to the first butt, where newly distilled brandy is added. This blends together a variety of vintages and results in a speeding up of the maturation process. As far as the quality designation system goes, the basic brandy can be sold after only six months, but 'Reserva' indicates the brandy has had a minimum of a year of maturation in a cask in a solera system, and 'Gran Reserva' indicates three years in solera. Most producers mature for much longer than the minimum requirement, with the best Reservas and Gran Reservas frequently having been aged for more than a decade. Spanish brandy is far sweeter than Cognac or Armagnac, with the sweet and fruity notes coming not only from ageing in Sherry casks, but also from the judicious use of caramel, fruit based flavors and oak essence (*boise*). Pot still brandy is also produced in Spain, an example being Penedès brandy from the Catalonia near Barcelona in northeastern Spain. It is made by two local producers (Torres and Mascaro) from a mix of regional grapes and locally grown Ugni Blanc. The Torres company ages its distillates in solera systems using casks made from French Limousin oak, whereas the Mascaro company matures its brandies in the standard Cognac manner, in Limousin oak vats.

The Balkan countries (especially the states of the former Yugoslavia, and Greece) produce notable examples of brandy, although pomace spirits (Chapter 3.7), plum spirits (Section 3.8.4) and aniseed flavored spirits (Section 3.5.6) are also important here. Good brandies are made by several companies (e.g. ETKO, Keo and Haggipavlu) around Limassol, in the southern half of the island of Cyprus. They are generally produced from the white wine of the Xynisteri vine variety, the island's major white cultivar (Robinson, 1986b), using double distillation in pot stills and maturation in oak casks, often for more than three years.

The United States

American brandy derives in the main from the state of California, also the center of the thriving wine industry, with the San Joaquin Valley producing notable examples. Cultivars used include a table grape

variety, Thompson seedless, as well as smaller quantities of the more traditional brandy cultivars like Ugni Blanc, Colombard and Folle Blanche. Pot stills are rare in the USA (notably, Jepson and RMS use original pot charentaise), with most brandy being distilled continuously in column stills. The process is more efficient than the pot still process and some producers claim that the product is softer and more finished, so shorter periods of maturation are required. A single column is usually most frequently used. The strength of the product varies from 78 to 85% v:v, which will be diluted to between 50 and 55% alcohol v:v before maturation. Rules governing American brandy production are not quite as strict as the French procedures, but there are still criteria in place for quality, for example, the maximum new make spirit strength allowed in production is 85% v:v, and the minimum age before release is two years. If less than this, 'unmatured' should be indicated on the label. It is more usual for producers to release brandies after four years maturation in either used American oak casks or casks that have been used to store Bourbon whiskey (Section 3.3.4). Brandy character can be overpowered by the wood flavor if newer American oak wood barrels are used. Frequently blends will include older brandies. Well known producers include the Christian brothers, Paul Masson, Schenley Distillers and E. & J. Gallo.

South Africa

Brandy is South Africa's top selling spirit drink, with average sales as at 2009, of around 45 million liters per year. Most South African grape brandies are, by law, made in the same way as Cognac, using a double distillation process in copper pot stills. Current European Union legislation demands that any product labelled as 'brandy' must be grape based and aged for at least six months in oak. South African legislation is even more stringent and requires ageing in oak barrels for a minimum of three years. Grapes for brandy production are mostly grown in the warmer wine production areas like Breede River Valley, Orange River region, Robertson, Worcester and Rawsonville. Cultivars include Colombard and Chenin Blanc, which account for around 90% of production, with other cultivars, Cinsaut, Palomino, Ugni Blanc and Sultana, making up the remainder. Distillates are aged in French oak, specifically Limousin and Tronçais for a minimum of three years by law for a 100% 'Pot Still' designation.

'Blended' brandies must contain 43% of three year old distillate and 'Vintage' brandy needs to be at least eight years old. Well known 'Blended' brandies include Klipdrift, Oude Meester, Richelieu, and Viceroy. These brandies are not as strongly flavored as pot still brandy, and are usually used as mixers. 'Vintage' brandies are composed of at least 30% pot still brandy, and should be matured for at least eight years. It is bottled at 38% alcohol by volume, and is generally served neat, or with a little water. 'Pot still' brandies must have at least 90% of their volume from pot still distillate, and be matured for a minimum of three years. These are the most complex of the brandies, and are often drunk neat. 'Pot Still' brands include Flight of the Fish Eagle, and Van Ryn (a distilling company that was established in Cape Town in 1845). The latter company makes 12, 15 and 20 year old brandies that are internationally recognized as excellent examples of their categories. Individual wine estates such as Backsberg, Laborie, Uitkyk and Avontuur also produce their own house brands, and the Solera system of brandy production is employed by some houses.

Pisco

Pisco is the name given to a certain kinds of brandy produced in a number of South American countries, but notably Chile and Peru (Faria *et al.*, 2003). There are two basic versions, depending on whether the production is based on nonaromatic wines of varieties such as Common Black, Mollar, Quebranta (especially) or Uvina, or on aromatic wines of a very wide range of Muscat varieties. The nonaromatic version is important in Peru, whereas the aromatic version is predominant in Chile, and because production procedures and regulations are rather different in these two countries, there is a considerable range of styles and qualities associated with this brandy. Much of the cheaper versions are used to make 'pisco sour,' consisting of brandy, lemon or lime juice, egg white, sugar syrup and bitters.

In Chile, the grapes used to make pisco are mostly of Muscat cultivars (although there are versions made from Pedro Ximénez or Torontel grapes), grown in warmer viticultural regions, so that the wine to be distilled is usually of *ca.* 14% ABV. Double distillation takes place in copper stills, with or without rectifying columns, so that the new make distillate is collected at 55–60% ABV. It is aged in oak casks for a few months, with higher quality versions being given much longer oak contact. After maturation, the spirit is diluted with deionized water (according to brand category), filtered and bottled. The four brand categories are (with % ABV) are Regular (30–35%), Special (35–40%), Reserve (40–43%) and Great (>43%).

In the production of Chilean pisco, the emphasis is not on varietal character, all versions having at least some wood maturation and most being made from the grapes of several vine varieties. The Chilean government has been keen to work with the producers to ensure a consistency of character and quality, especially regarding the basic versions, which receive only minimal wood maturation. To this end, special consideration has been given to the design and operation of stills used to make pisco (Osorio *et al.*, 2005).

In Peru, pisco production is regulated to preserve the character of the spirit, which must be made from a single vine variety, otherwise it is known as *acholado* ('half breed'). Maturation is carried out for a minimum of three months in glass (like many *Eau de Vie de Fruits* – see Section 3.8.3) or stainless steel, neither of which alter the character of the distillate, unlike oak, which allows oxygen ingress and leeching of phenolic substances into the spirit. A particularly fruity and highly perfumed distillate, *Mosto Verde*, is produced from a partially fermented must. Peruvian pisco is not diluted prior to bottling, so is generally of higher average alcoholic strength (~38–48% ABV) than Chilean pisco.

3.6.5 Quality Control in Brandy Production

Other than measures that are embedded in the legislation to ensure brandy producers adhere to consistent quality standards, Léauté (1990) noted that the distiller has three means to check the quality of wines for distillation in an alembic: tasting, analysis and microdistillation. Microdistillation can be conducted in a small-scale laboratory still with copper shavings placed in the boiler. This technique is very useful to detect butyric smells, oxidation characters, high levels of volatile phenols and/or sulfur-containing compounds. Sometimes a wine will seem fine until it distills, whereupon chemical interactions during heating may release components that were bound, and volatilize them. Checking the quality of distillates before maturation is also crucial. Column distillates are usually relatively odorless and tasteless, the only real quality control required is to check their alcohol content. The quality control process for fine brandies involves trained tasters with years of experience sampling brandy. A large Cognac house might have thousands of barrels in its cellars, each containing maturing spirit, which must be assessed for quality and maturation characteristics. A lot of brandy 'tasting' involves only smelling, as problems will very quickly be detected in the odor of the sample and tasting as such very quickly overwhelms the palate and impairs sensory analysis (see Section 3.2.5 and 4.7.4). The barrels may actually only be properly tasted once a year to assess how it is ageing and to evaluate it for its blending qualities. Brandies that contain off flavors will be discarded. Large establishments will have access to well trained panels of tasters who are used to tasting and assessing off odors and problems in raw distillates, barrel samples and finished products, and will also be used for assessing the success of blends and brands for the market. Interestingly, the standard sensory analysis procedures for Scotch whisky involve only 'nosing' (sniffing) the sample (Section 3.2.5).

3.6.6 Evaluating Brandy

Brandy, like whisky and red wine, exhibits more pleasant aromas and flavors at a lower temperature, e.g., 16 °C (61 °F). In most environments, this would imply that brandy should be cooled rather than heated for maximum enjoyment. Furthermore, alcohol (which makes up 40% of a typical brandy) becomes more volatile

as it is heated (and more viscous when cooled). Thus, cool brandy produces a fuller and smoother mouthfeel and less of a 'burning' sensation. Often it is slightly warmed by holding the glass cupped in the palm, but heating may cause the alcohol vapor to become pungent so that the aromas are overpowered. Brandy is best drunk from a tulip shaped glass or a snifter, which gives a large surface area for maximizing the release of volatiles. The wide base of the glass narrows towards the top, allowing the aromas to concentrate, and the tulip shape will act to funnel aromas up to the nose. The color of the liquor should be assessed against a white background and will be indicative of the age and blend of the brandy. Generally, the darker the brandy, the longer it has been matured in the oak casks, hence the stronger the woody aroma. It is also good to note that the color can also indicate the style of the brandy. Darker brandies are usually fuller and richer in taste. Unlike wine, which is often swirled in the glass to release its aromas, brandy should be kept stable in the glass as the liquid is very volatile and flavors can be lost if it is moved vigorously. In addition, the alcohol strength of brandy, neat, is approximately three times that typically found in wine, which means that the alcohol concentration in the volatile layer is far higher, and can be quite overpowering if swirled. The aromas in brandy are also presented in layers, according to brandy connoisseurs, with an initial impression, and then deeper layers of aroma, and the effect will be lost if the product is swirled.

Table 3.6.3 *A short glossary of brandy terminology*

Brandy term	Meaning
A.C.	Brandy that has been aged two years in wood.
Alambic	The copper pot still used in the Cognac area for the production of distillate
Armagnac	Brandy produced in the Gascony region of France using a specialised column still. Usually between 2 to 6 years old when released.
Brandy de Jerez	Brandy from the Jerez region of Spain, produced using the solera method, at least a year old on release.
Brandy de Jerez Reserva	Brandy from the Jerez region of Spain, produced using the solera method, at least three years old on release
Brandy de Jerez Gran Reserva	Brandy from the Jerez region of Spain, produced using the solera method, at least ten years old on release
Cognac	Brandy produced in the Charente region of France using a potstill. At least two and a half years old when released.
Heads	The first fraction of alcohol and other volatile components to distill over- usually rejected or redistilled due to poor quality
Heart	The major proportion of the alcohol (around 70%) recovered in a distillation
Hors d'age:	Brandies are too old to determine the age, although ten years plus is typical, and are usually of great quality.
Millesimes	Armagnac that is more than ten years old.
Tails	The last fraction of alcohol, usually containing high boilers like fuselols, may not be good quality and usually redistilled.
Vieil Armagnac	Armagnac that is over six years old
Vintage	Stored in the cask until the time it is bottled with the label showing the vintage date.
V.S.	"Very Special" or 3-Star, aged at least three years in wood.
V.S.O.P.	"Very Special Old Product" or 5-Star, aged at least five years in wood.
X.O.	"Extra Old", Napoleon or Vieille Reserve, aged at least six years in wood, Napoleon at least four years in wood.

Aromas associated with brandy can range from herbaceous (grass, mint or eucalyptus) to fresh or dried fruits as well as gooseberry, apple, citrus and even flowers. The impact of oak wood will be detected as vanilla, cedar wood, nuttiness, toast, cloves or cigar box aromas, as well as chocolate and mocha. Generally the younger the components, the grassier the flavor of the brandy, while older brandies tend to exhibit sweeter, fuller aromas and flavors. Additionally, brandy that has spent more time in oak casks tends to have a smoother, more harmonious mouthfeel. Some terminology associated with brandy styles and production can be seen in Table 3.6.2.

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3.7
Grape and Other Pomace Spirits

3.7.1 Introduction

Pomace is the name for the mass of fruit skins, pips and other debris left behind after pressing the crushed fruit before or after fermentation (Sections 2.9.1, 2.9.2 and 2.8.3). Although many wine, cider and perrymakers return the pomace to the land to act as a fertilizer, it is generally considered a waste and for centuries in some long established wine producing regions some of it has been converted to a value added product known as pomace spirit. Furthermore, pomace, especially that from red wine production, contains high concentrations of polyphenols, which may not be desirable ingredients to add to the land as fertilizer (Amico *et al.*, 2008). Indeed, disposal of large quantities of pomace and other winery residues on land in more recently established wine producing areas, such as Oregon or New York State is viewed as an increasingly serious problem (Rice, 1976; Hang and Woodams, 2008).

To give some idea of the problem, consider a 1 ha vineyard (a very small vineyard in commercial terms) with a yield of 5000 kg of grapes. This will produce around 3500 l of wine, leaving some 1200 kg (1.2 tonnes) of pomace to be disposed of. Putting this into greater perspective, the countries of the European Union produce around 30 million tonnes of wine grapes each year, from which winemaking leaves about 6 million tonnes of grape pomace (Laufenberg *et al.*, 2003).

Despite the extensive use of grape pomace to give pomace spirit in some areas, such as Sicily, there is some interest in finding further ways of obtaining high added value products from red wine pomace. Fractions rich in antioxidant polyphenols have been obtained in Sicily for possible use as drug or cosmetic formulations or as food additives, although further work is required before these fractions can be exploited commercially (Amico *et al.*, 2008). Additionally, even after the production of pomace spirit from the pressed grape pulp, there remains behind a residue known as distilled grape pomace, which is also rich in polyphenols of high antioxidant activity. Traditionally, this is used as fertilizer, animal feed or is burned as fuel in cogeneration plants to produce energy. Recently, polyphenols from the pressed liquor of distilled grape pomace have been adsorbed on activated charcoal or polymeric resins and recovered in high yield, suggesting a possible commercial use, as for red wine pomace (Soto *et al.*, 2008).

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

3.7.2 Production of Pomace Spirits

The majority of pomace spirit is derived from pressed red wine or grape pulp and is known in France as eau de vie de marc or more simply as marc (e.g. marc de Bourgogne or marc de Champagne, depending on the region of production), grappa in Italy, orujo/aguardente in Spain, tsipouro/tsikoudia/raki in Greece, zivania in Cyprus and bagaceiras/aguardiente in Portugal. Small amounts of pomace spirit are also made from pressed apple or pear pulp during cider and perry production; these are generally known as eau de vie de marc de cidre or eau de vie de marc de poiré, respectively.

Red wines are made by fermentation of grape juice in contact with the skins and pips, and sometimes stalks (Section 2.9.2). After a certain time, the pomace is separated from the wine by pressing. It is normally a compact mass of fibrous cellulose, lignin material and moisture, in which are impregnated original grape constituents and the products of alcoholic fermentation, including ethanol. The volatile components, and in particular ethanol, can be obtained from the pomace by adding a controlled amount of water (which acts as an extractant and helps break up the solid mass) and then distilling. The heart fraction of the second distillate (if using pot alembic stills) is the pomace spirit and usually contains ~48–58% ethanol (v:v). If a pot still with a rectifying column (such as an alquitara), or more especially, if a columnar still is used, then the pomace spirit usually contains ~64-74% ethanol (v:v) (Cortés *et al.*, 2005).

In the case of spirits being made from pressed white grape pomace (which will be unfermented, since the big majority of white wines are made by crushing and pressing the grapes, and then fermenting the juice: Section 2.9.1), water is added to the pomace, often followed by a cultured yeast starter. After the close of fermentation, which lasts only a few days, the weak wine, with about 4% ABV, is distilled.

As an alternative to both of the above procedures, which are essentially traditional methods, larger distilleries, equipped with rectification columns, often distill the dry (or even partially dried) wine pomace using entrainment with steam; essentially a steam distillation process.

Pomace spirits were once mostly made either by the vineyard owner in his own small distillery or more likely by a travelling distiller ('le bouillier de cru' in France), who visited the vineyards at vintage time with a portable still (le distillerie ambulant), usually a simple alembic. Latterly, the traveling distillery has largely disappeared (mainly because of prohibitive legislation) and most pomace spirit is now either made by the vineyard owner/winemaker or by distilleries set up by the local or state authorities or private companies for that purpose. The larger distilleries often use (mainly) stainless steel column stills, as mentioned above, which many believe gives a different spirit to the smaller traditional copper pot stills (but see the next paragraphs).

The sheer mass of wine pomace at harvest time, located at many different vineyards or winery sites, necessitates storage for a period of time (ensilaging) prior to distillation if the winemaker makes use of the local distillery, rather than distil the pomace himself. During this time, depending on the method and length of storage, further alcoholic fermentation and other microbiological reactions take place in the wine pomace. It is not unusual for pomace to be stored at the distillery for three months (e.g. from the time of pressing at the start of October to the end of December) (Da Porto *et al.*, 2004). It is this extensive storage period that is thought to cause the greatest differences between pomace spirits from industrial distilleries and those from small artisanal distilleries at the vineyard or winery (distilled very soon after pressing; with no storage period) (Cortés Diéguez *et al.*, 2001; Cortés *et al.*, 2003; 2005; Corich *et al.*, 2005), rather than because of different distillation methods (column stills versus alembics), although this will have a certain influence.

The 'traditional' way of storing pomace is in wooden or concrete tanks, holding up to 50–100 tons. When the tanks are full, they are covered with plastic sheets weighed down with sand to prevent exposure to air. Alternatively, smaller quantities of pomace (50–200 kg) have been stored in plastic bags or containers (Cortés Diéguez *et al.*, 2001). Larger quantities of pomace (up to 500 tons) can be stored in concrete rooms (about $20 \times 6 \times 5$ m and closed on three sides). More recently, vertical closed stainless steel containers (silos) of

capacity 200–500 tons, and salami-like plastic bags (like a polytunnel) of capacity ~300 tons have been used to store pomace (Da Porto *et al.*, 2004).

The temperature at the center of a large quantity of stored pomace can exceed 30 °C at times, due to the heat released as a result of microbiological activity. This can lead to a 'stuck' fermentation (thereby giving a low alcohol yield) and also to infection by bacteria or fungi that produce organoleptically undesirable volatile compounds in large quantities, particularly 2-butanol, ethyl esters and volatile acids, such as butanoic acid. Although levels of 2-butanol tends to be high in fruit spirits made from pomaceous fruit (Section 3.8.2) and stone fruit (Sections 3.8.3 and 3.8.4), if concentrations of allyl alcohol, ethyl acetate and the propanols are also high and if the 2-butanol:1-butanol ratio is higher than 6, then it is likely that microbial contamination has occurred (Reinhard, 1981).

Moreover, if the container is not well sealed from the air, acetaldehyde and acetic acid can be formed at levels that are organoleptically detrimental. The spirit resulting from the distillation of unrefrigerated pomace of long storage can have high concentrations of 2-butanol (solvent notes), butanoic acid (sweaty, cheesy notes), ethyl acetate (fruity, solvent notes), ethyl lactate (rancid butter notes), 1-propanol and unsaturated aldehydes (grassy/herbal notes) (Cortés Diéguez *et al.*, 2001), whereas spirit produced from oxidized pomace can have high levels of acetaldehyde, acetic acid and methanol (Cortés Diéguez *et al.*, 2001). Various steps can be taken to either reduce fermentation temperature (by refrigeration) or to prevent oxidation/microbial contamination (by compression/lowering pH by adding acid or addition of metabisulfite). Unfortunately, compression, whilst denying the ingress of excess oxygen, makes refrigeration by conventional cooling ineffective.

Refrigeration of wine pomace in stainless steel silos by traditional methods is ineffective, because of poor heat transfer through the mass of fibrous material. Da Porto *et al.* (2004) have described an effective way of cooling fermenting pomace by trickling wine at 0 °C or 4 °C through the pomace twice during the 90 day storage time. The first distillate (low wines) made from the cooled pomace had a higher ethanol content (22.8–23.3% by volume) than that from the unrefrigerated pomace (18.8% by volume). Moreover, levels of acetaldehyde, 2-butanol, ethyl acetate, ethyl lactate, 1-hexanol (grassy notes), higher alcohols, methanol and unsaturated aldehydes were lower in the first distillates from cooled pomace. Da Porto *et al.* (2004) also showed that pomace stored in plastic salami-like containers, unrefrigerated, but well sealed from the air and relatively uncompacted, also gave low wines of good alcohol content (24.5% by volume) and with low levels of congeners that are detrimental in higher concentrations.

Using a multifactor variance analysis method (ANOVA), Cortés *et al.* (2005) were able to show that significant differences between the mean concentrations of volatile components (determined by GC/FID/MS analysis) of seven industrial and seven homemade orujo samples from Galicia existed only for components whose levels were known to be qualitatively related to processing and storage of pomace. These compounds included ethyl acetate, ethyl lactate, 1-hexanol, methanol and 1-propanol. Methanol and various hexanols in particular were predominant in orujo spirits, and it was evident that the use of rectification columns in the industrial distilleries did not effectively eliminate negative sensory compounds (such as ethyl acetate and ethyl lactate) when they are present in the pomace at such high concentrations because of poor storage. Other compounds, such as allyl alcohol and ethyl myristate, were found (at low levels) only in industrial orujo and so were regarded (without statistical evidence) as being indicative of long storage of wine pomace, with concurrent microbial contamination. Cortés *et al.* (2005) also suggested that the large pneumatic presses used by large-scale winemakers gives a dry pomace, with low alcohol potential, and the typically high pressure exerted on the pomace causes rupture of the pectins from the solid pomace material, making hydrolysis to methanol easier during fermentation and distillation.

Thus much qualitative evidence and at least one statistical study suggest that the length of storage time and the mode of storage of wine pomace can have more impact on the organoleptic character of pomace spirits than the mode of distillation or grape variety. However, the method of distillation, particularly with

regard to the mode of heating the still and with regard to separating head and tail fractions from the heart cut, plays a significant role in determining certain sensory characteristics of the spirit. Furfural is formed by thermal decomposition of sugars during distillation, particularly if the still is heated by direct fire, as in the production of some brandies (Ebeler *et al.*, 2000). Artisanal pomace spirits are more likely to be made using directly fired alembic stills and so tend to contain higher levels furfural (Cortés *et al.*, 2005; Kokkinofta and Theocharis, 2005), especially since furfural mostly distils in the tail fraction. Likewise, 2-phenylethanol is concentrated in the tail fraction and tends to be found at higher levels in homemade pomace spirits as a result of improper separation of this fraction from the heart cut. This observation was found to be commensurate with the lower ethanol content of homemade orujo (~47–57% by volume), as opposed to industrial spirits with 53–74% ABV (Cortés *et al.*, 2005).

As with other spirits, the type of distillation system used and the manner in which it is used can have a considerable influence on the organoleptic character of pomace spirits. In a study of grappa production using traditional batch stills with and without a rectifying column, Da Porto and Longo (1997) found that without the rectifying column, the distillation was best carried out at the lowest possible temperatures in order to ensure a partial rectification of the distillate. They suggested that if a column with several plates is used, a higher quality grappa will be more easily produced.

3.7.3 Types of Pomace Spirits and Sensory Characteristics

Pomace spirits are produced all over the world – almost anywhere that vines are grown and wine is made. In most areas it is a minor product compared with wine and brandy and in some it is very rare: marc de Bourgogne and marc de Champagne are difficult to obtain outside their area of production. Even in areas (such as Cyprus, Galicia or Greece) where a significant volume of pomace spirit is still made by artisanal distillers, most of it is either consumed as such locally, or is used to make fruit and coffee liqueurs (Cortés *et al.*, 2005). In some areas, such as northern Portugal (Minho: the vinho verde region) and northwestern Spain (Galicia), Cyprus and parts of Greece, production of pomace spirit is of significant economic importance and was once of considerable traditional value.

The best known pomace spirits are probably those of Italy, which have the special name of grappa, designated by the European Union (or the European Economic Community as it was in 1989) (EEC, 1989) for spirits produced only in Italy. In recognition of the traditional production in specific regions within the EU, designated names are used for other pomace spirits, such as bagaceiras (Portugal), orujo (Galicia, Spain) and tsikoudia and tsipouro (Greece). The general regulations regarding the production of these spirits (EEC, 1989) indicate procedures to be used and give minimum levels for volatile substances (140 g/hl of 100% alcohol by volume) and maximum levels for ethanol (86% by volume) and methanol (1500 g/hl of 100% ethanol by volume).

Governmental bodies within the member countries of the EU that produce these spirits have awarded special denominations that protect them with regard to origin of raw material, distillation techniques and aspects of the composition of the final product. For each denomination, a regulatory body has been set up to ensure that any spirit labeled under its control complies with the requirements for that spirit. Thus in Galicia, the autonomous government, following the EU ruling, awarded the local pomace spirit the specific denomination of 'Orujo de Galicia/Aguadente de Galicia' in 1993. The government stipulated the origin of the raw material and the limits of several substances in the spirit: ethanol, acetal, acetaldehyde, methanol, total higher alcohol content (1-butanol, 2-butanol, isoamyl alcohols, isobutanol and 1-propanol), copper and total acidity. A regulatory committee was set up to ensure that the spirit in any bottle of 'Orujo de Galicia' complies with these regulations. In addition to chemical tests, the official tasting panel of the regulatory committee assesses the sensory characteristics of each representative sample of spirit from Galician distilleries.

Pomace spirits in the past have had a reputation for variable quality, roughness and fiery character. Compared with many other spirits, concentrations of acetaldehyde, methanol, 2-butanol and a number of carbonyl compounds are often high, as sometimes are furfural and 2-phenylethanol levels. In many cases, these characteristics can be explained by variability in the fermentation (including spectrum of fermentative microbes) and distillation techniques of small-scale winemakers/distillers, as described in Section 3.7.2. Likewise, it has been shown (also in Section 3.7.2) that modern distilleries with relatively sophisticated equipment can produce pomace spirits with high levels of certain less desirable components due, in particular, to overpressing the pomace (giving rise to higher methanol levels) and faulty storage of pomace (giving rise to 2-butanol, carboxylic acids and ethyl esters). The major carboxylic acids are acetic, butanoic, isobutanoic and isovaleric acids, which impart notes of vinegar, cheese and rancid butter. It has been found that Galician pomace stored under deficient conditions produced a defective distillate with high levels of all the above-mentioned acids (Diéguez *et al.*, 2002). Storage of the distillate for more than seven months caused a reduction in total concentration of acids (irrespective of temperature of storage; 5 °C or 25 °C), although quality was still poor. In cases like this, it was recommended that the pomace spirit should be redistilled.

Other than the above features, the aroma and flavor compounds of pomace spirits are similar to those found in most other spirits, with higher alcohols and esters playing a particularly important role (Da Porto, 1998). Carbonyl compounds are also thought to make a significant contribution to grappa aroma (Flamini *et al.*, 2005). Acetaldehyde was found in two commercial and two homemade grappa samples at levels (208–700 mg/l of 100% ethanol by volume), that are similar to those in other pomace spirits, such as orujo (48.5–709 mg/l) (Cortés *et al.*, 2005) and zivania (mean value 375 mg/l of 100% ethanol by volume) (Kokkinofta and Theocharis, 2005). Although high acetaldehyde content of spirits may not make much positive contribution toward organoleptic quality, acetaldehyde is nonetheless considered important in the spirits that are used to make Port wine because of its ability to stabilize the wine color (Section 2.10.7). Flamini *at al.* (2005) also found benzaldehyde, 2,3-butanedione, heptanal, hexanal, 3-hydroxybutanone, 2- and 3-methylbutanal and propanal in the grappa samples. The homemade grappa distilled from partially dried pomace of the Raboso variety had especially high levels of carbonyl compounds, probably the result of greater oxidation occurring as a result of partial drying.

The methanol content of pomace spirits (and others, such as fruit spirits – see Section 3.8.3) has always been a matter for concern. It is formed mainly from the enzymic hydrolysis of fruit pectins during fermentation and once present in the wine, it is distributed throughout all the fractions upon distillation (Claus and Berglund, 2005). The EU upper limit for the methanol content of spirits is 1500 g/hl (= 1500 mg/100 ml) of 100% ethanol by volume, but the USA legal limit is much lower, at 280 mg/100 ml 40% (= 700 mg/ml 100%) of 100% ethanol by volume (US Code of Federal Regulations, 2003). Several factors can influence the concentration of methanol in pomace spirit during its manufacture, including grape variety, acidity of pomace, strain of yeast used (Hang and Woodams, 2008) and wine press pressure. A laboratory-scale study of the methanol content of spirits made from the sweet pomace of white grape varieties (Cayuga White, Chardonnay, Gewurztraminer, Niagara and Riesling), grown in a Finger Lakes (New York) vineyard, showed methanol contents of 38, 55, 138, 44 and 112 mg/100 ml of 40% ethanol, respectively – all well below the legal limit (Hang and Woodams, 2008). The sweet pomace samples were fresh, moist (∼64%) (and hence had been lightly pressed) and fairly acidic (∼0.60% as tartaric acid), with sugar contents (7.81–16.17% as glucose) that gave pomace wine of ethanol content of 4.8–7.4% (v:v). The pomace was treated with sulfur dioxide and then fermented with cultured wine yeast. Distillation was carried out using a small-scale simple laboratory still. The results of this study suggest that clean fermentation of fresh sweet, moist pomace is capable of producing pomace spirit with low methanol levels and that the methanol content is dependent on the grape variety.

Higher alcohols are important contributors to the aroma and flavor of distilled spirits. They are present at higher concentrations than other volatile compounds, their levels in pomace spirit depending on grape variety,

fermentation method and distillation technique (Gerogiannaki-Chrisopoulou *et al.*, 2004). The prominent alcohols in many pomace spirits are 3-methyl-1-butanol (isoamyl alcohol) and 2-methyl-1-butanol. The mean concentration (415.9 mg/l) of the former in nine orujo samples (the pomace spirit of Galicia) was found to be lower than that of Portuguese bagaceira (915.5 mg/l), but higher than that of Italian grappa (243.5 mg/l) (Peña *et al.*, 2008). Other alcohols, such as 3-methyl-1-butanol and 1-propanol had high mean values in orujo (169.7 and 161.3 mg/l, respectively), although the mean 2-phenylethanol content was low (3.2 mg/l) compared with bagaceira and grappa, possibly indicating careful distillation techniques for these particular samples of orujo.

The volatile compounds that contribute most to the pomace spirit's sensory quality are (as with other spirits) probably esters of fatty acids. They are produced by fermentation and are found in the resulting distillate, their concentrations in brandy generally varying according to the grape variety (Milicevic *et al.*, 2002), and generally increasing on aging. Ethyl hexanoate and octanoate levels in orujo were found to be similar to those reported in bagaceira and grappa, but ethyl decanoate and ethyl dodecanoate were present at much higher levels in orujo (Peña *et al.*, 2008). These two esters contribute fatty or waxy notes to spirit aroma. Acetate esters, such as hexyl acetate, isomayl acetate and 2-phenylethyl acetate, are responsible for flowery/fruity notes; all have been found in pomace spirits.

Terpenic alcohols citronellol, geraniol, linalool, nerol and α-terpineol were identified in orujo, linalool (citrus notes) having the highest concentration (2.041 mg/l) (Peña *et al.*, 2008).

Zivania is a pomace spirit produced mainly by artisanal distillers in the Greek speaking part of Cyprus from Maratheftikon, Ofthalmon, Xynisteri and other local grapes varieties.

A study of 20 zivania samples and comparison with a large number of other spirits, including samples of Greek tsipouro, revealed that the mean levels of 2-methyl- and 3-methyl-1-butanol were similar to those of tsipouro, but were higher than those of many other spirits, including grappa (Kokkinofta and Theocharis, 2005). Mean methanol content was low (∼127 mg/l of 100% ethanol by volume), but acetaldehyde contents varied wildly and on average were higher than levels in grappa, tsipouro and other spirits. Copper concentrations were higher (mean value 2.87 mg/l), but varied widely from sample to sample, possibly indicating a wide range of still conditions and distillation techniques.

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3.8
Fruit Spirits

3.8.1 Introduction

Fruit spirits, often known as fruit brandies, are distilled fruit wines. Their generic French name is eau de vie de fruit and in German their family name is Schnaps, although there are subdivisions of Wasser and Geist, as discussed later. Individually, they tend to be named after the fruit from which they are derived (e.g. Kirsch(wasser) from cherries and Williams Birne from Williams pears). They are more often than not aged in glass or earthenware containers, rather than in oak casks, and hence are usually colorless, giving rise to yet another generic name, alcools blancs. An important exception to both the above general rules is Calvados, apple brandy named after a French département (in the old province of Normandy), rather than after the fruit, and aged in oak casks. Another exception is Obstler, used in German speaking countries to describe a spirit derived from apples and pears.

Like other alcoholic beverages, there is some confusion of nomenclature regarding fruit spirits and some other beverages. For example the English term cherry brandy can mean either a cherry spirit or more likely a liqueur made by infusing cherries in sweetened brandy (Section 3.9.2). A way around this would be to use the name that contains the word brandy for brandy based fruit liqueurs and to use the name that includes the word spirit for distilled fruit wines – or to use the French or German names for the fruit spirits, since the majority are made in French or German speaking countries. Another confusion is the use of the word schnapps, derived from the German Schnaps. In Germany, Schnaps is an unsweetened fruit spirit, whereas in the USA, schnapps refers to sweetened spirits flavored with fruit or fruit extracts. These drinks (sometimes known as cordials) are more akin to fruit liqueurs (Section 3.9.2) (Lichine, 1982). Additionally, one should be aware that sometimes in Germany the word Schnaps is used loosely to describe any kind of German made spirit.

In France, almost any fruit wine is distilled to produce a very wide range of eau de vie de fruit, whereas in Germany, Schnaps is nearly always made from apple, cherry, pear or plum wine. In French and German speaking countries, the most common fruit spirit is that of the cherry, but in eastern European countries, such as the former Jugoslavia, plum spirit (slivovitz) is the most common.

The most important areas of fruit spirit production are firstly the French Provinces of Normandy, Britanny and Maine for eau de vie de cidre (including Calvados) and eau de vie de poiré. Secondly, areas stretching

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

from Lorraine and Alsace (France), through Baden (Germany) and northern Switzerland (mostly quite close to the upper Rhine) into the Valais canton of Switzerland (close to the upper Rhône), produce a wide range of fruit spirits, with those from cherry and plum tending to predominate. Thirdly, Croatia, Serbia and other countries of the former Jugoslavia, as well as other central and eastern European countries, produce a clear spirit from blue plum wine known generally as slivovitz. Apart from the above, notable fruit spirits are made in a number of other countries, including the UK and the USA.

3.8.2 Apple and Pear Spirits

Apple spirit is distilled cider or apple wine, and pear spirit is distilled perry or pear wine. By far the most important producers of apple spirit are to be found in northwest France, in Brittany, Maine and Normandy, with the last named predominating. Pear spirit is also made in this part of France from perry, but it is also produced from pear wine in many other parts of Europe. The provinces of Brittany and Normandy are the major producers of cider and perry in France, using special sharp, bittersharp, bittersweet and sweet apple or pear varieties (Section 2.8.2), many of which are of ancient lineage and few of which are grown in any quantity anywhere else in the world. Apple and pear spirits made elsewhere in France (and in most other countries) are distilled from wines produced from noncider apples and pears, the Bartlett or Williams (Bon Chretien) pear variety being especially popular.

A substantial quantity of Norman and Breton cider is distilled each year; a number of cider and perry makers are also distillers, but cider spirit is also produced in northern Spain, and a little in England. Calvados is the most important cider spirit of Brittany and Normandy, although poiré and ordinary eau de vie de cidre are also made. Additionally, a small amount of eau de vie de marc de cidre is made from the cider apple pressings (Section 3.7.2).

Although there are records of cider distilling in the sixteenth century on the Cotentin Peninsular, near Cherbourg, today's most important spirit, Calvados, is named after the département of that name, whose major city is Caen (Figure 3.8.1) (Johnson, 1977). However, the best Calvados usually comes from the valley of the Auge in the eastern sector of Calvados: Calvados du Pays d'Auge was given Appellation Contrôlée

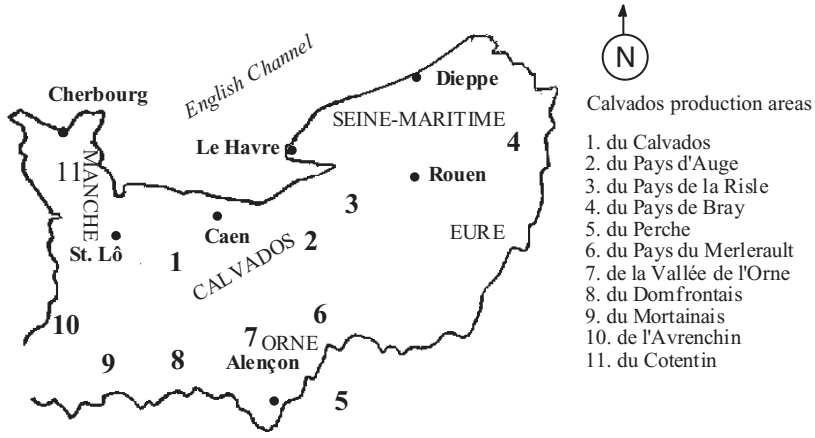


Figure 3.8.1 *Sources of Calvados and eau de vie de cidre*

status in 1942. It therefore must be made in accordance with a strict set of regulations, which are outlined below:

- Calvados du Pays d'Auge must be made in the designated region from cider apples growing in that region
- The cider must be prepared according to traditional practices and fermentation must occur over at least six weeks
- The cider must be double distilled in pot stills to give a spirit of ~72% ABV (in this way the production of Calvados resembles that of Cognac – see Section 3.6.2)
- The spirit must be aged for at least two years and bottled for sale at 40–50% alcohol (v:v).

In general, according to EU regulations, the distillation of fruit spirits must occur at less than 86% ABV, so that the spirit retains some character of the fruit from which it is derived (see for example Council Regulation (EEC) No. 1576/89 of 29 May 1989). In relation to this, there are also minimum and maximum limits on the levels of volatile substances and methanol (respectively) in the spirit; these are discussed more generally in Section 3.8.3.

The volatile components of samples of freshly distilled Calvados have been extensively investigated by a variety of separation techniques followed by analysis using GC-MS (Ledauphin *et al.*, 2004). Liquid–liquid extraction with dichloromethane, preparative GC and silica gel fractionation together allowed characterization of 331 volatile compounds (162 at trace levels) in freshly distilled Calvados and Cognac of which 130 trace aroma compounds were found in Calvados. The nontrace compounds included acetals, alcohols, aldehydes, carboxylic acids, esters, ethers, ketones, norisoprenoids, organosulfur compounds, phenols and terpenoids. Included in the alcohols were the unsaturated prop-2-en-1-ol and 3-methyl-2-en-1-ol, both of which were not found in Cognac. Likewise, hydroxyesters ethyl 2-hydroxybutanoate, ethyl 2-hydroxypentanoate and butyl 2-hydroxypropanoate were unique to Calvados, as were a number of aromatic compounds and phenols (including various guaiacols), 4-terpineol and 3,3-diethoxypropanol. Of the trace compounds, esters ethyl and methyl tiglate; secondary alcohols pentan-3-ol, hexan-2-ol and octan-2-ol; unsaturated alcohols pent-4-en-1-ol, hept-4-en-1-ol and four others; acetals 4-methyl-1,3-dioxane and 2-methyl-1,3-dioxane; unsaturated aldehydes pent-2-enal, oct-2-enal and tridec-2-enal; terpenoids camphor and eucalyptol; and phenolic compounds guaiacol, isoeugenol and various salicylic esters, were unique to Calvados and thus could be used as markers for this spirit. The sensitive preparative GC separation technique allowed identification of many trace compounds such as methional, oct-3-en-one and 4-oxoisophorone, which have low odor threshold values (Section 4.7.3) and which may make significant contributions to the aroma of freshly distilled (cooked potato, mushroom and wood, respectively).

Organosulfur compounds have been found to be much less in evidence in freshly distilled Calvados than in freshly distilled Cognac and terpenoid/norisoprenoid ketones were almost absent from Calvados (Ledauphin *et al.*, 2006). Nevertheless, many saturated and unsaturated carbonyl compounds were found in Calvados, which may provide grassy and mushroom aromas and several sulfur compounds were probably present at high enough levels to provide aroma contribution, particularly dimethyl disulfide (cabbage-vegetal) and methional (potato). The ratio of 3-thiophenecarboxaldehyde to 2-thiophenecarboxaldehyde in Calvados was found to be more than 10 for Calvados, but less than 0.5 for Cognac.

In practice, Calvados du Pays d'Auge is often aged in large oak casks, resembling small vats in size. Because these casks are only rarely replaced (in general like cider casks – see section 2.8.5), relatively few volatile compounds or nonvolatile tannins in the aged spirit are derived from wood contact. Even after several years in cask, many examples of Calvados are almost colorless and are hence given a mild coloration by the addition of a small amount of caramel, the only allowed additive.

Other (specified) regions outside of the Pays d'Auge are able to make cider brandy under the more general AOC Calvados appellation. These areas are in all of the Normandy departéments: Calvados, Eure, Manche,

Orne and Seine-Maritime, plus small areas of Eure-et-Loir, Mayenne and Sarthe départements, which are now outside Normandy (Figure 3.8.1). This AOC is similar to the AOC calvados Pays d'Auge, but is less restrictive and allows single column (batch column) distillation. Cider brandy made outside these areas, using methods outside those required for AOC status is known simply as eau de vie de cidre. Additionally, there is the AOC Calvados Domfrontais for spirit made from apples and pears, using single column distillation the minimum pear content being 30% and minimum required aging in oak being three years. This spirit is made in southern Normandy around the town of Domfrontais in the Orne département on farms which must include 15% of pear trees in the orchards.

Cider brandy or apple spirit is produced in several other European locations, including northern Spain (especially Asturias), northern Italy (especially Alto Adige and Trentino), Germany and England, from a wide range of apple varieties – not solely from classic cider varieties that are grown in the major cider manufacturing areas. Similarly, these apples are grown in different climatic conditions, which for each region vary from year to year. Thus it is expected that the spirit aroma profiles should reflect the apple varieties and the vintage from which the spirit is derived. Versini *et al*. (2009) were able to show apple distillates made from native Sardinian varieties can be distinguished from those produced from locally grown apple varieties in the traditional cider brandy provinces of Trentino, using similar fermentation/distillation equipment/methods. Using GC/FID/MS (Section 4.3.2) to characterize the aroma compounds, univariate statistical analysis (ANOVA) was used to evaluate where significant differences existed between Sardinian distillates of different vintages and between Sardinian and Trentino distillates, depending on variety.

Variables that showed high significance were then used in principal component analysis (PCA). ANOVA and PCA highlighted that ethyl octanoate, hexyl 2-methylbutanoate, 1-hexanol, benzaldehyde and furfural were correlated with apple varieties and were able to differentiate them. Of these, the first named may provide an index of fermentation characteristics, while the last named may be linked to distillation and sugar residues. The remaining three were considered to be linked to apple variety. On the other hand, 3-methyl-1-butanol, total aldehydes, ethyl acetate and 6-methyl-5-hepten-2-ol variables in Sardinian varieties were correlated with vintage.

Recently (Rodríguez Madrera *et al.* (2010) in Asturias have investigated the influence of duration of cider maturation on the aroma quality of the resulting distillate. It was found that the most mature cider gave a distillate of superior aroma (with more sweet and spicy character), with higher levels of ethyl acetate, ethyl lactate and ethyl succinate, and volatiles derived from bacterial metabolism (which is more prevalent in extensively matured cider), such as 2-butanol, 4-ethylguaiacol, eugenol and 2-propen-1-ol. On the other hand, the same group has demonstrated that higher ethyl carbamate (EC) levels were associated with spirits produced from well matured cider (up to 67 μg/l), although use of alquitara (a batch column still) in place of pot stills gave cider spirits with the lowest EC content (Rodríguez Madrera and Suárez Valles, 2009). EC is a carcinogen produced by the reaction between HCN and ethanol during distillation; the HCN in cider spirits is probably derived from urea from alcoholic fermentation (see Section 5.11.5).

Apple juice concentrate (AJC) is widely used in the production of cider, usually large-scale factory cider (Section 2.8.6) in many parts of the world, although its use is strictly regulated in many areas. Analysis by GC/MS of a range of cider spirits from Asturias, Calvados and Herefordshire distilled in either alembic (Charentais) stills or column stills was able to differentiate those derived from fresh apples and those derived from AJC (Rodríguez Madrera and Mangas Alonso, 2005). The analysis was based on the variability of 27 aroma compounds, including nine major volatiles: acetaldehyde, methanol, ethyl acetate, 1-propanol, 2-methyl-1-propanol, 1-butanol, acetal, 2-methyl-1-butanol and 3-methyl-1-butanol. Methods used to prepare AJC (Section 2.8.6) and the way in which it is fermented (normally using *S. cerivisiae* cultures) were considered to account for certain differences in aroma profiles. Thus, spirits derived from AJC tended to be higher in methanol content (due to pectin hydrolysis occurring during the preparation of AJC)) and lower in varietal (prefermentative) aroma compounds, such as 1-butanol and 1-hexanol. Likewise, spirits derived

from fresh apples tended to be higher in acetate esters (except 3-methyl-1-butyl acetate) (due to the action of non-*Saccharomyces* yeasts), whereas ethyl esters from the middle aroma fraction tended to be associated with AJC spirits.

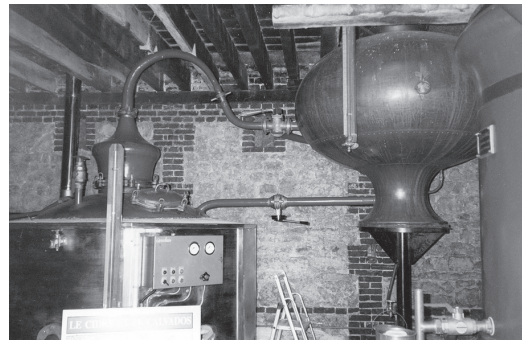
The above paragraphs have discussed how apple distillate aroma profiles differ between vintages of the same apple cultivars, between different cultivars (all aged and with similar fermentation and (alembic) distillation methods), between distillates made from unaged and aged cider and between spirits produced from AJC or fresh apples (with and without aging and using a variety fermentation regimes and stills). However, it seems that the method of distillation and extent of aging, although influencing the overall aroma profiles of apple distillates, do not significantly influence AJC/fresh fruit correlations, provided the aroma compounds chosen for correlation do not vary significantly with distillation methods and aging.

The two major distillation methods for producing apple distillate are pot still distillation (using alembics or 'Charentais' stills – resembling Cognac production: used in Asturias and Normandy/Brittany) (Section 3.1.2) and column still distillation (used in Asturias, England and Normandy/Brittany) (Sections 3.1.3 and 3.8.3). A third method uses the alquitara, a kind of direct heated copper pot still of around 130–350 l capacity that has a pear shaped bulge or reflux condenser at the top coupled to a recipient filled with cold water to condense the vapors. This type of still is used by some Asturian distillers, but is a traditional distillation system more often used in the production marc distillates in Galicia (Section 3.7.2). A study of Asturian cider distillation using alquitaras showed that the resulting spirits compared well with those distilled using alembics, having methanol, ethanol, furfural, lead, copper and zinc below maximum permissible levels, and with the minimum permissible levels of volatile flavor substances (congeners) surpassed (Rodríguez Madrera *et al.*, 2006). However, double distillation (with tails from first distillation) was necessary to give a spirit with adequate ethanol content and so was costly in energy, although double distillation gave reduced furfural and metal levels in the final distillate.

As with all alcoholic beverages, aging in wooden casks alters the organoleptic characteristics of apple spirit: it loses its rough edges, becomes softer in mouthfeel and more rounded and harmonious in aroma and flavor. The processes that cause these changes are discussed in more detail in Sections 2.9.5, 3.2.5, 3.3.4 and 3.5.3. Thus, apple distillates aged in oak generally become richer in certain acetals, ketals, medium and long chain ethyl esters, lactones (e.g. oak or whisky lactones) and phenolic compounds, while losing some aldehydes, ketones, nonethyl esters and fatty acids (except acetic acid) (Mangas *et al.*, 1996). Various attempts have been made to relate the presence of certain components in distilled spirits (both in absolute and relative terms) to maturation history – particularly with regard to aging in oak casks. Low molecular weight phenols and coumarins cannot be used as sole indicators of cask maturation of apple distillates, because of the use of oak extracts and (more commonly) caramel in the production of these spirits (Fernandez Izquierdo *et al.*, 2001). Similarly, furfural, 5-methylfurfural and 5-hydroxymethylfurfural are unreliable markers of oak aging when used alone, because of their presence in caramel and also because their concentrations depend on the distillation method and type of cider or apple wine distilled (Rodríguez Madrera *et al.*, 2003). Chemometric (principle component, linear discriminant and Bayesean) analysis of extracted aroma compounds, furans and phenolic compounds from many samples of cider brandies allowed classification of aged and unaged spirits according to main discriminant variables ethyl caprylate, ethyl isovalerate, 1-propanol and hexyl acetate (Rodríguez Madrera *et al.*, 2003).

Although there are records mentioning cider brandy production in England in the seventeenth century, it seems that the art died out, like many other things, around the time of World War 1. Cider distilling was revived using a small still installed at Bulmer's museum (Hereford) (Section 2.8.6) in 1984, although not on a commercial scale. It wasn't until 1989, that a well established cider producer at Burrow Hill (Somerset) obtained a distiller's license, and using two single column copper stills (Section 3.1.2), began distilling cider at the Somerset Distillery (see Figure 3.8.2). The column stills give a fresher, fruitier spirit, like some AOC calvados examples. The cider is made from around 40 apple varieties by traditional methods and is matured

Batch columnar stills at the Somerset distillery, UK. *Photograph by courtesy of the Somerset Distillery.*



Cognac-style alembic still at Etienne Dupont's Calvados distillery, France. *Photograph by courtesy of SARL Domaine Familial Louis Dupont.*

Figure 3.8.2 *Cider spirit and Calvados stills. Photo courtesy of SARL Domaine Familial Louis Dupont. Photo courtesy of Julian Temperly*

(without sulfiting) into the year following the vintage before being distilled. As with double pot distilled malt whiskies (Section 3.2.4), the new make spirit is collected at ~70% ABV. It is aged in oak casks of various sizes, typically 500 l, for three to 15 years and is diluted with pure water to ~40% ABV before bottling.

Like apple distillates, pear spirits are made in many locations, but especially in northwestern and eastern France, southwestern Germany and Switzerland. They are made from a wide range of pear varieties, although the Williams or Bartlett cultivar is popular in noncider or nonperry producing regions. Other pear varieties used include Gelbmostler (Austria and Switzerland), Schweizer Wasserbirne (Switzerland) and Theilerbirne (Switzerland). Perry pears (e.g. De Cloche, Plant du Blanc and others) are used to make pear spirit or Calvados Domfrontais in northwestern France.

The components of freshly distilled pear spirit are generally similar to those of other fruit wine distillates – including (apart from ethanol and water), methanol, acetaldehyde, furfural, higher alcohols, 2-phenylethanol, acetate esters and ethyl esters (García-Llobodanin *et al.*, 2007). The first three are toxic at higher levels and hence their concentrations must fall below specified maximum permissible levels, but the remaining components (provided that concentrations are moderate), along with many other low level and trace components, contribute to flavor quality. In particular, 2-phenylethanol contributes a floral, rose character to the spirit, but higher levels of this compound indicate poor separation of heart and tail distillation fractions, since it is a typical tail component.

The most characteristic pear aroma/flavor compounds are ethyl and methyl 2-*trans*-4-*cis*-decadienoate. This compound is found in pear juice, wine and spirit; in the latter distilled from Bartlett pear wine, their typical concentrations were 68 mg/l and 27 mg/l (respectively), which were lower than those of fresh ripe pears, probably because of isomerizations and other reactions occurring during fermentation and distillation (Kralj Cigić and Zupančič-Kralj, 1999). Furthermore, if Bartlett pear spirit is stored in colorless bottles, the 2-*trans*-4-*cis* isomers partially isomerize to the 2-*cis*-4-*trans* and 2-*trans*-4-*trans* isomers, all of which have much less pronounced pear-like odors, so the flavor quality of the spirit decreases. No such isomerization was

noted for pear spirit stored in green bottles. Observation of isomerization of a ethyl 2-*trans*-4-*cis*-decadienoate standard in ethanol:water (40:60 v:v) when exposed to strong UV light suggested that loss of ethyl and methyl 2-*trans*-4-*cis*-decadienoate on storage of spirit in colorless bottles is due to photochemical isomerizations (Kralj Cigić and Zupančič-Kralj, 1999). It is interesting to note that some pear spirits are still sold in colorless bottles.

As with other spirits, it is in the distiller's interest to ensure that his product contains minimal concentrations of acetaldhyde, ethyl acetate, furfural and methanol. This can be done by careful double distilling in pot stills with proper separation of head and tail fractions from the heart fractions (Glatthar *et al.*, 2001). However, apart from this general consideration, there are few reports on the influence of various distillation conditions on the concentration of key components in pear spirit.

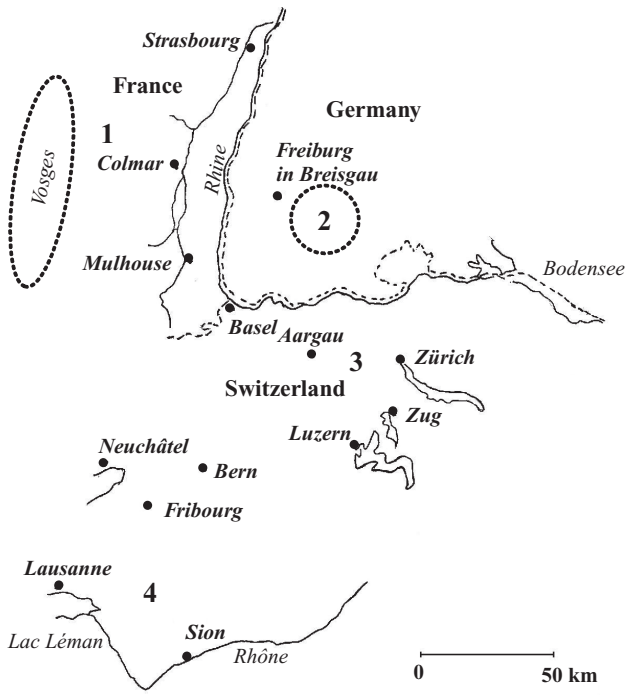
García-Llobodanin *et al.* (2007) investigated the effect the presence of wine lees and the material of the pot still on selected components of the whole first distillate and heart fraction of the first distillate of pear wine produced from Blanquilla pear juice concentrate. It was found that wine distilled from its lees in a copper alembic (as opposed to a glass alembic and a glass alembic containing 5g/l of copper shavings) gave the best results. Under these conditions, concentrations of compounds in the heart fraction considered to have a negative effect on spirit quality (ethyl acetate, furfural and methanol) were minimized, whereas the levels of beneficial components (especially ethyl decanoate and ethyl 2-*trans*-4-*cis*-decadienoate) were maximized.

3.8.3 Fruit Spirits of Central Europe

Fruit spirits, made from a wide range of fruit wines, are produced on a line running parallel to the river Rhine from Lorraine and Alsace in the north through Baden, past the Black Forest area of southern Germany and into Switzerland (Figure 3.8.3). The major, but by no means all of the distilling activity occurs in Lorraine, Alsace, Freiburg in Breisgau and the Black Forest, northern and central Switzerland (as far as Zug and Luzern) and southeastern Switzerland, especially in the canton of Valais, around the wine producing town of Sion (Figure 3.8.3). The different regions specialize in different spirits, depending partly on tradition and partly on the local availability of particular fruits. Thus Lorraine produces in any quantity only one kind of spirit, mirabelle, made from a small yellow plum, Alsace produces the widest range of fruit spirits, whilst the Black Forest region of Germany and Switzerland tend to specialize in cherry, pear and plum spirits. Because of varying production methods, particularly with regard to distillation, the spirit derived from a particular fruit (say Williams pear) can vary markedly from region to region.

Lorraine, just north of Alsace produces mirabelle from yellow plums of that name. The major production areas are those surrounding Nancy and Metz; here the spirit is entitled to the Appellation d'Origine Contrôlée (AOC) Mirabelle de Lorraine, provided it is made according to specified regulations. The best is probably Mirabelle Fine du Val de Metz, made by double distilling (in pot stills) wine made from ripe plums that has fermented/matured for at least two months in wood. The spirit is aged in oak casks (unlike many fruit spirits of the other regions) and, like most others, is diluted to ~45% ABV before bottling. In general, the word Fine in the name of a fruit spirit indicates that it has been aged in wood (vieillie en fûts).

Just like its wines, Alsace is an Aladdin's cave of fruit spirits, with regard to variety and diversity. Distilling activity tends to be centered on the wine producing areas between Obernai in the north, through Ribeauvillé and Colmar to Thann in the south, with many small distilleries situated in the Vosges mountains above the vineyards. Spirits are made from apple, apricot (abricot), bilberry (myrtille), blackberry (mûre sauvage), blackcurrant (cassis), blue plum (quetsch), cherry (Kirsch), elderberry (sureau), holly berry (baie de houx), peach (pêche), pear, quince (coign), raspberry (framboise), redcurrant (groseille), rowanberry (sorbier), serviceberry (alisier), sloe (prunelle), strawberry (fraise), wild plum (prune sauvage) and wild



1. Alsace/Vosges
Noted for a wide range of fruit spirits (alcools blancs and some oak aged): apricot, bilberry, blackberry, cherry, holly berry, peach, pear, plum (various), raspberry, rowanberry, strawberry.
Also noted for fruit liqueurs (Section 3.9.2).

2. Freiburg/Black Forest
Spirits from cherry, peach, pear, plum mainly. Also liqueurs and flavoured fruit spirits, such as *Himbeergeist*.

3. Northern Switzerland
Spirits from mainly cherry, peach and blue plums. Also Enzian, a fruit spirit made from gentian roots.

4. Valais/Southeast Switzerland
Mainly noted for cherry, peach and blue plum (*eau de vie de pruneaux*) spirits.

Figure 3.8.3 *Principal regions of fruit spirit production in central Europe*

strawberry (fraise de bois). Many of these spirits are made by fermenting the fruit pulp and then, after some maturation time, distilling the wine. Fruit spirits of this kind in Germany have the generic name of Wasser. A few fruit types with low natural sugar contents (e.g. holly berries) are sugared before fermentation. Some spirits are made by soaking the fruit (especially soft fruit) in neutral spirit (1 l of spirit to 4 kg of fruit) for several weeks and then distilling. The latter method when used in Germany gives a spirit known as Geist.

There are still many small-scale distillers who sell some of their products to wine producers for sale under the wine producer's name and there are some wine producers who make their own spirits (e.g. Preiss-Henny in Mittelwihr and Trimbach in Ribeauvillé). Many of the producers of fruit spirit in Alsace also make a certain amount of liqueurs (Section 3.9.2). Notable distillers include Jacobert (Colmar), Massenez (Dieffenbach-au-Val), Meyblum (Albé), Miclo (Lapoutroie) Nusbaumer (Steige) and Sainte-Odile (Obernai).

The most famous of the Alsace fruit spirits is Kirsch. Fresh cherries are crushed and fermented on some or all of their stones and then left for a while in contact with the stones before distillation. The stone contact gives rise to the 'almond' flavor common to all Kirsch.

In Germany and Switzerland, Kirsch (sometimes called Kirschwasser) is also the most widely produced and is a most popular fruit spirit. It is made in much the same way as the Alsace spirit, the treatment regarding the cherry stones varying from distiller to distiller. In the Black Forest area, clear spirits of the Wasser type are made from cherries (both wild and cultivated), pears (e.g. Williams-Birne), blue plums (Zwetschenwasser) and yellow plums (Mirabelle) by pulp fermentation, followed by distillation by distillers such as Elztalbrennerei (G. Weis), Kammer, Schladerer and Sutterer. Generally, around 8–10 kg of these fruits are required to produce a single bottle of spirit. Spirits of the Geist type are also made, by steeping soft

fruit, such as raspberries, in neutral agricultural alcohol for a few weeks, and then distilling. Himbeerengeist (from raspberries) is a typical example of this type.

In Switzerland, the majority of fruit spirit distillers are to be found in the central northern part of the country, especially in the area surrounding the towns of Luzern and Zug. The latter is the home of Etter, one of the best known of all producers of fruit spirits, making (apart from the usual range) Apfel Gravenstein (Pomme Gravin) and Birnenquitte, a spirit made from pear-quince wine. Other distillers include Fassbind of Oberarth making Kirsch, Pfümli (plum spirit) and Williams-Birne spirits.

German and Swiss fruit spirits are usually made by distilling the wine or macerate in batch column stills (rather than pure alembics as used in Cognac, Calvados du Pays d'Auge and Lorraine), which are typically copper pot stills with a dephlegmator (a partial condenser) attached at the stillhead (see Section 3.1.2), and with an enrichment section between the still and the condenser. The enrichment section usually consists of three or more bubble plates to control reflux and a dephlegmator that takes water from the top of the main condenser. Some systems also have a catalytic converter or cyan catalyst (a cylinder packed with copper of high surface area) between the enrichment system and the condenser. The purpose of this is to catalyze reaction between ethanol and any cyanide containing components in the vapor to produce ethyl carbamate (EC) (Section 5.11.5), so improving its separation from the heart cuts. Such distillation systems allow the distiller considerable control over the distillation process, which in Germany and Switzerland is used to give a lighter, cleaner spirit, but at the same time one that contains essential congeners to capture the fruit character. Usually two trays are used during distillation (three would give the lightest spirit:highest in ethanol concentration and lowest in congener levels), but if a catalytic converter is also used it acts as a third tray (Claus and Berglund, 2005). This had the effect of reducing *iso*amyl alcohol and 1-propanol congener levels and increasing the levels of ethyl acetate and methanol, whereas there was little effect on acetaldehyde concentrations, in the production of cherry spirit (Claus and Berglund, 2005).

Maturation is generally carried out in glass or earthenware, but sometimes, as in Alsace, in ashwood casks. Like oak casks, the latter allow a certain amount of controlled oxidation (due to slow ingress of oxygen) and the leeching of a certain amount of phenolic compounds into the spirit, but unlike oak casks (especially new, toasted casks), there is virtually no color extracted from the wood, so that spirits matured this way are essentially colorless.

The widespread practice of including stones in the manufacture of cherry and plum spirits, although claiming to give a higher concentration of congeners, some of which provide 'bitter almond' character (which is popular), may also lead to the formation of compounds that present health risks. The compounds of greatest concern are ethyl carbamate (EC – see Section 5.11.5) and hydrogen cyanide. The former may be formed during distillation, when prunasin (in the flesh) and amygdalin (in the stones) (see Figure 5.11.4, in Section 5.11.5) are subjected to heat. They decompose first to mandelonitrile, whence that compound is hydrolyzed to benzaldehyde (responsible for 'bitter almond' character) and HCN. Some of the latter may then react with ethanol to produce EC, although another possible source is urea, which is formed from arginine by some yeast strains during fermentation. Recently Schehl *et al.* (2005) have studied the influence of yeast strain and the inclusion of fruit stones on the organoleptic character, sensory analysis and EC/HCN levels in cherry and plum spirit, using a batch column still. The identity of yeast strain used (a commercial strain CGC62 and a laboratory strain HHD1) made only minor differences to the flavor profiles and little difference in quality (as perceived in the sensory analyses). The levels of EC and HCN in the spirit were similar for each strain, but generally somewhat lower when fruit without stones were fermented. Interestingly, plum stones when fermented alone with Uvaferm yeast gave the highest levels of EC and HCN in the resulting spirit. Also of interest was the observation that, although the panel tasters could detect the differences between spirits from whole mashes (with stones present) and destoned fruit mashes, there was no overall preference, indicating that qualities added by the presence of stones during fermentation are largely a matter of personal taste, rather than a matter of quality difference.

3.8.4 Plum Spirits of Eastern Europe

The Slavic countries of central and eastern Europe have long specialized in the production of colorless plum spirits, usually known as slivovitz. The spirits are generally considered to have originated in the Balkan Peninsular in one of the states of former Yugoslavia, although some authors maintain they were first distilled further northwest in Slovakia. Today, slivovitz is made in all of the former Yugoslavian states, the Czech Republic, Hungary, Poland, Romania and Slovakia, and its equivalent is produced in many other countries in Europe (as discussed in Section 3.8.3) and elsewhere (see below).

Slivovitz is generally made from local cultivated blue plum (*Prunus domestica* L.) varieties, where it is recognized that fresh, good quality fruit of high sugar content produce the best spirit. The plums are crushed with about 15% of the stones being cracked and then fermented on the pulp by natural fermentation (as in some artisanal distilleries) or by inoculation with a yeast culture (as in most industrial distilleries). At the end of fermentation, the mash is usually distilled using a batch column system, (Section 3.1.2) where fractionation (often using a rectifier with four trays or plates) is used to separate heads and tails from the heart fraction and to increase the ethanol content of the latter to 60–75% (v:v). Some artisanal distillers use simple pot stills, with no rectification column, although sometimes with an air dephlegmator above the still. The heart fraction is generally matured in oak casks for 2–5 years and is usually diluted with distilled water to 45–50% ABV before bottling and sale, but 'cask strength' examples exist, with alcohol contents in excess of ∼65–75% (v:v) (Satora and Tuszyński, 2008). As with other spirits, chill filtration may or may not be practiced prior to bottling.

Slivovitz, like any other fruit spirit, varies in aroma profile depending on the mode of fermentation (natural versus inoculation: Satora and Tuszyński, 2005; 2008), distillation process (Tešević *et al.*, 2005) and ageing process (duration, type of oak and size of cask: Tešević *et al.*, 2005). Within the European Union (EU), the levels of methanol and hydrogen cyanide in plum spirits should not exceed 1200 mg/l and 100 mg/l (respectively) for 100% ethanol (EEC, 1989). In the USA, the maximum level of methanol is 700 mg/l for absolute ethanol, meaning that a distilled beverage of 40% ABV should have no more that 280 mg/l of methanol. The maximum allowable level of ethyl carbamate in fruit has not been established by the EU, but most countries use 400 mg/l, the maximum level recommended by Canada and the USA.

An earlier work on the volatile components of industrial and homemade or artisanal Yugoslav slivovitz (Filajdić and Djuković, 1973), using packed column gas chromatography was able to identify and quantify 23 esters and 21 carboxylic acids, as well as a number of higher alcohols. Seventeen carbonyl compounds were also identified. The methanol contents varied enormously, between 300 and 4390 mg/l, being generally higher for the homemade samples. Variation of other components was not so wide, the samples generally having concentrations of 1-propanol, 2-methyl-1-propanol, 1-butanol, 2-methyl-1-butanol and 3-methyl-1-butanol of 400, 190, 50, 100 and 280 mg/l, respectively. The brandies also had ∼500mg/l of ethyl acetate, 30 mg/l of acetaldehyde and 1500 mg/l of carboxylic acids, of which ∼90% was acetic acid. Artisanal samples generally had lower acetic acid contents than commercial samples.

More recent analysis of the volatile components of old slivovitz samples (again homemade and industrial), by continuous dichloromethane extraction followed by GC/MS, allowed the identification of 99 compounds (Tešević *et al.*, 2005). The oldest spirit (an industrial sample) had higher concentrations of some components, such as benzaldehyde, benzyl alcohol, ethyl benzoate, ethyl salicylate, ethyl cinnamate and 2-phenylethanol. Eugenol was found in all the samples, but α-pinene and β-myrcene were found only in the oldest brandy. Sensory analysis by experts showed high scores for all the brandies, but with a significant preference for the oldest one, which had been distilled in a 300 l still fitted with a rectifying column with four trays, whereas the artisanal spirits came from either simple smaller pot stills or a pot still fitted with an air dephlegmator. Also, the oldest spirit was aged in the smallest oak casks (∼500 l) of *Quercus petreae* L. wood (*Q. sessiliflora*), as opposed to *Q. pedunculata* (*Q. robur*).

Śliwowica Łącka is a traditional Polish plum spirit made by artisanal distillers in the area around Łącko, in mountainous country near the Czech border. It is made by natural fermentation of local blue Węgierka Zwykła plums. Comparison of the aroma profiles of Śliwowica Łącka samples and commercial slivovitz samples from Poland, Romania and Slovakia (Satora and Tuszyński, 2008) indicated generally higher levels of methanol (up to 8740 mg/l), 1-propanol (up to 1516 mg/l) and 1-butanol (up to 335 mg/l), and lower levels of 2-butanol (up to 491 mg/l), 1-pentanol (up to 15 mg/l) and 2-phenylethanol (up to 68 mg/l). The figures all relate to 100% ABV. In particular, the amyl alcohols:1-propanol ratio was close to unity for Śliwowica Łącka and closer to 2 for the commercial samples, except the Romanian sample, which had the lowest ratio of all (0.72). This ratio was suggested as a marker to distinguish between plum spirits produced by natural fermentation and those made by cultured yeast fermentation. The acetic acid contents of the samples in this study (14–625 mg/l 100%) were much lower than those reported in an earlier study (Filajdić and Djuković, 1973) on Yugoslavian plum spirits (550–3220 mg/l 100% for artisanal samples and 650–4670 mg.l 100% for industrial samples) and were more in line with other European fruit spirits. Hydrogen cyanide levels (0.67–5.91 mg/l 100%) were well below the EU maximum allowed limit (100 mg/l 100%). Cluster analysis of the chemical composition of the plum brandies clearly differentiated Śliwowica Łącka spirits from the others, suggesting that fermentation methods may play an important role in determining flavor profile, along with plum variety and distillation techniques. Sensory analysis generally favored the commercial foreign samples over the Polish samples and it may be significant that the top scorer (the Slovakian slivovica) and the bottom scorer (the Polish commercial śliwowica) had (respectively) the lowest and highest levels of acetaldehyde and amyl alcohols, with the artisanal spirits having intermediate values.

The yeast microbiota conducting fermentation of Węgierka Zwykła plums in the production of Śliwowica Łącka are diverse, originating from both plant material and distillery equipment (Satora and Tuszyński, 2005). As with wine fermentations (Section 2.2.4), *Kloeckera apiculata* and *Candida pulcherrima* dominate the first 48 h of fermentation, reaching a population of 1.4×10^6 cfu/ml. After this, the ethanol level kills off these species and fermentation is rapidly taken over by 14 *Saccharomyces cerevisiae* strains that changed quantitatively and qualitatively throughout the fermentation period. The ones that dominated the end of the fermentation (~30 days) were those with greatest killer toxin resistance.

3.8.5 Other Fruit Spirits

Fruit spirits are made by both industrial and artisanal distillers in many other European locations, including Austria (e.g. Brennerei Franz Bauer), Hungary (e.g. Pecsetes Distillery) and Saxony (e.g. Augustus Rex Erste Dresdner Spezialitäten Brennerei). Many of these spirits are produced from fruit wine or distilled macerated fruit by methods and from kinds of fruit already discussed in Sections 3.8.2–3.8.4 and are generally available outside the region of production. Others, described next, are either well known local spirits produced by artisanal distillers and are worth considering for standardizing and scaling up for greater distribution, or are novel spirits made from an overproduction of certain fruit in order to cut waste and provide a profitable sideline.

In Greece, a fruit spirit known as 'mouro' or 'mournoraki' (Crete) is made from black mulberry (*Morus nigra* L.) by artisanal distillers (Soufleros *et al.*, 2004). The ripe fruits (with a natural sugar content of (9–22.5% dry weight) are collected and fermented naturally, sometimes with the addition of a little pure water. The pomace is double distilled in copper pot stills, often of 130 l capacity, whence the spirit is usually offered for consumption at 35–45% ABV. Allowing for a wide variation in flavor profiles (typical of artisanal products), concentrations of acetaldehyde, ethyl acetate, ethyl lactate and methanol (which can be detrimental to spirit quality at high levels) were either below official limits or qualitative odor threshold limits (Soufleros

et al., 2004). On the other hand, high levels of aroma/flavor beneficial higher alcohols (total > 1400 mg/l; greater than the authorized minimum for spirits in the EU), esters such as ethyl octanoate (∼13 mg/l) and ethyl decanoate (∼14 mg/l), as well as 2-phenylethanol (trace-127 mg/l) gave the spirits agreeable organoleptic character. If greater attention is paid to the distillation process and maturation, giving more quality control and leading to greater consistency, it was felt that mouro may gain greater overall fame.

There is considerable interest in Spain on producing spirits and liqueurs (Section 3.9.2) from an over-production of melons (especially in the Castilla-La Mancha region), thereby reducing waste and providing an additional valuable outlet. Hernández Gómez *et al.* (2005) investigated the production of melon spirit on a semi-pilot scale using cultured yeast inoculation to ferment either melon juice, paste (without skin) or skins/paste (each pH adjusted or unadjusted) and distilling the wine firstly in an alquitara (reflux still) and then in an alembic still. The paste distillates had higher levels of ethyl butyrate than the others, a component that adversely influences the organoleptic character of spirits. This could explain the rejection of the paste distillates on the basis of sensory analysis. The other melon spirits, derived from juice and skins/paste were not well differentiated by sensory analysis (there was a slight preference for pH unadjusted skins/paste spirit). In a comparison of the volatile components of melon spirits (from juice and skins/paste) with those of commercial fruit spirits (cherry, pear and raspberry) and pomace spirits (grappa and orujo), it was observed that fusel alcohol levels were slightly higher in melon spirits, pear spirit and grappa, methanol content was lowest in melon spirits, 1-propanol levels were higher in melon spirits and pomace spirits and the ethyl acetate contents of melon spirits were the highest. Organoleptically, the juice and skins/paste spirits compared favorably with commercial spirits, although from a commercial viewpoint, the skins/paste substrate was considered preferable, because it gives less waste and does not need to be pressed prior to fermentation/distillation.

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3.9

Liqueurs and Their Flavorings

Each and every liqueur, in his opinion, corresponded in taste with the sound of a particular instrument. Dry curaçao, for instance, was like the clarinet with its piercing, velvety note; kümmel like the oboe with its sonorous, nasal timbre; crème de menthe and anisette like the flute, at once sweet and tart, soft and shrill.

—J.K. Huysmans

3.9.1 Introduction and Scope

For the purposes of this book, liqueurs are defined as alcoholic beverages with alcohol contents from as little as 15% to over 40% (v:v) and with sugar contents of at least 10% (w:v). Although this is a good working definition, like many definitions concerning alcoholic beverages, it has a certain amount of arbitrariness attached to it and authors do not always agree where the dividing line is between liqueurs and apéritifs (Sections 3.5.5 and 3.5.6) and bitters, for example. Some authors even describe distilled fruit wines (fruit spirits or *eau de vie de fruits*) (Chapter 3.8) as liqueurs.

In practice, the alcohol in liqueurs is derived entirely from distilled spirits and their sweetness is derived partly or entirely from added sugar (or honey), a fact that sets liqueurs apart from sweet, alcoholic wines, such as the liqueur Muscat and Tokay wines of Australia (Section 2.10.9), where some (if only a little in some cases) of the final alcohol content comes from fermentation of the must, but which never have added sugar. Similarly the French vins de liqueurs (Section 2.10.10): although all their alcohol comes from added spirits, their sugar is all derived from the grapes.

There are two basic ways of making liqueurs:

- By mixing of an infused or macerated flavor base (extract) with sweetened distilled spirit(s) and water. Sometimes these two steps are combined: the flavor base is directly macerated with the sweetened spirit(s)
- By maceration of a flavor base with distilled spirit, followed by distillation and mixing of the distillate with sweetened distilled spirit(s) and water
- In either case, additional flavorings, colorants or other additives can be added at the final compounding stage.

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

The flavor bases can be fruits, vegetables, flowers, roots, kernels, nuts, beans, herbs, leaves, barks, powdered spices, fruit concentrates, essences, distillates, nature identical flavorings or artificial flavorings, or mixtures of these (Clutton, 2003; Zach, 2007). The spirit can be any single spirit (e.g. grain neutral spirit, brandy, eau de vie de fruit, gin, marc, rum or whisky/whiskey) or a mixture of two or more spirits. The spirit component(s) are sometimes chosen to match the flavoring (e.g. Kirschwasser used to make a cherry liqueur) (Section 3.9.2), although often a grain neutral spirit predominates to allow for maximum expression of the flavorings (Section 3.9.4). Spirit distillates (e.g. minimally aged undiluted or cask strength brandy of *ca.* 60% ABV, or rum of *ca.* 75% ABV) tend to be used to make liqueurs, but some liqueurs are made using more mature spirits for additional flavor. The sweetening agent is usually sugar syrup, but glucose syrup can also be used and some liqueurs are sweetened with honey.

In reality, because liqueurs are essentially flavored, sweetened spirits, there is almost no limit to the number of recipes that can be concocted, and indeed there are a great many commercial liqueurs of a similar type, but whose individual characters vary according the composition of their flavor bases, their sweetness, their spirit(s) components and their methods of production. Thus there are many brands of cherry liqueur (often known confusingly as cherry brandy) – Bols, De Kuyper, Heering etc., but they are all made by carefully guarded recipes. The approximate recipes of many liqueurs are known, but it is the minute details of flavoring ingredients, compositions, spirit types and production methods that are kept secret.

The first liqueurs were probably made in Italy in the thriteenth century, as a result of the researches of Arnáu de Vilanova and Raimundo Lulio on the extraction of herbs by alcohol (Lichine, 1982a). Originally, these sweetened, herb flavored spirits were used as remedies for diseases, just like other kinds of herbal preparations at that time. Arnáu and Lulio were the first to write extensively on the production of ethanol by distillation and their recipes for healing liqueurs became widely known throughout Italy. Later, they introduced fruit pulp and skins, flower petals and even particles of gold into recipes for liqueurs (gold in those days was considered a universal panacea for illnesses). Liqueurs became popular throughout the land and by the fifteenth century; Italy was the world's leading producer of liqueurs. The art was then taken to France by Catherine de' Medici at Montpellier, where there was easy access to grapes (for wine and brandy), fruit and herbs, so this city soon became a major center of liqueur production. By this time, liqueurs were being consumed primarily for enjoyment, although their medicinal properties were still much in mind. Indeed, monks of various orders took up the production of liqueurs for medicinal and restorative purposes at an early stage, especially in France. In the main, these liqueurs were consumed originally only by the monks, along with various people who had connections with the monastery, as well as important visitors. Benedictine D.O.M. (*Deo Optimo Maximo*) liqueur was reportedly first formulated around 1510, the recipe being passed on from generation to generation of Benedictine monks at Frécamp (Normandie) until about the time of the French Revolution. The recipe survived and the liqueur is still made by a secular company of that name; there are no longer any religious connections (Lichine 1982b).

Chartreuse was first formulated about 1605 by Carthusian monks at Voirin (France) purely for consumption by the monks, until it became commercialized in 1848 as the result of the efforts of a group of enthusiastic army officers who had tasted it (Lichine, 1982c). The Chartreuse liqueurs are now made at Tarragona, as well as Voirin, due to two expulsions of the Carthusian order from France and its return in 1932.

Nowadays, some of the best liqueurs still come from Italy and France (there are even some with gold particles), although many other countries produce a very wide range of liqueurs from many different flavor and spirit bases.

The aim of this chapter is to discuss a variety of commercial liqueur types, their approximate recipes, their production methods and their flavor ingredients, while at the same time expanding on what has been written in these introductory paragraphs.

3.9.2 Fruit and Fruit Flavored Liqueurs

These beverages are highly popular and constitute a major category of liqueurs. In fruit liqueurs it is the fruit pulp, juice and/or extract that forms the flavor base and these beverages are often simply named after their fruit ingredient: cherry brandy, strawberry liqueur, sloe gin, liqueur de framboises, crème de pêches, to name but a few. More examples can be seen in Table 3.9.1. Depending on the philosophy or commercial expectations of the manufacturer, fruit liqueurs are made with or without a distillation process that involves the flavor base. Also, depending on the country of manufacture's regulations, additives, such as fruit essences, natural or artificial flavorings, colorants and acidulants, can be found in some fruit liqueurs. A general scheme for the production of fruit liqueurs is shown in Figure 3.9.1. Many manufacturers of fruit liqueurs use only the ripest and healthiest fruit, subjecting them to a period in the freezer (at $\sim -20\ ^{\circ}C$) before macerating them in spirit. The freezing process preserves the essential aromas and also helps soften otherwise rather hard stone fruit such as greengages, damsons and sloes, also making the pulp and skins more easily detached from the kernels. The maceration process (with or without kernels) can take several months, even up to one year. In larger, more modernized companies this process is sometimes carried out in slowly rotating stainless steel drums in the absence of oxygen.

Some of the finest fruit liqueurs are produced in France: in Alsace, Burgundy and the Loire Valley, in particular (Table 3.9.1). Notable amongst these is crème de cassis made originally from blackcurrants growing on the Cote d'Or, near Dijon, but now produced in many locations in France. This liqueur is popular as the minor ingredient of a Burgundian aperitif known as kir, the major ingredient being a light white

Table 3.9.1 *Examples of fruit liqueurs and liqueurs of the Curaçao type*

Liqueur type	Examples of brands or companies producing these liqueurs	Comments
Apricot	Giffard (Loire), Maraska (Croatia)	
Banana	Bols (Grune Bananes) (Holland), Giffard	
Bilberry	Boudier (Liqueur de Myrtilles) (Burgundy)	
Blackberry	Boudier, Chambord[1] (Loire), Combier (Loire), Marchand (Burgundy), Marie Brizard (France), Nusbaumer (Alsace), Tremontis (Italy), Védrenne (Burgundy)	[1] Contains a number of other components
Blackcurrant (Créme de Cassis)	Bols, Boudier, Giffard, Massenez (Alsace), Védrenne	
Cherry	Bols[1], De Kuyper (Holland), Heering (Denmark), Luxardo (Italy)[1], Marnier (France), Maraska	[1] Make both red (infused) and clear (distilled) types
Peach	Boudier, Combier, La Gina (Italy), Marie Brizard, Massenez, Védrenne	
Pear	Boudier	
Raspberry	Boudier, Schladerer (Germany)	
Strawberry	Boudier, Védrenne	
Curacao style and citrus peel	Alize (France), Arancello (Italy)[1], Bols[2], Cointreau (Loire), Grand Marnier, Marie Brizard, Materdomini (Italy)[3], Senior[4]	[1] Orange liqueur [2] This company also makes a blue liqueur [3] Made with lemon peel [4] Made with laraha

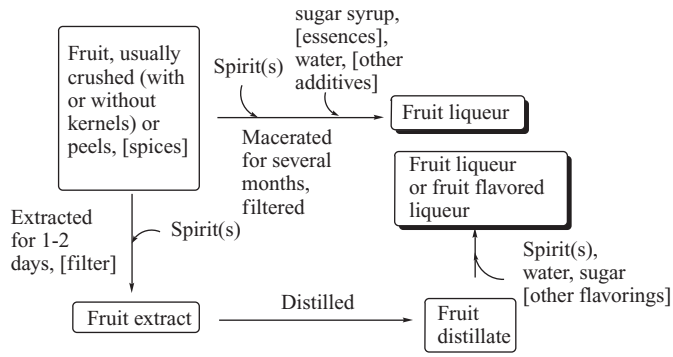

Figure 3.9.1 *General scheme for fruit liqueur and fruit flavored liqueur production. Optional ingredients are in square brackets. Water is distilled or deionized. Based on Zachs (2007).*

Burgundy wine, usually an Aligoté. The apéritif is named after Canon Félix Kir, a leader of the French resistance in Lyon during the Second World War, and a former mayor of Dijon.

Cherry liqueurs, often called cherry brandy if brandy is the spirit base, are amongst the most popular of fruit liqueurs. They are usually made with more flavorful bitter/sour varieties of cherries, such as Morello (as with Cherry Marnier) or Maraschino (as with Luxardo Maraschino and Maraska Maraschino). The liqueurs are usually bright red because of the long maceration time in spirit, but there are also some colorless versions produced by distillation of alcoholic cherry extract, followed by sweetening and dilution to the required alcoholic strength before bottling. Examples of these include the two mentioned above, plus Bols Kirsch liqueur – not to be confused with the eau de vie de fruit, Kirsch, which is unsweetened (Section 3.8.3).

The Asian countries of China, Japan and Korea make many beverages from fruit, particularly blackberry (black raspberry), green or yellow plums and raspberry. Although some of these beverages are fermented and hence fall into the category of fruit wines (Chapter 2.11), the majority of them are made like liqueurs, by macerating fruit in (usually) sweetened rice spirit (soju: see Section 3.4.4) and then filtering before bottling. Their ethanol content is usually around 16% (v:v) (but can be higher), so they are often used to accompany meals, as wines accompany meals in European countries.

Korea has a number of traditional fruit 'wines', produced by combining fruits or berries with alcohol For example, podoju (포도주) is made from soju that is mixed with grapes and sugar. The most popular fruit wines are made from green plums (maeshil), Korean black raspberries (bokbunja, 복분자), Chinese quinces, cherries, pine fruits, mulberries and pomegranates. Bokbunjaju (복분자주, bokbunja wine) is said by many to be especially good for sexual stamina (although many drinks and foods in Korea make this claim!). A particularly popular Korean fruit liqueur known as maeshilju, is made from maeshil, a small greengage-like plum that is gathered hard and unripe in May/June. Maeshilju has a pronounced gage-like flavor overlaid with an almond flavor from the kernels, and some versions of this beverage are sold wide necked bottles containing one or more fruits at the bottom.

Japanese plum wine is essentially a sweetened spirit (shochu – see Section 3.4.4) flavored with partially ripened 'Japanese apricot,' a blue plum containing much acid (citric and malic acids) and minerals. It improves the appetite and aids digestion and is said, like maeshilju of Korea, to be a good restorative with anti-aging properties that help the drinker keep a clear skin.

Sloe gin is a popular liqueur in England and Europe. It is made from the small bitter/sour black fruit of the wild blackthorn tree (*Prunus spinosa*). Traditionally, it was not made until November, after the first frosts of winter, because these have the effect of softening the otherwise hard fruit and hence easing the maceration

process. Nowadays, the fruit is often gathered before the first frosts and kept in the deep freezer until required. The thawed fruit is then bruised and added to sweetened gin at the rate of 450 kg of fruit to 730 l of gin and 200 kg of sugar, to give about 730 l of liqueur. Brandy can be used in place of gin, to give sloe brandy. The mixture is agitated at regular intervals while left to macerate for about three months, after which time it is filtered, adjusted if necessary and bottled. Blanched almonds (30 kg) can also be included in the recipe, and bullaces (yellow wild plums), damsons or greengages can be used in place of sloes.

The production of distilled spirits (or even industrial alcohol) or liqueurs is sometimes used as a cost effective way of using surplus agricultural produce (as in the wine industry, making spirit from excess wine) or commercial process waste (as in the wine and cider industries, making pomace spirits from pressed grape or apple mass). Melon (*Cucumis melo* L.) fruits are produced in large quantities in many warm climate countries, including Mediterranean countries. Consequently, there has been interest in converting excess fruit to a melon distillate and this has been carried out on a pilot scale in Spain (Section 3.8.5) (Hernández Gómez *et al.*, 2005). Further interest has been shown in using melon distillate as a base for a melon liqueur, bearing in mind the general overall popularity of liqueurs. Investigation of maceration of melon pieces, seeds and placenta in melon spirit, showed that, by analysis of color and aroma, as well as sensory analysis, seeds and placenta were more suitable than melon pieces. Apart from the above reasons, liqueur made with seeds and placenta only was more viable because of lower production costs, less waste and easier filtration (Hernández Gómez *et al.*, 2009). The total volatile component content, based on acetaldehyde, ethyl acetate, ethyl butyrate, ethyl lactate, methanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-methyl-1-propanol and 1-propanol was highest for melon spirit macerated with seeds and placenta at 0.28 kg/l. The liqueur (with alcohol content 28% (v:v) and sugar content 100 g/l) made from this scored highest in sensory tests.

Fruit flavored liqueurs are made using natural and/or nature identical flavorings, the former usually as distillates or extracts (Section 3.9.6) (Figure 3.9.1). Liqueurs of this type can be made from stone fruits, such as apricots and peaches, but probably the best known and most popular types are the Curaçao liqueurs, involving citrus peel as the major flavor base. These liqueurs were originally made from oranges growing on the Dutch colonial island of Curaçao in the Caribbean Sea. The orange trees, known as lahara (*Aurantium currassuviensis*) and descended from the 'Valencia orange tree,' were introduced by the original Spanish explorers in the fifteenth and sixteenth centuries. Very little Curaçao liqueur is now made from lahara (see Table 3.9.1), other citrus fruit (especially bitter oranges from tropical islands like Haïti or from Spain) or extracts and essences being used instead. Nowadays, there are many brands of Curaçao made by many companies, but the best known are probably Cointreau and Grand Marnier. Further examples can be found in Table 3.9.1. A general scheme for the production of a Curaçao style liqueur can be seen in Figure 3.9.2; detailed recipes can be found in Zachs (2007).

Another well known orange liqueur is the South African Van der Hum, produced using the peel of the nartjie, a local orange, and a number of other ingredients that are closely guarded secrets.

Limoncello is a related Italian liqueur based on lemon. Its manufacture commonly involves maceration of lemon peels in 95% ethanol for a week or so, after which the filtered extract (without distillation) is diluted with sugar syrup to give a final product of ~32% ABV. In the European Union, addition of natural or synthetic flavors or essential oils to limoncello is regulated by Council regulation No. 1576 (EC 1989), as for other spirit beverages.

Limoncellos have been characterized by the presence of selected volatile compounds (mainly alcohols, aldehydes and terpenoids) and nonvolatile compounds (acids, coumarins, phenolic compounds, psoralens and sugars) (Andrea *et al.*, 2003). α-Pinene, myrcene, *trans*-β-bergamottene and β-bisabolene were the most characteristic of terpenoids of limoncellos made using lemon peel maceration. Likewise, the presence of coumarins and psoralens (Figure 3.9.3) gave a likely indication of the use of macerated lemon peels, since these are absent from essential lemon oil produced by steam distillation. Limoncello made using essential oils only can thus be distinguished from the genuine article.

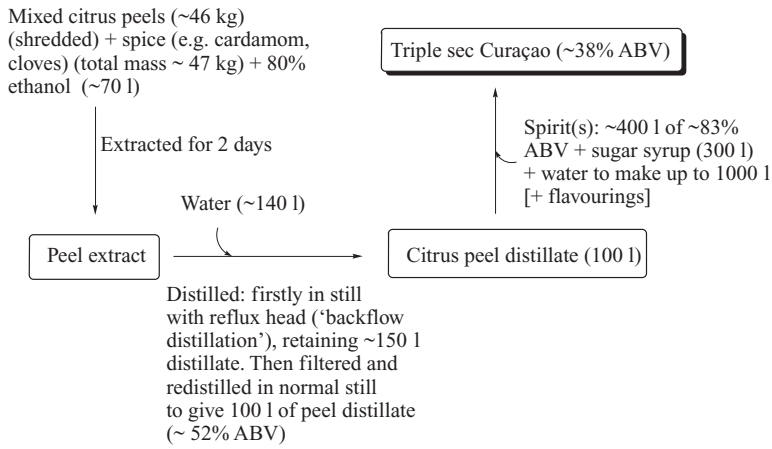

Figure 3.9.2 *General scheme for the production of a Curaçao style liqueur. Distilled or demineralized water is normally used. Optional ingredients are in square brackets. Based on Zachs (2007)*

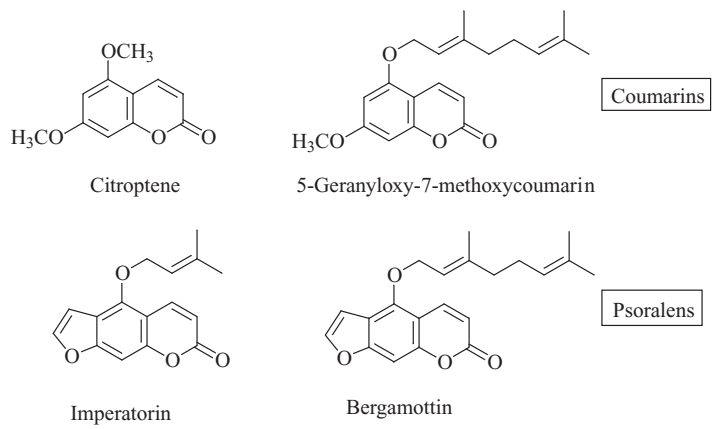

Figure 3.9.3 *Characterstic coumarins and psoralens in authentic limoncello liqueur. Andrea et al. (2003).*

3.9.3 Flower, Herb, Spice and Bitter Liqueurs

This very large and diverse family of liqueurs involves the use of botanicals, natural essential oils and natural essences as the flavor bases. The earliest recorded liqueurs (see Section 3.9.1) were of this type, produced for medicinal purposes in Italy in the thirteenth century. Today, Italy still produces some of the best versions. In time, the manufacture of herbal liqueurs in monasteries spread throughout Europe, the carefully preserved and guarded recipes being handed down from generation to generation of monks over many centuries. In many cases (e.g. Benedictine), the monks' original herbal/spice recipes are still used in the extraction processes used to make modern versions of the liqueurs.

The range of botanicals used in liqueurs is very wide, as in the case of vermouths and other aromatized wines (Sections 2.12.2 and 2.12.3). They can be subdivided into a number of categories that depend on

the flavor, taste or mouthfeel sensation contributed to the liqueur. These are listed next, with a number of examples of each:

- *Herbal botanicals*. Usually leaves, stems, sometimes flowers. Artemesia (e.g. mugwort, wormwood), basil, camomile, hyssop, lemon balm, mint (many varieties), saffron, sage, thyme, woodruff
- *Spice botanicals*. Usually bark, buds, roots or seeds. Ambrette, angelica, cardamom, caraway, celery (seed), cinnamon, cloves, coriander, ginger, mace, turmeric
- *Bitter botanicals*. Usually roots, sometimes stems or flowers. Elecampane, dandelion, gentian, orris, thistle
- *Saponin botanicals*. Usually bark. Cascarilla, Cinchona, quillaja
- *Tannin botanicals*. Bark, oak
- *Flower (glycoside) botanicals*. Elderflower, heather, lime tree flower, orange flower, rose
- *Essential oil botanicals*. Citrus peel
- *Miscellaneous botanicals*. Nuts (e.g. almonds, hazelnut and walnut: they supply oils as well as flavor), beans (e.g. cocoa, coffee, vanilla), mucilaginous botanicals (e.g. fenugreek seeds), silicic acid botanicals (e.g. corn horsetail).

Many of these materials will also contribute to the color of the final liqueur; for example, green if the leaf content is high and an extract rather than a distillate is used to make the liqueur (see next), or yellow if saffron flower extract is added to the mixture of colorless spirit(s), syrup and botanical distillate.

One method for producing herbal type liqueurs involves the preparation of a botanical extract and then mixing this with a mixture of spirit, sugar syrup (or honey) and, in some cases, specific extracts or additives. The botanicals are extracted in ethanol/water for a number of days, after which time the extract is decanted off the debris, stored at low temperatures to effect precipitation of finely suspended matter, after which it is chill filtered before use. A quantity of the herbal extract is mixed with spirit, water and sugar syrup and, since grain neutral spirits are often used in both the extract and the spirit/syrup mixture, the color of the final liqueur will be determined by that of the botanical extract, unless permitted colorants are added.

A more common method involves several days maceration in neutral spirits/water, followed by single pot still distillation to give a herbal distillate of around 65% ABV. This is then mixed with spirit(s)/sugar syrup/water. Additives and colorants (e.g. saffron extract to give a deep yellow color) can also be added at this time if required. Grain neutral spirit is always used for the preparation of the herbal distillate, but brandy, rum or whisky distillates (or mixtures) may be used in place of or as well as neutral spirit in the final compounding of the liqueur.

There are many variations of the above two methods, particularly with regard to the herbal extract or distillate. Some recipes call for both extract and distillate of the same botanical mixture (see Figure 3.9.4), others (e.g. Chartreuse) require the compounding of a number of different botanical distillates, and there are various ways in which the distillation or extraction may be carried out (Section 3.9.6). Additionally, some recipes make use of specific essences or concentrates (e.g. elecampane root extract) at the final compounding stage (Figure 3.9.4). These, like elecampane extract (from *Inula helenium* or horse-heal) (Section 5.11.2), are used to add specific flavors or bitterness, while others such as saffron are added primarily for color. Interestingly, various components of *I. helenium* roots have been found to have antimicrobial activity (Stojakowska *et al.*, 2005) and *in vitro* anticancer activity (Chen *et al.*, 2007). Although there is no direct proof that the presence of these components in liqueurs has any beneficial effects, it should be remembered that liqueurs were originally formulated as medicines and extracts of *I. helenium* and many other herbs used in liqueurs have been used in European and Asian folk medicine for centuries.

Most herb liqueurs are made from a large number of herbs and spices, using secret recipes, although a few of the main components are known or can be guessed in some cases. Probably the best known are Bénédictine, Chartreuse, Galliano, Jägermeister and Strega. The first mentioned claims to be made from only 27 botanicals,

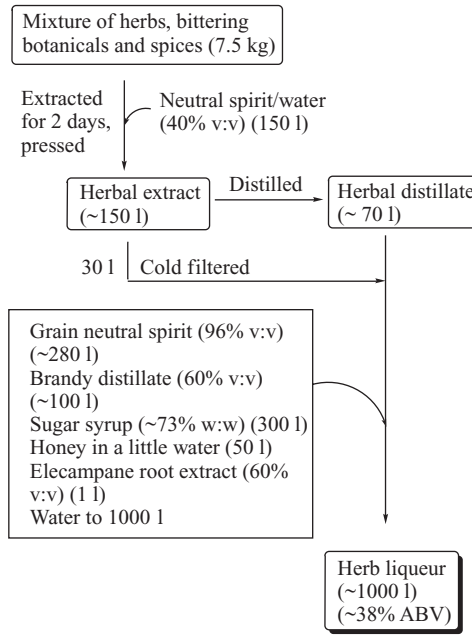

Figure 3.9.4 *Scheme for the production of a monastery type herb liqueur. Based on Zachs (2007).*

as opposed to over 130 for Chartreuse. Nevertheless, neither liqueur has been successfully recreated by competitors or copied by counterfeiters. The standard ethanol content of these liqueurs is around 43% (v:v), but Green Chartreuse has 55% ABV and the Elixir Végétal de la Grande-Charteuse has an exceptional alcohol content of 71% (v:v). Galliano is a bright yellow liqueur made in Italy that contains anise, citrus, ginger, star anise and vanilla, amongst other botanicals; it is the base ingredient of Harvey Wallbanger type cocktails. Its color is provided by tartrazine, in contrast to Strega, another Italian herb liqueur, whose golden color is derived from saffron. Mint and fennel are the major botanical contributors to the flavor of Strega.

Other examples of multiple herb liqueurs include Aiguebelle (France), Angelica (Pays Basques), Bärenfang or Bärenjäger (Germany), Becherovka (Czech Republic), Drambuie (Scotland), Fior D'Alpe (Italy), Glayva (Scotland), Izzara (Pays Basques), Krupnikas (Lithuania), Trappistine (Doubs, France; based on Armagnac), Unicum (Hungary) and Vieille Curé (Cenons, France), to which can be added Amaretto (Italy) and Crème de Noyaux (France and other countries). The last two are sometimes described as almond liqueurs, relating to their dominant flavor of bitter almonds, but this flavor comes from apricot kernel oil or apricot kernel distillate rather than almonds and both contain other botanicals. Honey, rather than (or as well as) sugar syrup is used to sweeten several of these liqueurs: Bärenfang, Drambuie, Glayva and Krupnikas. The first and last named are made using grain neutral spirit or vodka and nectar honey: Bärenfang varies between manufacturers, but is often flavored with few botanicals, including carnation flowers and vanilla pods; Krupnikas is made with clover honey and a greater number of herbal botanicals. Drambuie and Glayva, on the other hand are made with aged Scotch whisky and heather honey, the former having anise, nutmeg and saffron in its botanical make up as well as herbs, the latter having anise, cloves, herbs, citrus peel and almonds. More recent additions to this category of liqueurs include Lochan Ora (Pernod Ricard) made with a base of Scotch whisky and Irish Mist (C&C Group plc) made with Irish whiskey.

Relatively few herb liqueurs are made of just one or a small number of strongly flavored herbs or spices. The foremost example is Crème de Menthe. It is made all over the world, ideally from Corsican mint. The

simplest method involves mixing mint essence with grain neutral spirit (or vodka), sugar syrup, water and a green colorant if a green color is required. Usually, better green versions of this liqueur are produced by macerating mint leaves in neutral spirit/water for several weeks, filtering, and then adding sugar syrup and water to adjust sweetness/alcoholic strength. Alternatively, the mint extract can be distilled and the mint distillate mixed with spirit/sugar syrup/water to produce a clear ('white') Crème de Menthe.

Other examples of single herb or spice dominated liqueurs include the aniseed liqueurs Anisette, Sambuca and Sassolino (both Italian), using star anise as the major flavor ingredient and Kümmel, with caraway as the major flavorings. Kümmel is popular in northern Europe (especially the Baltic countries) and Russia. It is often made by distilling grain spirit over caraway seeds and then diluting to strength (~40% ABV) by adding sugar syrup. In some cases, (Kümmel Crystallize), sugar is allowed to crystallize in the bottle. Sambuca also uses other herbs, including elderflower essence (for the white version) and elderberry extract (for the black version). The Greek liqueurs Tentura and Zamoura, made by distillers such as Chris and Polykala, are dominated by cinnamon/clove flavors, although they may have other botanicals, such as nutmeg and citrus peel, in their recipes. A more recent addition to this group of liqueurs, with a dominant cinnamon flavor, is After Shock from Canada.

Certain herb liqueurs are about the only liqueurs to undergo some aging in cask, the prime example being Chartreuse, especially the version known as VEP – Vieillissement Exceptionnellement Prolongé. Other examples include Bénédictine and Unico.

In Korea, various medicinal alcoholic beverages are made from sweetened spirit and bark, roots or shoots, commercially and at home. For example, ginseng, bamboo shoots, and the root bark of various araliaceous shrubs are soaked in sweetened soju (>35%) (Section 3.4.4) and aged, usually in earthenware pots. The ratio of soju to herbal root is usually 3:1.

Ginseng liqueur is made with ginseng, soju and sugar, after six months aging it becomes suitable to drink. After three years maturation, ginseng liqueur is often a deep golden color and the alcohol aroma becomes subdued, with the unique aroma of ginseng, sweet and slightly earthy/bitter, being dominant. Additionally, jujube (Chinese date) or ginger can be added to the mixture and honey can be used instead of sugar (Jo, 2004).

Korean medicinal liqueurs are known collectively as yagyongju (약용주) (the Korean syllable yak means medicine) and are produced by combining medicinal seeds, herbs, and roots with sweetened soju, although some are made by fermentation and so are more appropriately classed as flavored rice wines (Section 2.7.1). They include Dosoju (도소주 or 屠蘇酒), a popular herbal liqueur, traditionally served only on New Year's Day and Songsunju (송순주 or 松筍酒) made with glutinous rice and soft, immature pine cones or sprouts. Similarly, Ogalpiju (오갈피주) is made from the bark of *Eleutherococcus sessiliflorus* blended with soju and sugar and Jugyeopcheongju (죽엽청주 or 竹葉青酒) is a traditional liquor made with bamboo leaves.

Chuseongju (추성주) is a traditional wine made from glutinous and nonglutinous rice and herbs, including omija (*Schisandra chinensis*) and *Eucommia ulmoides*; it is commercially available in bamboo shaped bottles. Likewise, Daeipsul (대잎술) is another traditional folk wine from South Jeolla Province, made from glutinous rice, brown rice, and bamboo leaves, along with 10 medicinal herbs.

Liqueurs based solely or mainly on flower infusions or macerations are relatively few. The best known is probably Millefiore (Italy), its name implying the use of a thousand (Alpine) flowers and its bottles typically contain a small twig around which sugar has crystallized. Liqueurs made with rose petals include Rosolio (Italy), Rosolis (France) and Roza (Bulgaria).

There are several Korean traditional liqueurs (although they are known as wines in Korea) made from flowers. These include chrysanthemum (gukhwaju, 국화주, marketed by Jinro as Chun Kook), acacia flower, maesil blossom (maehwaju, 梅花酒), peach blossom (dohwaju, 桃花酒), honeysuckle (indongju, 인동주), wild rose and sweet briar petal liqueurs. Dugyeonju (두견주) is a liqueur produced in Chungcheong Province from azalea petals. It is sweet, viscous, and light yellowish brown in color, with a strength of about 21% ABV. Myeoncheon Dugyeonju is protected, being designated by the South Korean government as Important

Intangible Cultural Property No. 86-2. Another variety of flower liqueur, called baekhwaju (백화주 or 白花酒), is made from 100 varieties of flowers, the Korean syllable 'baek' meaning 100.

3.9.4 Cocoa, Coffee and Tea Liqueurs

These liqueurs are made from percolates of the flavor base in neutral spirit:water (30% v:v, typically). The percolate is then filtered and mixed with the spirit(s)/sugar syrup/water mixture, which usually contains vanilla extract or essence, that flavor blending well with cocoa or coffee flavor. Sometimes the coffee percolate will also contain some spice, such as cinnamon or coriander, but this will not be dominant.

Most cocoa liqueurs are simply named Crème de Cacao, although one or two have specific names, such as Liqueur Fogg (Brazil) and Sabra (Israel), a cocoa liqueur flavored with Jaffa orange essence or distillate. Chocolate liqueurs, on the other hand, usually include cream in the recipe and so can be found in Section 3.9.5.

The most famous coffee liqueurs are the Mexican Kahlúa (from Pernod Ricard; and the largest selling liqueur brand in the USA), made from rum, coffee beans, sugar syrup and vanilla, and the Jamaican Tia Maria, made with Blue Mountain coffee beans, vanilla, sugar syrup and cane spirit. There are many other coffee liqueurs (some claiming to be made from freshly brewed coffee), based on a variety of spirit bases, including Kapali Coffee (Mexico), Komora Coffee (Mexico), Oblio Caffe Sambuca (Italy), a liqueur flavored with espresso coffee, anise, fennel and elderflower essence, and Pasha (Turkey).

3.9.5 Nut and Emulsion Liqueurs

Nuts tend to have rather delicate flavors that need careful extraction into ethanol/water. Other flavors, such as vanilla and/or cocoa, are sometimes used in combination and nuts are sometimes used in cream based emulsion liqueurs, again often flavored with cocoa, coffee or mocha. Nut liqueurs include Frangelico, made with hazelnut, cocoa and vanilla, Hazelnut Polykala (Greece), Davis Walnut liqueur (Lurgashall, UK) and Cashew Liqueur (Korea). So called almond liqueurs, such as Amaretto, are actually made from apricot kernels, oil or essence. One potential problem with nut liqueurs is the often large proportion of lipids in the nuts or skins, as with peanuts, whose skins can yield up to 35% of their weight as oil (Sobolev and Cole, 2003). However a palatable liqueur has been made on a laboratory scale from hexane-defatted and dried peanut skins, by adding them ($\sim$ 2% by weight) to a warm alcohol/sugar syrup mixture (of $\sim$40-70% ABV) at 70-90 °C and allowing the mixture to stand overnight (Sobolev and Cole, 2003). The result, after filtration, was a pink peanut flavored liqueur. It was suggested that this may be a way to give some added value to the peanut industry, where 750 000 tons of skins are produced annually, their main use being as a limited ingredient of cattle feed.

The production of dairy cream emulsion liqueurs has rocketed in recent years. These liqueurs depend on the homogenization of dairy cream and sweetened, flavored ethanol/water, using caseinate or other emulsifier, to form a stable liquid with very little or no separation. Flavorings are often chocolate or coffee, but butterscotch, caramel and noisette are also known and more recently, fruit flavorings have been used. Spices (e.g. cinnamon, mint) are also used to flavor many of these liqueurs. Separation of the fatty cream component into a plug of cohesive cream that is difficult to redisperse by mild agitation, is a potential storage hazard associated with these liqueurs. It has been found that cream plug formation during storage can be minimized by ensuring the liqueur has moderate pH and calcium levels, with high levels of sodium caseinate (Dickinson *et al.*, 1989). In experiments with soya oil/aqueous ethanol emulsions stabilized by either sodium caseinate or β-lactoglobulin surfactants, it was found that the kinetics of pH induced aggregation could be explained by

orthokinetic flocculation, whereas ethanol induced aggregation kinetics suggested a mechanism involving Ostwald ripening (Agboola and Dalgleish, 1996).

The best known cream liqueur is Bailey's Irish Cream (Diageo), based on Irish whiskey; it was the first in this field and is one of the most notable market successes in the recent history of distilled spirits. Much more recently, Mussu Crema de Pacharan (Spain) is claimed to be the first cream liqueur made from pacharan, a traditional Spanish liqueur made from sloes soaked in anisette. There is now (2009) a cream based liqueur that contains a variety of flavors, rather than chocolate, coffee or fruit: Voyant Chai Cream (Holland) is composed of aged Virgin Island rum, Dutch spirits, cream, black tea (from India) and herbs (from Asia).

In recent years, milk and yogurt, rather than cream, have been used to make liqueurs. For example, Crème Likier Mleczny (Poland) is made from milk and wodki zoladkowej gorzkiej (bittersweet digestive vodka) and possesses 18% ABV. Chylls Yoghurt Liqueur (Germany) is based on maracuja (passion fruit) liqueur and yogurt.

Advocaat is an egg emulsion liqueur made in the Netherlands from brandy, egg yolk, vanilla, sugar syrup or honey. There are many versions of this liqueur (e.g. those produced by Bols, Cooymans, De Kuyper or Warninks), but they all have around 18% ABV and are frequently used in cocktails such as Bombardier.

One emulsion liqueur based on bananas rather than cream or egg is Licor De Banana Crème (Brazil), made with real bananas (rather than extract or essence) and cane spirit.

3.9.6 Production and Use of Essences and Concentrates

Fruit concentrates, essences, essential oils and extracts are important in the manufacture of liqueurs. Concentrates refer to fruit juices (apricot, cherry, peach, etc.) from which much of the water has been removed by, for example, a ramped low temperature vacuum concentration process (see Section 2.8.6), thus avoiding substantial loss of aroma/flavor compounds. Fruit liqueurs can be made using concentrates as well as fresh fruit (or more likely freshly thawed frozen fruit – see Section 3.9.2), but many liqueur manufacturers, especially smaller companies aiming at a niche market, prefer to use only the latter. Some typical recipes for fruit liqueurs, using both fruit and concentrates, can be found in Zach (2007).

Essences, extracts and essential oils are used to make a wide range of liqueurs. Many liqueur distilleries prefer to use their own formulas, in which case they make an extract of the specific botanicals by infusing them in alcohol/water (Sections 3.9.3 and 3.9.4). This extract can be used as such or can be distilled; in some cases both extract and distillate are used (Figure 3.9.3). However, many liqueur recipes call for the use of essences and essential oils, as well as home produced extracts or distillates. The term essential oil generally refers to a concentrate of aroma compounds from the skins of citrus fruits, whereas essence usually refers to a concentrate of aroma compounds from other botanicals (bark, flowers, leaves, nuts, pods, seeds, etc.), either separate or as mixtures. In practice, however, the two terms are often used interchangeably. Many essential oils and essences are commercially available, including clove essence, essential oil of lemon, essential oil of lime, hop essence (or oil), rose essence (or oil), vanilla essence, violet essence (or oil) and walnut essence.

Essences and essential oils can be made by distillation (including steam distillation for essential oils and vacuum distillation for essences – see Section 3.4.2), soxhlet extraction into ethanol, supercritical fluid extraction, followed by fractionation (as for hop essence – see Section 2.6.3) and the spinning cone column technique. The latter (see Section 2.13.3) is particularly useful in separating volatile compounds from their host materials. It is a (usually heatless) distillation or 'stripping' technique that uses a turbulent counterflow of gas and liquid to give enhanced interphase contact, resulting in efficient extraction. The host materials must be supplied at the top of the column as slurry of finely ground botanicals in ethanol/water.

The soxhlet method (Figure 3.9.5) is a well established continuous extraction technique, in which aroma components of a wide range of solid materials are extracted into ethanol. The solvent is allowed to percolate

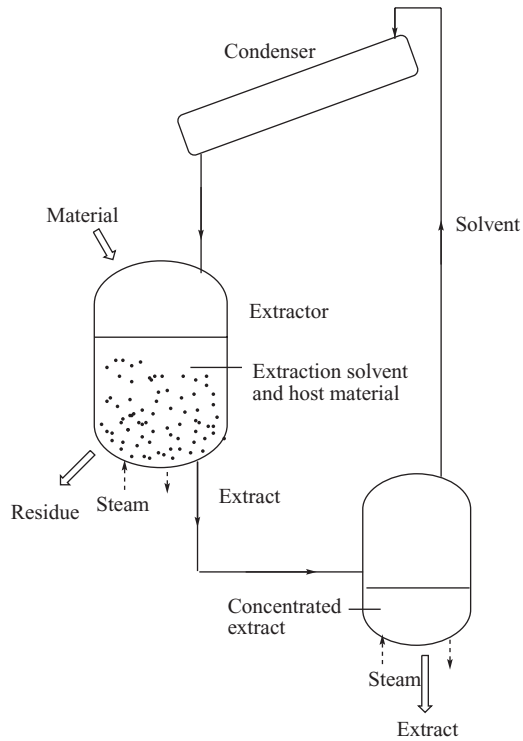

Figure 3.9.5 *Schematic diagram of a Soxhlet extraction device. The extractor vessel will be fitted with a stirrer. Both the extractor and the vapour generator are heated with steam* via *either internal vanes or an external jacket.*

continuously through the ground material, becoming more and more concentrated in flavor components at each pass. Eventually, the concentration of flavor compounds in the solvent vessel will be sufficient and the extraction process is terminated, collecting the concentrated extract solution and discarding the residues.

A more recent technique involved the adsorption of the pear aroma compound ethyl 2,4-decadienoate on granular activated carbon and its consequent solvent desorption, giving a solution 40 times more concentrated than the original (Diban *et al.*, 2008). The authors concluded that this low energy consuming, mild and ecologically friendly method was viable for the concentration of aroma compounds; it is possible that such a method may be used to produce fruit and other concentrates in the food and alcoholic drinks industries.

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Part 4
Analytical Methods

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Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

4.1
Introduction

Analytical science is applied to just about the entire range of aspects associated with the production and consumption of alcoholic beverages. Such methods not only enable producers to maintain a consistent level of quality, but by increasing general knowledge of beverage components and details of biological, chemical and physical processes occurring during manufacture, they can provide the producer with the opportunity to increase the overall quality of his or her products. There is no doubt that the application and development of analytical methods (in research and development, as well as process day to day monitoring) in the alcoholic beverage industry continues to play a crucial role in raising the overall quality of the products.

This chapter reviews the major methods for the analysis of alcoholic beverages and their raw materials, the methods available for the analysis of particular components or physical parameters and the contexts within which such measurements are made. Knowledge of analytical chemistry is assumed at undergraduate level. In particular, it is assumed the reader has a grasp of such concepts as sampling techniques, chemometric methods for the design of experiments, accuracy, recovery, precision, reproducibility, repeatability, robustness, sensitivity, limit of detection, limit of quantification and basic statistical methods for the treatment of results. However, short explanations of some of these terms are given in the Glossary.

Although outline general accounts of important techniques are given in each Chapter (apart from Chapter 4.1) for the sake of background and also to render some perspective to these techniques when applied to the analysis of alcoholic drinks and their raw materials, it is simply not feasible in a book of this kind to give detailed theory. Instead, at relevant points, the reader is referred to a selection of many excellent textbooks in analytical chemistry, chromatography, electrochemistry and spectroscopy.

Chapter 4.1 is meant to serve as a general background to Part 4 and is also a summary of the applications of analytical methods to a variety of aspects relating to alcoholic beverages. Some of the major uses and benefits of the analytical methods applied to alcoholic drinks described later are put into a more general context in Chapter 4.1.

Chapter 4.2 considers the separation and focusing techniques that are necessary for preparing the sample for analysis of very low concentration and trace components. This section also relates how these methods can be linked with specific methods of analysis, particularly chromatographic techniques.

The next chapter discusses the application of chromatographic techniques to the separation, identification and quantification of beverage components. The emphasis is on liquid and gas chromatography and consideration is given to mobile phases, stationary phases, experimental conditions and detector types for particular kinds of analyses. Chapter 4.4 discusses the applications of spectroscopy, where particular

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consideration is given to the different methods available for the analysis of particular components or families of components.

Chapter 4.5 relates how electrochemical methods, including miniaturized sensors, can be applied to the analysis of drinks. Electrophoretic methods, techniques using flow injection and physicochemical methods are dealt with in Chapter 4.6, and finally sensory analysis is the subject of Chapter 4.7.

4.1.1 Why Analyze Alcoholic Beverages? An Overview of the Need for Analysis

Understandably, a considerable amount of analysis of alcoholic beverages is commerce driven and is biased toward the elimination of defects and the maximization of quality and therefore of profit. However, there are many more ways in which analysis is being used for the benefit of drinks producers, the general public and the scientific community. The principal areas of contemporary chemical analysis of alcoholic drinks are summarized below, although the reader will realize that there is a certain amount of overlap amongst some of these areas:

- Process monitoring and quality assurance/control. Here, determination of the levels of key components in raw materials, to check whether they are within specification, and regular measurement of specific process parameters are used to maximize quality and consistency of the product.
- Determination of authenticity and identity. Determination of geographical origin (e.g. winegrowing region, etc.), process methods (e.g. pot still versus column still distillation, pasteurization versus filtration or maturation in oak casks versus maturation in concrete vats) and material origin (e.g. beer grain composition, grape variety or origin of CO_2 in sparkling wine) can be reliable indicators of authenticity and can lead to the detection of certain kinds of fraud.
- Detection of fraudulent (illegal) additives. Chemical analysis can be used to detect adulteration (e.g. use of artificial sweeteners in Spanish cider or addition of elderberry juice to grape must to boost color), illegal chemicals (e.g. potassium ferricyanide – in some countries), illegal ingredients (e.g. sweet flag in vermouth) and extraneous sugar; the extent of chapalization of wine is carefully regulated from region to region and from year to year.
- Satisfaction of regulatory requirements. Analytical methods are used here to ensure that the levels of certain components (e.g. ethanol, methanol, residual sugars, sulfur dioxide (a legal preservative), pesticide residues and others) fall within regulatory limits. Regulations differ from country to country, region to region and can differ even between products of a particular region.
- Development of new methods of analysis. Research here is orientated toward the enhancement of one or more of the following aspects of analysis: accuracy/precision, automation, cost, environmental aspects, robustness, safety, speed, selectivity and sensitivity. In many cases, improvements made in methods in the general field of alcoholic beverages are helpful to analytical science as a whole.
- Identification of components or measurement of parameters in relation to quality, character, biochemical history, nutritional or health aspects. Analysis of such components as acids, amino acids, proteins, aroma compounds, carbohydrates, phenols, pigments and others are important here.
- Characterization of new components (e.g. aroma/flavor compounds, phenolic substances or pigments), especially in the context of elucidating process chemistry (e.g. fermentations, maturation or oxidation) or metabolic pathways.
- Investigation of pollutants, natural toxins and their metabolites.

Outline examples of applications of analytical science in some of the above areas will be found in the following sections. More detailed accounts will be found in the other chapters of Part 4, and indeed elsewhere, especially Parts 2 and 3.

4.1.2 Process Monitoring

The pursuit and maintenance of quality are the most important goals for any producer of alcoholic drinks – large or small. At the very least, application of analytical methods at the key stages in the manufacturing procedure (process monitoring) should ensure consistent quality and hence help the market performance of the product. All other things being equal, if it's consistently good, it'll sell consistently well.

This does not mean to say that all breweries, distilleries and wineries need an on site array of sophisticated and expensive analytical and ancillary equipment to carry out routine and special analyses. Although most of the largest producers of alcoholic beverages are part of some giant international company and do indeed have on site or easy access to such equipment, some medium to large- and many small-scale producers make world renowned products using only minimal analytical procedures during the production process. Their secret is to combine the right kinds of analyses at the right times with a time honored production method (i.e. one that is known to give a high quality product), that is *never* altered, unless analytical results early in the process suggest certain modifications must be made to the procedure later on. Their routine analytical methods may be little more than specific gravity, sugar, acid, ethanol or tannin determinations, combined with the all important sensory/organoleptic (including color/clarity) analyses.

At one extreme, Chateau Latour at Pauillac in the Haut Medoc (Bordeaux) manages to make one of the indisputably greatest red wines in the world without the regular use of expensive analytical instruments. Any variation in quality from year to year, is due mainly to differences in climatic conditions, but even so, an overall very high level of quality is achieved. At the other extreme, a particular large brewery company may produce consistently high quality beers, always making routine, but judicious use of a wide range of sophisticated analytical equipment.

There is no hard and fast rule correlating quality with extent or sophistication of process analytical monitoring; consistently high quality beer can be produced by brewers whose regular analytical equipment consists only of balances, thermometers, hydrometers, volumetric equipment and simple microbiological equipment (such as a microscope and an ATP-bioluminescence kit), as well as by brewers who use a much wider and more sophisticated range of equipment.

However, it is generally agreed that, within reason, consistent quality of product is more easily achieved with the use of a rather more than basic range of analytical equipment. A small to medium size brewery, distillery or winery could do well with the equipment mentioned above, plus a simple colorimeter or inexpensive UV-visible spectrophotometer and a basic chromatograph; HPLC and/or GC. To these basic items, one could add a friabilimeter and foam assessment equipment for a brewery, a viscometer for a grain distillery and equipment for measuring total nitrogen content for any laboratory associated with alcoholic beverage production. A Fourier transfer infrared spectrometer (FTIR) could also be included in a malting plant or large brewery/distillery, for the determination of carbohydrates, proteins and moisture in grains. On top of all of this, sensory analysis should be much in evidence, sensory sampling being conducted at key stages during the manufacturing process.

Whether small-scale or large-scale, for consistent production of quality products, irrespective of the range or sophistication of analytical monitoring methods, the common denominator is taking all necessary steps to ensure quality and doing nothing – absolutely nothing – to compromise this.

Quality has been mentioned many times already, but what does it mean? It is necessary to adopt an objective definition of quality, since subjectivity can be unhelpful and even misleading. Consider the situation where a particular brewery has been producing a beer that has sold well over a number of years (i.e. it is one that many people like). Consumers of that beer will come to expect the presence of certain pleasing key characteristics in future purchases of the same brand of beer, in which case quality can be defined as the closeness of match between expectation and reality. Built into this definition is the idea of consistency; an important factor to take care of in the production of 'all the year round' beverages, which includes everything, except vintage wines (and certain fortified wines) and vintage or single cask spirits.

Absolute consistency (from year to year) should not be expected for the latter kinds of drinks, especially vintage wines; instead something approaching this can be found in nonvintage blends. But even here, consumers of a vintage wine would expect consistency from bottle to bottle of the same vintage, provided the wine is not of an ancient vintage, in which case bottle quality variation can be enormous. Additionally, a wine of two different vintages, say Chateau Latour 1960 and 1961, should still be recognizable as products of the same source. In the example above, the wine of the former vintage will be lighter (in all senses) and less well balanced than the 1961 wine; its quality will obviously not be as high, but it should still be a quality product and it should have features recognizable as a product of Chateau Latour.

There are many such ways of defining quality (Bamforth, 2002), but there is no universal way of measuring it and expectations can be quite different for different versions of the same drink. For example, drinkers of Hefeweissen in southern Germany or cask-conditioned ciders in England expect a cloudy drink, whereas drinkers of pale ale, Pilsner beer (and most other beers) or factory cider expect their drinks to be bright.

The commitment to quality pervades throughout a good company from top management to process operators and cleaners. This is sometimes known as total quality management (TQM); simpler for smaller outfits employing a few people and rather more exacting for larger concerns, where coordination and communication are of special importance. It can be achieved by implementing international standards such as ISO 9000 or simply by the adherence to a predetermined and agreed set of protocols and procedures.

The major advantage of ISO 9000 (or similar) accreditation is that it indicates quality consciousness in the company for all to see; companies of like mind will do well to trade with each other. For example, a quality minded winery may buy all its finings from an ISO accredited supplier, and a brewery or distillery may purchase all its malt from an ISO accredited malting company.

There are two basic approaches to quality management in a company, although both require the application of monitoring procedures at various stages during the manufacturing process. Quality assurance (QA) requires the setting up of process specifications all the way along the production stream (from raw materials to finished product) and the implementation of monitoring methods and programs with which to check these. The specifications should be realistic and will have been designed specifically for a particular product or process. The monitoring program will include protocols for recording, disseminating and archiving the information. Quality control (QC) is a regime that is more orientated toward 'reaction to circumstances,' and as such can often be associated with waste and hence expense. A good company will operate a regime that is geared more to the QA side of things, although such regimes are often recorded generally as quality control in the literature.

Monitoring is the process of checking specification parameters at key points in the process, sometimes manually, as in small companies, or sometimes with in line sensors with feedback facilities, as in some larger concerns. Either way, the QA protocol will be clear on what is being monitored, by which method, how frequently and by whom. The QA personnel collect the results, sometimes process them and act upon any indication that part of the process is tending toward an 'out of control' situation; the particular process parameter is heading toward rejection values (Figure 4.1.1). In such a situation, remedies will be put in place to prevent the parameter value from reaching a reject level or the process will be stopped, checked and restarted. It may seem that temporary closure of part of the process is a rather drastic step, but this is infinitely better than allowing an out of specification and possibly defective product to reach the market place.

The kind of tolerance associated with a process parameter depends on how precisely it can be measured, which in turn depends upon the amount of variability associated with measurement of that parameter. A high precision parameter is one whose variation is small compared with the specification value; monitoring of this type of parameter is performed with a narrow tolerance. Typical parameters of this type are acidity (winemaking), clarity or haze value (brewing and winemaking), color (brewing and winemaking; sometimes distilling), density (brewing, distilling and winemaking), diacetyl content (brewing), gas (dissolved CO_2 and O_2) content (brewing and winemaking), temperature (brewing, distilling and winemaking) and viscosity measurement (distilling).

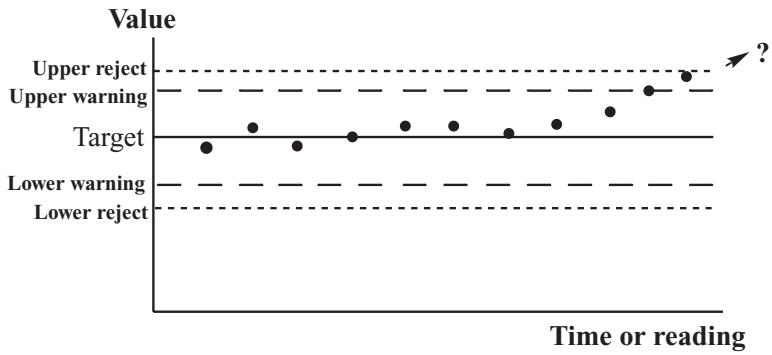

Figure 4.1.1 *Control parameter monitor, showing tendency toward out of specification situation*

Conversely, low precision parameters can usually be monitored with wider tolerances, because of greater natural variability. These parameters include dimethyl sulfide (or its precursor) levels (brewing), phenolic content (winemaking) and the levels of certain congeners (distilling).

Table 4.1.1 displays the parameters that are typically monitored in breweries, distilleries and wineries, but it should be remembered that some producers monitor fewer parameters than these, yet others will include more.

The overall trends in the application of analytical chemistry to this and other areas of alcoholic beverage production include miniaturization of instrumentation, faster method development, simplification and speeding up of sample preparation, increased sample throughput and faster data processing. If more replicate

Table 4.1.1 *Selection of parameters suitable for monitoring during production processes*

Parameter	Examples of typical specifications	Reasonable tolerances (±)
Acidity	<45 μg/l (beer)	1 μg/l
	6 g/l (wine grapes) as tartaric acid	0.1 g/l
Clarity/haze	<50 FTU (0.5 EBC) (beer)	10 FTU (0.1 EBC)
	<2 NTU (red wine)	0.1 NTU
Colour	1.6 ASBC (Pilsner malt)	0.2
Density	Sugar content: 1045 (expressed as OG, beer)	0.5
	12.5% ABV (wine)	0.2
	70% ABV (pot still run off)	0.05 (ultrasound)
Diacetyl	0.1 mg/l (normal beer)	0.01 mg/l
	0.2–0.5 mg/l (wheat beers and some others)	
Dimethyl sulphide (DMS)	40 μg/l (lager)	10
	<20 μg/l (ale)	
Dissolved CO_2	4 g/l (beer)	0.1 g/l
Dissolved O_2	<0.5 mg/l (beer)	0.1 mg/l
SO_2 (free)	<30 mg/l (beer)	1 mg/l
	50 mg/l (white wine with more than 5% (w:v) sugar)	
Temperature	65.5 °C (mashing)	0.1 °C
	12.0 °C (white wine fermentation)	
Viscosity	350 cP (peak viscosity for distillery malt)	10

analyses can be carried out in a fixed time, measurement precision will be increased, thus helping process control. One example involves the use of temperature sensors (linked to a computer with appropriate software; see Section 3.1.2) in stills of large distilleries to monitor the concentration of ethanol in the distillate, as a quality assurance parameter of the spirit. Such sensor systems allow simultaneous monitoring of several distillations, where conventional specific gravity methods would be more difficult, slower and less accurate, especially if there are few process still operators present (Ossorio *et al.*, 2008).

Another example is the use of fast GC to monitor beer flavor compounds (Horák *et al.*, 2009). Short, narrow bore columns (10 m × 0.18 mm, as opposed to conventional columns, such as 25 m × 0.32/0.53 mm) reduced the sample run time by 60%, without compromising resolution.

4.1.3 Identification of Origin, Determination of Authenticity and Detection of Fraud

Fraud is defined as the deliberate production or trading of merchandise that is not true to claimed origin or type. Fraud has existed for as long as alcoholic drinks have been produced, although up to the twentieth century many practices were carried out that were not considered to be fraudulent at that time, since most of the antifraud and protection of origin laws were not formulated until the twentieth century. For example, until well into the nineteenth century, it was common practice to openly 'fill out' the wines of Bordeaux and Burgundy with stronger, fuller and cheaper wines of the Rhône Valley and further south, since there was no law against it. Similarly, wine must was sometimes heavily sugared to boost the alcohol content resulting from fermentation and some wines were mixed with brandy and/or herbs and spices before shipping. Others were blended with elderberry juice to increase the strength of color.

On the other hand, the practice of adding wine spirit to grape must, or fermenting wine, was thought by many in the eighteenth century as fraudulent, yet today, such practice is designated as an essential part of the production process of fortified and liqueur wines (Chapter 2.10). A number of other practices that at one time were considered dubious, if not fraudulent, have become in the fullness of time accepted parts of certain manufacturing processes, sometimes resulting in new or changes in regulations. For example, the use of oak chips in place of oak casks in the production of wine is allowed for many different types of wine, but would be a fraudulent practice in the production of Scotch whisky.

Antifraud laws did exist before the twentieth century, but they were comparatively few and left much room for the manipulators, blenders and counterfeiters. A famous early piece of legislation regarding beer production is the Reinheitsgebot, imposed by Duke Wilhelm of Bavaria in 1516 to ensure that commercial beer in that country was brewed with water, malted barley, hops and yeast only. The law is still in force today for all German domestic beers, excepting wheat beers and rye beers.

Toward the end of the nineteenth century and into the twentieth century, greatly increased legislation in all countries and in all areas of alcoholic drinks production and sales led to much more clearly defined regulations regarding the designation of origin, classification, production processes (including allowed additives or adjuncts) and labeling of alcoholic drinks. At the same time, analytical methods for discovery of fraud improved enormously and policing the regulations became more effective. Thus, much of the open blending, adulteration and general manipulation of wines in the eighteenth and early nineteenth century became illegal and such wines became fraudulent, except in clearly defined cases. Consequently, nowadays there are fewer fraudulent beverages on the market than in previous centuries, but fraud does surface from time to time, much of it merely relatively minor infringements of regulations, although there are still occasional counterfeits of rare and expensive bottles of wine and spirits.

Regulations exist for all commercial alcoholic beverages and in all countries that produce them, although these often differ for a particular drink from country to country. These regulations also naturally vary from drink to drink, but generally include stipulations regarding specific geographic origin (for most wines and

spirits), acceptable cultivars (for many wines, Cognac, Tequila), methods of viticulture, including maximum yields (for many wines), limit of sugar enhancement (for wine – chaptalization – and some cider), minimum alcohol levels (as for most wines and spirits and some beer categories), production practices, including use and levels of additives (for all alcoholic beverages) and maturation (for most spirits), and labeling (for all alcoholic drinks).

Fraud nowadays falls into two main categories; label fraud and adulteration fraud. Adulteration here is defined as the addition of extraneous substances against regulations and does not include the blending of like beverages or additions of sugar or water. In a sense, most drinks frauds are label frauds – the bottle content is not what the label claims – but the most serious kind is counterfeit, because of the large sums of money that are usually involved. Label fraud ranges from the extreme, the completely bogus, to simple infringements of regulations. The first type can involve large-scale counterfeit of a well known, but rather scarce and expensive product, such as the Tuscan red wine Sassicaia (Frank, 2007) or small-scale counterfeit of extremely rare, very old and possibly unique wines that no person alive today has tasted, as alleged in the case of the Jefferson bottles (Keefe, 2007). Here, the authenticities of a number of Bordeaux wines of late eighteenth century vintages that were supposed to have been bottled for Thomas Jefferson and were engraved with his name, were put into serious doubt, the buyer of four such bottles (at *ca.* $100 000 each) claiming they were fakes.

Examples of infringements of regulations (excluding adulterated drinks) include dilution of most types of wine or must with water (and overdilution of spirits), wine with too little of the named grape variety in its composition (e.g. an Oregon Pinot Noir wine with less than the required minimum of 90% wine of that variety), a Chambertin sold as the 1985 vintage, but containing 20% of wine from 1984, wine made from oversugared must (e.g. a Premier Cru Chablis of 12.5% ABV made from grapes with sugars giving potential alcohol of only 10% (v:v) instead of the required 11%), Asturian cider made with local apple juice concentrate and a German Bockbier brewed using wort of only 15 °Plato original gravity, instead of the 16 °P minimum. These are only hypothetical examples of how infringement of regulations might result in fraud, although fraud similar to these is uncovered from time to time, mostly involving wine (see for example Prial, 2001; VinoWire, 2008).

In practice, fraud of this kind may go unnoticed for some time, since organoleptic detection is often quite difficult, although tasting may arouse suspicion in the first instance. Detection usually comes from either the leaking of inside information, from the finding of direct evidence, from the results of analytical investigations or from a combination of these.

Analytical methods fall into two major categories; those that determine a specific beverage component, such as ethanol or water, and those that compare data profiles (or 'fingerprints') of different beverages.

In the first category, analysis of isotope ratios by site specific isotopic fractionation-NMR (SNIF-NMR) (Section 4.4.1) and isotope ratio mass spectrometry (IRMS) (Section 4.4.5) are especially useful tools in the detection of fraud, via determination of the origin of specific components. In particular, these techniques can be used to detect a number of illegal processes: dilution with water, addition of excessive sugars to unfermented must, addition of ethanol from other sources to distilled spirits, presence of exogenous CO_2 in sparkling wine claiming to be 'Méthode Traditionelle' and addition of exogenous glycerol to wine. These methods are based on the fact that certain isotope ratios (e.g. H/D, $^{12}C/^{13}C$, $^{16}O/^{18}O$) of key components (e.g. water, ethanol) depend on whether those components originate from the natural base of the beverage or have been added from some other source.

SNIF-NMR has been used extensively in the detection of fraud and the authentication of wine origin, usually by measurement of $^{2}H/^{1}H$ ratios at the methyl and methylene sites of ethanol, after it has been distilled from the wine (Ogrinc *et al.*, 2003). IRMS has been used in similar ways, often in combination with SNIF-NMR (Ogrinc *et al.*, 2003). It has also been used to analyze the type of sugar (beet, cane or corn) used to produce the bubbles (carbon dioxide, via secondary fermentation – see Section 2.9.3) in sparkling wines (Martinelli *et al.*, 2003).

There are several studies of isotope ratios in the literature using GC-IRMS, in either combustion or pyrolysis modes, including an early one on Tequila authenticity (Aguilar-Cisneros *et al.*, 2002). Here, combustion mode was used to determine $\delta^{13}C_{VPDB}$ values of ethanol and pyrolysis was used to $\delta^{18}O_{VSMOW}$ values of ethanol in authentic and commercial Tequila. The method was able to distinguish between the two gross classes of Tequila, but not between subclasses (see Section 3.5.5) within these. See below for Tequila authenticity determined by another method. See Section 4.4.5 and Glossary for explanations of VPDB and VSMOW.

In the second category, a common procedure is to determine a number of beverage constituents simultaneously and the 'fingerprints' are used to decide authenticity by comparison against authentic samples, via the use of a variety of statistical chemometric methods (see Section 4.1.5), mostly discriminant function analysis. Typical techniques are those that are rapidly and easily applied, such as fast GC/GC-MS (Section 4.3.2), ion chromatography (Section 4.3.3), infrared spectroscopy (Section 4.4.2), Raman spectroscopy (Section 4.4.2), electrochemical methods (Chapter 4.5) and inductively coupled plasma mass spectrometry (ICPMS) (Section 4.4.4).

As an example, the four subclasses of Tequila and mezcal have been differentiated on the basis of chemometric analysis of the concentrations of 12 elements determined by ICPMS (Section 4.4.4) (Ceballos-Magaña *et al.*, 2009), where probabilistic neural networks analysis gave 100% success in the differentiation of extra aged, aged, gold and silver Tequila, and mezcal.

However, some workers have used univariate statistical methods to correlate data with sample origin/authenticity, as in the differentiation between subgroups of Mexican Agave spirits (including Tequila) by application of one way analysis of variance (ANOVA) to GC and ion chromatographic data (Lachenmeier *et al.*, 2006). It may also be possible to differentiate between spirits (e.g. unaged cachaça, aged cachaça and rum) by comparison of aroma 'fingerprints' obtained from a two dimensional GC procedure, such as GC×GC/TOFMS (Cardeal and Marriott, 2009).

Adulteration is defined here as the illegal addition of extraneous substances (those that are not normally present in the beverage or its raw materials). It includes addition of colorants, flavoring substances and sweeteners, but does not include the fruits and 'botanicals' used to flavor beer (Section 2.6.13), vermouth, other aromatized wines (Chapter 2.12), gin (Section 3.4.2), vodka (Section 3.4.3), arak (Section 3.5.6) and liqueurs (Chapter 3.9). Nor does it include the use of caramel as a colorant in the production a wide range of alcoholic beverages, nor the use of artificial sweeteners in the production of some cider (Section 2.8.6) and certain spirits, such as some soju (Section 3.4.4). The many cases where these additions can be made are clearly defined by law, but are otherwise illegal; they are then classed as adulterations. Governments all over the world have constructed regulations and published lists of chemicals and substances that may be legally added to wine and other drinks (Robinson, 2006), and increasing regulatory requirements will mean that, for some of them at least, their presence must be stated on the label.

A common and long standing adulteration of wine is the addition of deep red juice or fruit wine (e.g. those of elderberry), or even an artificial colorant, to pale red grape wines; deepening the color of red wine is a profit enhancer, since the public generally equates deep color with high quality. This kind of adulteration is not so common nowadays, because once suspected, it can be detected easily, especially by HPLC with UV-visible detection (Section 4.3.3). Nowadays, using such a technique it is possible to determine all common artificial colorants in drinks in less than 20 minutes chromatography run time (Yoshioka and Ichihashi, 2008). Likewise, the presence of anthocyanins from the skins of elderberries or blueberries in red grape wine is easily demonstrated by this technique, since anthocyanins tend to be characteristic of their origin (see, for example, Jordheim *et al.*, 2007).

Unfortunately, toxic substances have been used to adulterate wines throughout the years; lead acetate was occasionally used as a wine sweetener in the eighteenth and nineteenth centuries, and even as late as 1986, a particularly unfortunate case of adulteration occurred in Italy, when 23 people died as a result of consuming wine adulterated with methanol to boost its alcohol content.

However, the biggest recent wine adulteration scandal was that concerning diethylene glycol. In 1985, several Austrian wine producers, as well as smaller numbers of German and Italian producers were found to have added diethylene glycol to dry wines in order to give them a smoother mouthfeel and greater sweetness, allowing the producers to sell their wines at higher prices. Fortunately, the diethylene glycol was present in concentrations that were too low to be toxic, but the fraud darkened the reputation of all Austrian wine for many years. Diethylene glycol is readily detected by gas chromatography (Section 4.3.2), but in the 1980s planar chromatographic methods were much in vogue (see, for example, Klaus and Fischer, 1987).

4.1.4 Determination and Characterization of Beverage Components and Processes

Many of the hundreds of compounds known to be present in alcoholic beverages are crucial to the quality of those beverages. Many of these compounds are already present in the raw materials or precursors, but many more are derived from the various production processes, such as fermentation, boiling/distillation, maturation (especially in wood), pasteurization and so on. Moreover, composition inevitably changes during the manufacturing process, with some of the original components rapidly or slowly disappearing to trace level (e.g. being metabolites of fermentation, or as a result of precipitation) and others appearing at various later stages (e.g. fermentation metabolic end products or compounds derived from contact with wood).

The balance of components is very important; too much or too little of certain flavor compounds can profoundly influence beverage quality. For example, the presence of dimethyl sulfide is unwanted in pale ales and other top-fermented beers, but at certain levels, is beneficial to the flavor of pale bottom-fermented beers (Sections 2.6.5 and 2.6.6). Likewise, certain flavor compounds are desirable in one beverage, but not in others – for example β-(*E*)-damascenone is generally desirable in white wine, but not in beer. The balance of many other components can have an effect on the quality of beverages in other ways – for example: proteins and certain carbohydrates (β-glucans) on clarity and foaming characteristics of beer, cider and wine; metal ions on oxidative stability of beer; phenolic compounds on color, taste and mouthfeel of cider and wine; organic acids on the taste of beer, cider and wine. Analytical chemistry, often combined with sensory analysis, can determine key quality factors and can point the way to making changes in the production processes that maximize desirable factors and minimize undesirable ones.

Although it is 'tasting,' or more properly organoleptic or sensory assessment (using sight, smell/flavor, taste and mouthfeel in a scientific way – see Chapter 4.7) that is ultimately used to determine the beverage quality, analytical chemistry plays a crucial role in identifying favorable and unfavorable components, as well as shedding light on the origins of those components and on the huge variety of pathways that produce or degrade them during the course of many different kinds of production processes. Furthermore, analytical chemistry is important in the detection, determination and characterization of components that do not necessarily influence quality, but may have health implications. These include additives such as sulfites (Section 5.9.2), contaminants such as heavy metals (Section 5.10.3) and toxic fungal metabolites (Section 5.11.4).

Table 4.1.2 gives a list of the types of compounds most often determined by analytical methods, along with some examples of the contexts in which these they are analyzed. The list by no means exhaustive; indeed it is fair to say that any component may be the subject of analysis if its presence has (or is suspected of having) some bearing on beverage character or quality.

Analytical investigations include the following broad types. Firstly there is quantitative or semi-quantitative determination of known components. These methods need internal or external standards (for direct determinations) or calibration models (for indirect determinations) and involve the whole spectrum of analytical methods, from chromatography and spectroscopic methods to physicochemical methods. Increased chromatographic resolution can be achieved using one dimensional GC with rapid mass spectrometry and deconvolution or by two dimensional techniques (e.g. GC×GC) (see, for example, Cardeal and Marriott, 2009)

Table 4.1.2 *Some important components of alcoholic beverages and the major analytical methods used for determination or identification*

Component(s)	Raw material/ precursor/beverage	Method of determination/ identification	Contexts
Acids	Mostly must (for wines and spirits) and wine	Physicochemical methods. CZE, HPLC, UV-vis spectroscopy (inc. enzymic methods)	Total acidity. QA/QC, assessment of MLF progress
Alcohols (inc. ethanol and polyols)	All beverages	GC, FTIR, physicochemical methods, IRMS/SNIF NMR (for isotope ratios)	QA/QC, flavor analysis, detection of fraud
Amino acids, peptides and proteins	Cereals, wort (for beer and spirits), must and wine, cider	Kjeldahl, Dumas, etc., FTIR. HPLC, UV-vis spectroscopy, CZE. HPLC, GPC, SDS-PAGE, MS	Total nitrogen content. Nutritional/health value, potential haze formation, foaming ability, production processes
Biogenic amines	All	GC, HPLC, CZE	Health issues, production processes
Carbohydrates	All, except pure distillates	FTIR, physicochemical methods. HPLC, GPC, enzymic methods (UV-vis spectroscopy), NMR, MS	Total carbohydrates. QA/QC, fermentability, health/nutrition, production processes
Esters	All beverages	GC	QA/QC, flavor analysis, production processes
Metal and nonmetal ions (inc. heavy metals and sulfites)	Most, inc. water for brewing and spirits dilution	AAS/AES, HPLC (IC), CZE, electrochemical methods, UV-vis spectroscopy, ICPAES/ICPMS	QA/QC, health issues/regulations, spoilage, bioavailability
Organic contaminants	All	GC, HPLC	Health issues/regulations, production processes
Phenolic compounds (inc. anthocyanins)	All	UV-vis spectroscopy, FTIR, HPLC, NMR, MS	Total phenolic content. QA/QC, sensory analysis, production processes
Sulfur compounds	Most	GC, HPLC	QA/QC, aroma/flavor analysis, spoilage
Terpenoids, carotenoids, norisoprenoids	All	GC, HPLC, NMR, MS	Flavor analysis, production processes
Water	As moisture in cereals In beverages	FTIR IRMS/SNIF	QA/QC. Authentication of origin/detection of fraud

KEY: GC (inc. GC-MS^n) (Section 4.3.2) HPLC (inc. LC-MS^n, IC and GPC) (Section 4.3.3); NMR/SNIF NMR (Section 4.4.1), FTIR (Section 4.4.2), UV-vis (Section 4.4.3) AAS, AES, ICPAES (Section 4.4.4), MS/IRMS (Section 4.4.5); electrochemical methods (Chapter 4.5); CZE, SDS-PAGE (Section 4.6.1), Kjeldahl, Dumas, physicochemical methods (Section 4.6.3). Production processes: examples include malting, mashing, boiling, distillation, fermentation, maturation, pasteurization/heating, fining/filtration.

(Section 4.3.2). Retention time locking is now being used to aid component identification, and quantification is often helped by the use of isotopically labelled internal standards (see, for example, Poisson and Schieberle, 2008; Cooke *et al.*, 2009; Grant-Preece *et al.*, 2010). GC separations are often used in conjunction with sensory analysis in order to correlate organoleptic properties with particular components or combinations of components (see generally Sections 4.3.2 and 4.7.3 for examples of GC-olfactometry and GC-aroma extraction dilution analysis).

Many sophisticated extraction methods are now available for the concentration (focusing) of trace components (Chapter 4.2), allowing their investigation by less sensitive techniques such as NMR spectroscopy. There are many statistical chemometric methods available, as computer software, for the design of experiments and for treatment of results. Multivariate analysis methods, in particular, are being used with rapid analytical methods, such as FTIR spectroscopy (Section 4.4.2) and electrochemical methods (Chapter 4.5) for the routine analysis of large numbers of samples. Electronic noses and tongues are being increasingly used in the classification/identification of origin of samples and miniaturized sensor devices are finding increased use as process monitors.

Secondly, there is the identification of newly discovered components. Sometimes comparison of chromatographic retention times with those of authentics, use of Kovats/other indexes or coinjection of authentic standards may be sufficient, but often structure determination is required. In this case, the two prominent methods are NMR spectroscopy and/or mass spectrometry. Nowadays, a multitude of NMR techniques, involving different pulse regimes, can be applied to solve highly complex structures, including terpenoids, oligomeric polyphenols, their glycosides and carbohydrates (Section 4.4.1). Techniques such as (homonuclear) correlation spectroscopy (COSY), heteronuclear correlation spectroscopy (HETCOR), homonuclear multiple bond coherence spectroscopy (HMBC) and heteronuclear multiple quantum coherence spectroscopy (HMQC), allow H–H and C–H connectivities to be determined and nuclear Overhauser effect spectrocopy (NOESY) gives information on near through space neighbors.

Likewise, mass spectrometry (Section 4.4.5), particularly if using atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) modes of ionization and often combined with chromatographic separation (e.g. HPLC; Section 4.3.3) or with capillary zone electrophoresis (CZE) (Section 4.6.1), can be used to determine large or complex molecules. Tandem mass spectrometry (MS^n) is more and more frequently used to give detailed fragmentation information and TOFMS lends itself to the determination of molecular weights of proteins and other large molecules.

Thirdly, there is the investigation of physicochemical processes involved in alcoholic beverage production, including metabolic and nonenzymic pathways. This includes following changes in composition that occur during malting/mashing, fermentation, distillation, heating (including pasteurization and the Maillard reaction), fining, maturation/ageing (including extraction processes from wood, redox reactions and the effects of light and other types of radiation). Investigations of fermentation have often been carried out using isotopically labeled substrates, the fate of the label being determined by either mass spectrometry (often GC-MS or LC-MS) or NMR spectroscopy. In this way, the origins of important components can be elucidated and important precursors can be determined. A recent example of this involved the use [^{13}C6] and [^{12}C6] glucose in a study of the Maillard reaction (see Section 2.6.2) between glucose and various amino acids (Wang *et al.*, 2009). One major pathway was shown to exist for the production of 2,5-dimethyl-4-hydroxy-3[2*H*]-furanone, in which the glucose carbon skeleton remained intact, whereas there were two routes to 2-acetylfuran, one involving glucose only and the other involving both glucose and glycine.

Similarly, analytical science has been directed toward physical and chemical processes that influence sensory perception of drinks. For example, mechanisms of the release of aroma compounds from beverages such as wine during dynamic headspace dilution conditions (similar to normal sniffing conditions) have been investigated by APCI-MS (Tsachaki *et al.*, 2009). Likewise, sensory analysis combined with analytical

chemistry has been used to investigate the release of odorous cysteine-*S*-conjugates from fruits by saliva enzymes (Starkenmann *et al.*, 2008).

4.1.5 Development of New Analytical Methods

There exist many prescribed methods in the literature for the analysis of important parameters and key components of alcoholic beverages and their raw materials (e.g. grapes, fruit, malted barley, water) or precursors (e.g. beer wort). They are known variously as established, official, recommended or reference methods (with quantitative methods predominating) and are published by a number of professional bodies (Table 4.1.3) from time to time, with updates and additions. The aim is to ensure that the determination of a

Table 4.1.3 *Scientific associations providing standard methods of chemical analysis of alcoholic beverages*

Association or Institute	Comments	Association or Institute	Comments
AACC American Association of Cereal Chemists	Provides standard methods for analysis of cereals	IBD Institute of Brewing and Distilling	Publishes recommended methods for cereal, wort, beer and spirit analysis
AOAC Association of Official Analytical Chemists	Publishes recommended methods for assay of many analytes in different matrices, inc. food and drink	ICC International Association for Cereal Science and Technology	Provides standard methods for analysis of cereals
ASBC American Society of Brewing Chemists	Publishes standard research methods for all aspects of beer	ISO International Organization for Standards	Includes many member bodies worldwide – e.g. AFNOR (France), ANSI (USA) and SABS (South Africa). Main interest is in standardization of methods
ASTM American Standards for Testing and Materials		JECFA Joint Expert Committee on Food Additives (FAO/WHO)	Provides scientific expert advice and publishes data and methods for food additive assay
Codex Alimentarus (FAO/WHO)	Main concern is with food and health, especially regarding additives and residues	JMPR Joint Meeting on Pesticide Residues (FAO/WHO)	Publishes toxicological data and references to methods of analysis
EBC European Brewery Convention	Publishes Analytica-EBC series on all aspects of brewing	OIV Office Internationale de la Vigne et du Vin	Publishes codes of enological practices, as well as methods for analysis of wines and spirits
EC European Commission	Joint Research Centre publishes methods for assay of food components, especially additives and residues	RACI Royal Australian Chemical Institute	

particular component is carried out in a particular way, so that results from year to year or between brewery, distillery or winery laboratories can be compared directly (i.e. the results have both high repeatability and high reproducibility), with a high degree of confidence. Some of these associations also publish recommendations regarding evaluation and development of alternative methods against a reference method (e.g. AFNOR, 1999).

Official methods have been carefully and thoroughly constructed for particular kinds of analyses for particular ranges of alcoholic drinks, where development of the methods was usually conducted by well respected experts (sometimes over a period of years), although they are often modified from time to time, as problems or suggested improvements come to light. However, these methods have a considerable range of advantages and disadvantages. Some involve lengthy and labor intensive steps, others lack precision (even though they may be regarded as the best available at the present time for particular analytes) or accuracy (i.e. they may have a bias) and others may lack robustness (i.e. they are susceptible to slight changes in conditions).

As in other areas of analytical science, there is a perpetual search for new methods, not necessarily to replace official methods, but often to provide alternatives that can be used together with existing or official methods. A new method should offer improvements in accuracy, automation, cost, environmental aspects, precision, robustness, safety, speed, selectivity, sensitivity or versatility, without itself having significant disadvantages. All new methods should be rigorously compared with existing and/or official reference methods. In reality, a newly developed and tested method may be used in routine or day to day analysis, whereas for important instances (e.g. providing high confidence data for presentation in a fraud court case or for providing data for an official body such as the Institut National des Appellations d'Origine des vins et Eaux-de-Vie (known as INAO), the official or reference method is used.

Apart from the search for new and improved methods to determine an individual component or parameter *directly*, as discussed above, there is much activity aimed at predicting one or more parameters *indirectly* using other more easily and rapidly measured parameters, the two being correlated by statistical analysis. See Cozzolino *et al*. (2009) for a useful introduction to multivariate statistical methods applied to grape and wine analysis. More generally, the reader is referred to the Statsoft electronic textbook on statistics (http://www.statsoft.com/textbook/) and Cserhàti (2008), Otto (1999), Alfassi *et al.* (2005) and Brereton (2005) for application of statistical methods to analytical chemistry.

Typically, rapid methods such as visible/NIR, MIR, NMR (all Fourier transform) spectroscopic, fast chromatographic or electrochemical methods (especially electronic noses and tongues) are used to predict such parameters as percentage ethanol (v:v), protein content, total N content, glycerol content, Cu^{2+} content, or many others that generally require more time consuming methods to measure directly. The normal methodology is to use multivariate regression analysis to develop a calibration model that correlates the measurement information (e.g. peaks in particular regions of the NIR spectra) contained in the set to the property or parameter of interest (e.g. protein content). Usually, a different data set is then used to test the predictive ability of the regression model, or sometimes the integrity of the model is tested by 'cross-correlation' (see below).

The most commonly used statistical methods are those of the multivariate type, which considers multiple variables (e.g. pH, total nitrogen content, ethanol content, etc.) simultaneously. Of these, principal component analysis (PCA) and partial least squares (PLS) regression analysis (a kind of discriminant function analysis) are probably the two most widely used procedures.

PCA is the main exploratory method, reducing large data sets into linear combinations of variables that can be used to describe and predict some aspect of the data matrix. The results are typically presented as PCA score plots, often centered, with the axes crossing at the origin (Figure 4.1.2). As an example, Nieuwoudt *et al.* (2004) used PCA to correlate ethanol, glucose, glycerol, reducing sugar and volatile acidity contents data (determined by standard methods) with FTIR spectra of South African Chenin Blanc fermenting musts and synthetic musts. Improved PCA score plots were obtained when outliers were removed and certain wavenumber regions (5011–2970; 1716–1543 cm^{-1}) were deselected. Refinements such as this are often

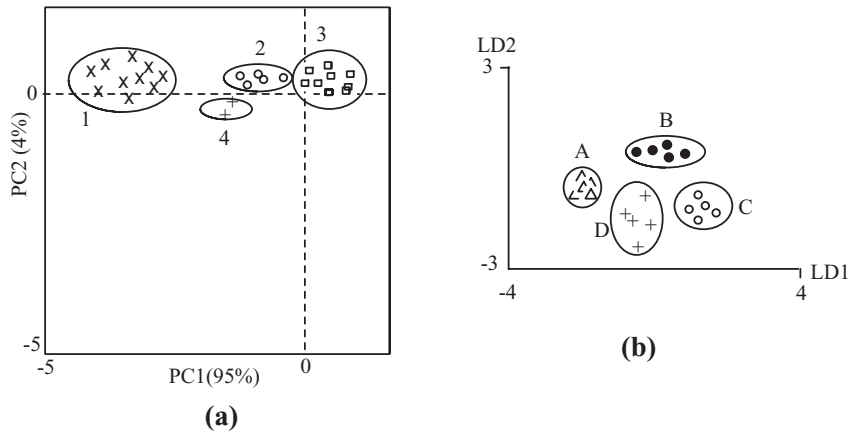

Figure 4.1.2 *Typical PCA (a) and PLS (b) disciminant analysis plots*

used to increase the effectiveness of PCA. See Section 4.4.2 for other examples of FTIR spectroscopy and statistical methods used in this and other ways.

Discriminant analysis gives a set of orthogonal (independent) linear discriminant functions (LD1, LD2, etc.) in order of discriminating aptitude (LD1 first, etc.), but in practice the first two are usually sufficient to describe most of the variation. Ideally, intragroup differences are minimized and intergroup differences are maximized, so that when LD1 is plotted against LD2, the points fall into distinct areas representing characteristic groups (Figure 4.1.2).

These functions are often used to predict group membership of new or unknown samples not used in the development of the original model (see also PLS, below), but if such samples are unavailable cross validation can be used. Here, data for each sample are removed sequentially from the model, whereupon the model is rebuilt without that sample. The categorization of the removed sample is then tested against the rebuilt model.

As an example, an electronic nose based upon 12 metal oxide sensors (Section 4.5.3) was able to discriminate not only between fruit and grape wines from different Ontario wineries, but also between certain different varietal grape wines (McKellar *et al.*, 2005). Here discriminant analysis was used to determine which sensor outputs (relating to the wine aroma profile) could differentiate between samples from several natural groups, which included (from 33 wineries) red and white grape wines from five different varieties and eight different kinds of fruit wines. It was possible to distinguish each wine variety based on different wineries, fruit and grape wines (75.9% correct), and red and white wines (92.2% correct), but overall classification by variety was poor (58.7% correct). The wines could be separated into four distinct groups (79.9% correct). See Section 4.5.3 for other examples of the use of sensor arrays in 'fingerprinting' beverage samples.

PLS regression is a discriminant analysis method that is used as a major qualitative calibration procedure in alcoholic beverage and raw materials analysis. Calibration is carried out using group membership (categories) rather than by use of a continuous variable. Typically, for two groups, A and B, an artificial Y-variable is created that has a zero value for say group A and a unit value for group B. A regression equation between Y and the X variable measurement is set up and is then tested on its ability to predict Y values of new or unknown samples. The magnitude of success is gauged by the standard error of prediction. PLS is a data reduction technique; it reduces the X variables to a set of noncorrelated factors describing the variation of the data.

As an example, Garde-Cerdán *et al.* (2010) used PLS analysis of NIR spectroscopic data to discriminate between Spanish red wines according to the extent of aging in oak casks and according to type of oak cask used: American, French or combination. This type of method could be used for the rapid assessment of

authenticity of new samples and hence in the detection of fraud (see Section 4.1.3). Another example involved the use of an electronic nose (zNose^{TM}; a fast gas chromatograph) to obtain aroma fingerprints of 339 Chilean red wines of known origin, of a number of grape varieties and vintages (Beltrán *et al.*, 2009). Here, the data was analyzed by application of wavelet transform for chromatogram feature extraction, and this was classified by analysis with support vector machines. The mean percentage of correct classification performed on the validation set was obtained by cross validation against the percentage of correct classification found on the test set. This is a good example of how a highly populated, accurate database leads to reliable classification; wines from four valley areas could be discriminated (>94% classification rates) and it was possible to discriminate certain wines from coastal and inland vineyards.

The major limitation of linear regression, PCR and PLS regression is their inability to deal adequately with nonlinear relationships between variables, although locally weighted regression can do this. An artificial neural network (ANN) is a multivariate data processing technique that simulates biological neurons by multiplication of the input signal (X), using the synaptic weight to derive the output signal (Y). This technique can handle nonlinear relationships between variables and hence outperforms standard PLS regression in these circumstances, its superiority arising from the high degree of interconnections.

As an example, capillary zone electrophoresis data on resveratrol levels in wines of eight varieties of three vintages when subjected to ANN allowed the prediction of vine varieties of unknown wines (but within the limits of the database), with a high (<90%) success rate (Pazourek *et al.*, 2005).

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4.2

Extraction and Focusing Methods in Sample Preparation

Logic is the last scientific ingredient of Philosophy; its extraction leaves behind only a confusion of non-scientific, pseudo problems.

—Rudolf Carnap

4.2.1 Overview

Apart from a few substances, the components of alcoholic beverages are present at very low levels (μg/l) or even trace levels (ng/l or less) and hence either require highly sensitive methods for their detection and quantification, or need to be concentrated (focused) by some extractive sample preparation technique prior to analysis. Highly sensitive methods are not always suitable and are often subjected to interferences from other components of the highly complex matrices that constitute most alcoholic beverages. This section considers the sample preparation techniques available for the focusing of specific groups of components in alcoholic drinks. The emphasis of the discussion is on volatile compounds responsible for the aroma and flavor of drinks, partly because of their special importance and partly because extractive sample preparation techniques for other analytes, such as non-volatile acids, amino acids, contaminants, mycotoxins, pesticide residues, phenols and others are mentioned elsewhere. Therefore, the analytical technique most widely discussed here in conjunction with the focusing techniques is gas chromatography (GC) (Section 4.3.2).

The quantitative analysis of the volatile compounds present in alcoholic beverages is extremely demanding due to: (i) the complex chemical composition of the volatile fraction and (ii) the fact that individual volatile compounds can be present in a wide range of concentrations. For example, Rapp *et al.* (1978) estimated that the concentrations of volatile flavor compounds in wines range between 1 g/l and 1 ng/l (a factor of 10^9).

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

The concentrations at which volatile compounds influence the flavor of foodstuffs are often extremely low – sometimes at concentrations below the detection limits of even the most sophisticated analytical instruments. It is therefore imperative that the volatile fraction of the alcoholic beverage be isolated from the bulk of the sample and subsequently concentrated (or focused).

The extremely low odor threshold values (OTV – concentrations just barely sufficient to achieve sensory recognition of substances – Section 4.7.2) of some of the more significant flavor compounds, demand that techniques be aimed at isolating these trace level compounds from other less sensory significant components, which may be present at concentrations several orders of magnitude higher. More than 99% of an alcoholic beverage extract is typically composed of fusel alcohols, fatty acids and some fermentation esters (Section 2.2.11), whereas the remaining 1% of the extract (which contributes significantly to the bouquet of a wine for example), is composed of hundreds of compounds which are present at concentrations about 10^6–10^8 times lower than the fusel alcohols (Maarse and Visscher, 1989, Etievant, 1991). The human sensory organs (Section 4.7.1) display somewhat sensitive and variable reactions to these amounts of aroma compounds. Boeckh (1972) and Guadagni *et al.* (1963) reported that threshold values differ considerably and could vary between 10^{-4} and 10^{-12} g/l. For example, Demole *et al.* (1982) quoted the odor threshold value for 1-*p*-menthen-8-thiol as 0.1 ng/l, whereas Guth (1997) gave the value of 0.00001–0.00004 ng/l for wine lactone. Therefore, methods for alcoholic beverage volatile analysis must be able to successfully qualify and quantify compounds over a wide range of concentrations. There are several main sample preparation techniques that have been used to study the aroma compounds of grape juice, wine, beer and spirits. These techniques fall into four general categories which include liquid extraction (or solvent extraction including supercritical fluid extraction or SFE, Section 4.2.2) distillation methods (Section 4.2.3), such as simultaneous steam distillation-extraction (SDE) and solvent assisted flavor evaporation (SAFE), the sorption techniques (Section 4.2.4) solid-phase extraction (SPE), solid-phase micro extraction (SPME), stir bar sorptive extraction (SBSE) and headspace sorptive extraction (HSSE), and headspace methods (including static headspace and purge and trap, Section 4.2.5). It is also very common to combine such sample preparation techniques to obtain alcoholic beverage aroma isolates of which SDE strictly speaking also fits (as it is a combination of both distillation and liquid extraction). Other combinations used in alcoholic beverage analysis also include the chromatographic methods such as a combination of liquid–liquid extraction and high performance liquid chromatography (HPLC) fractionation (Section 4.3.3), a combination of liquid–liquid extraction and countercurrent extraction (Section 4.3.4), a combination of liquid–liquid extraction and solid phase extraction using column/adsorption chromatography (for creation of aroma fractions) and also as a final example, a combination of solid phase extraction and HPLC fractionation.

Research of the available literature over the past five decades indicates that solvent extractions are among the most commonly used sample preparation steps for the analysis of beverage volatiles. However, recent environmental concerns over the use of chlorofluorocarbons such as freon-11 (trichlorofluoromethane, CFC-11, or R-11) have resulted in limitations in the availability of these solvent varieties for research purposes. Other solvent systems are now more commonly employed. However, increasing costs for solvent disposal, as well as safety and environmental concerns, are prompting research and industrial analytical chemists to search for sample preparation methods that minimize or better still eliminate the use of organic solvents. Hence, the sorptive extraction techniques have gained a surge in popularity, mainly because of advantages in sensitivity, speed, robustness, safety and 'greenness' over previously established methods.

4.2.2 Liquid Extraction Techniques

Liquid extraction techniques are based on the distribution of a solute between two immiscible solvents. In most cases, one phase is an aqueous solution of analytes (for example volatile aroma/flavor components) the

other an organic solvent. An analyte which is soluble in both water and solvent phases will distribute itself between these two phases. Equilibrium will be achieved when the free energy of the solute is the same in each phase. The extract is obtained by stirring or mixing and agitating a liquid sample with organic solvent and then separating the subsequent solvent phase. This can be carried out manually (simple extractions with a separating funnel) or automatically and can be either batch extraction or continuous flow extraction (commonly known as continuous liquid–liquid extraction).

The choice of extraction solvent is extremely important, as it must have a low enough boiling point so that it can be easily removed from the extract without a significant loss or change of the original sample aroma volatiles. Ideal extraction solvents must be able to extract polar (such as alcohols and carbonyl compounds) and non-polar (such as terpenoids and esters) components and provide an extract with an aroma/flavor profile reminiscent of the original sample (an important prerequisite of any isolation technique applied to the study of aroma/flavor profiles).

Two terms are employed to describe the distribution of a solute between two immiscible solvents: *distribution (partition) coefficients* and *distribution ratio.* These theoretical concepts have been widely covered from first principles in the literature (Skoog *et al.*, 1996; Holden, 1999; Meloan, 1999).

When the distribution ratio is low, but the separation factor is high, continuous methods of extraction are favored. This technique utilizes a continuous flow of immiscible solvent phase through the aqueous solution to be extracted. As volatile solvents are generally used for aroma analysis, this can be stripped and recycled via distillation and condensation. Although partition/distribution equilibrium may not be achieved during the limited time of contact of the two phases, solute is being removed continuously with the extracting phase and carried back to the solvent flask for subsequent concentration (by using for example a Vigreux column on a steam bath, a Kuderna–Danish evaporative concentrator or rotary evaporator following previous drying of the solvent extract over sodium or magnesium sulfate) and analysis by gas chromatography (Section 4.3.2). Efficiency depends not only upon the value of the partition ratio, but also the viscosity of the phases, the relative phase volumes (phase ratio), the area of contact between the phases and the relative velocity of the phases. As the extraction solvent passes through the solution being extracted, stirrers are used to bring the two phases into closer, more effective and prolonged contact.

Many continuous extraction devices operate on the same general principle (see Figure 4.2.1). This consists of distilling the extracting solvent from a round bottomed flask that serves as a boiler/receiving flask, condensing the solvent, and passing the condensate through the solution to be extracted that is held in an extractor vessel. A rotating distributor in the extractor vessel is driven by a magnetic stirring plate. The extractor solvent fed from the condenser to the distributor is centrifugally forced through the small holes in the distributor ring as fine droplets into the liquid to be extracted, producing optimum exchange of matter. The liquid to be extracted also rotates in the extractor. The rotary distributor on the inlet tube has a magnetic agitating rod, pivot nipple, bearings, antislip lock and separating ring with retaining ring. When the extracting solvent is lighter than the aqueous phase (see Figure 4.2.1, apparatus A) the rotary distributor (or dispersion disk) is placed near the bottom of the extraction vessel. During operation the solvent rises to the surface of the aqueous phase and in doing so allows the extractable analytes to partition between the droplets and the bulk solution. Eventually the extracting liquid flows back into the receiving flask (and boiler), from where it is evaporated and recycled. The extracted analytes remain in the receiving flask. For extraction solvents heavier than water (see Figure 4.2.1, apparatus B) the rotary distributor is placed towards the top of the extraction vessel. Here, the condensed solvent is allowed to drop down through the phase being extracted and then it flows to the outer part of the extractor. Spent solvent rises in the outer cylinder of the extractor and exits into the boiler for stripping and recycling.

Zurbrick (1997) reported a simple example of how analytes partition or distribute themselves between water and an organic extraction solvent. Below is a further example based on this calculation.

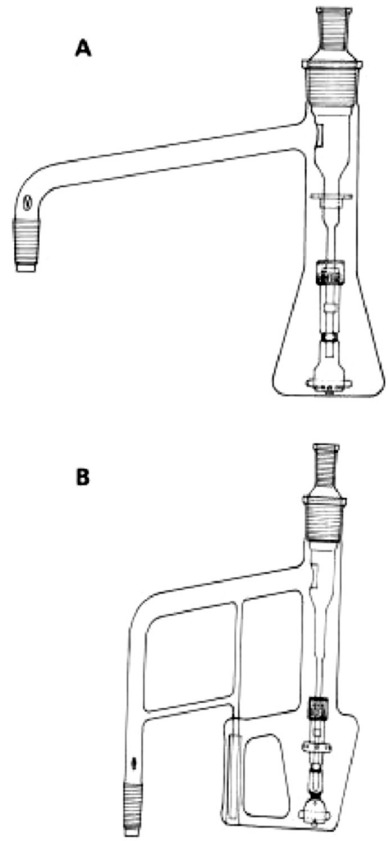

Figure 4.2.1 *Normag® liquid-liquid extractors (A) for extraction with solvents of lower density than water (B) for extraction with solvents of higher density than water. Reproduced with permission from Normag Labor- und Prozesstechnik GmbH, Ilmenau, Germany*

The solubility in water of compound A is 10 g/100 ml. The solubility in diethyl ether of compound A is 5 g/100 ml. From this solubility data, we can calculate firstly the partition (or distribution) coefficient (K_D) for compound A (Equation 4.2.1).

$$K_D = \frac{\text{Solubility of compound A in diethyl ether}}{\text{Solubility of compound A in water}} \tag{4.2.1}$$

This amounts to 5/10 or 0.5.

If we have 50 g of compound A in 1000 ml water and extract with 1000 ml of diethyl ether, compound A will distribute itself between the two immiscible solvents as follows:

$$\text{Weight of compound A in diethyl ether} = (1000\text{ ml})(0.5x\text{ g/ml}) = 500x\text{ g}$$
$$\text{Weight of compound A in water} = (1000\text{ ml})(x\text{ g/ml}) = 1000x\text{ g}$$

Therefore the total weight of compound A is 50 g (now distributed between two layers) and:

$$1500x \text{ g} = 50 \text{ g}$$
$$x = 0.03333$$

Hence:

$$\text{Weight of compound A in diethyl ether} = 500\ (0.03333)\ \text{g} = 16.67\ \text{g}$$
$$\text{Weight of compound A in water} = 1000\ (0.03333)\ \text{g} = 33.33\ \text{g}$$

This example highlights the distribution following a simple, single batch extraction as would be carried out using an ordinary laboratory separating funnel. To demonstrate the benefits of exhaustive extractions (either by multiple batch or continuous liquid–liquid extraction) if we again begin with 50 g of compound A in 1000 ml water and carry out three extractions with 300 ml of diethyl ether, after the first extraction compound A will distribute itself between the two immiscible solvents as follows:

$$\text{Weight of compound A in diethyl ether (300 ml) } (0.5x \text{ g/ml}) = 150x \text{ g}$$
$$\text{Weight of compound A in water} = (1000 \text{ ml})(x \text{ g/ml})\ 1000x \text{ g}$$

Therefore the total weight of compound A is 50 g (now distributed between two layers) and:

$$1150x \text{ g} = 50 \text{ g}$$
$$x = 0.04348$$

Hence:

$$\text{Weight of compound A in diethyl ether} = 150\ (0.04348)\ \text{g} = 6.52\ \text{g}$$
$$\text{Weight of compound A in water} = 1000\ (0.04348) = 43.48\ \text{g}$$

This diethyl ether layer is then removed and replaced with the next 300 ml of diethyl ether and so:

$$\text{Weight of compound A in diethyl ether} = (300 \text{ ml})(0.5x \text{ g/ml}) = 150x \text{ g}$$
$$\text{Weight of compound A in water} = (1000 \text{ ml})(x \text{ g/ml}) = 1000x \text{ g}$$

However, here we began with 43.48 g of compound A (now distributed between two layers) and:

$$1150x \text{ g} = 43.48 \text{ g}$$
$$x = 0.03781$$

Hence:

$$\text{Weight of compound A in diethyl ether} = 150\ (0.03781)\ \text{g} = 5.67\ \text{g}$$
$$\text{Weight of compound A in water} = 1000\ (0.03781)\ \text{g} = 37.81\ \text{g}$$

Finally, this diethyl ether layer is removed and replaced with the last 300 ml and so:

$$\text{Weight of compound A in diethyl ether} = (300\text{ ml})(0.5x\text{ g/ml}) = 150x\text{ g}$$
$$\text{Weight of compound A in water} = (1000\text{ ml})(x\text{ g/ml}) = 1000x\text{ g}$$

Here we began with 37.81 g of compound A (now distributed between two layers) and:

$$1150x\text{ g} = 37.81\text{ g}$$
$$x = 0.03288$$

Hence:

$$\text{Weight of compound A in diethyl ether} = 150\ (0.03288)\text{ g} = 4.93\text{ g}$$
$$\text{Weight of compound A in water} = 1000\ (0.03288)\text{ g} = 32.88\text{ g}$$

Therefore, from the three multiple batch extracts, the total quantity of compound A extracted = 6.52 g + 5.67 g + 4.93 g = 17.12 g.

This is compared against 16.67 g of compound A extracted via the previous single batch extraction into 1000 ml diethyl ether. Although these values are similar (due to the low distribution coefficient of compound A) this example demonstrates that continuous liquid–liquid extraction (using apparatus as previously mentioned) permits the potentially exhaustive extraction of analytes from an aqueous matrix because the extraction solvent is automatically distilled, condensed and forced through the sample before flowing back to its starting point where it is evaporated and recycled. Therefore, the equivalent of several hundred batch extractions with fresh solvent can be achieved with the same starting solvent in a few hours and requires minimal attention once underway.

The choice of solvent is extremely important, as it must have a low boiling point so that it can be easily removed from the extract without a significant loss of sample aroma volatiles and ideal extraction solvents must be able to extract polar and non-polar components.

Hardy and Ramshaw (1970) found they could isolate and quantitatively analyze minor volatile constituents of Riesling white table wine, using the solvent trichlorofluoromethane (freon 11). This solvent does not extract water or ethanol, and was reported to extract other alcohols, carbonyls and esters with recoveries of between 70 and 100% in 17 hours by continuous liquid–liquid extraction.

The bulk of the content of higher alcohols was then removed from the extract by treatment with propylene glycol, allowing the remaining volatile components to be concentrated further. The analysis of the wine extract was carried out by gas chromatography and gas chromatography-mass spectrometry (GC-MS). Forty-five compounds were successfully identified. Freon 11 was also used as extraction solvent for the examination of the volatile flavor fraction of Cabernet Sauvignon wines (Boison and Tomlinson, 1990). New designs of solvent extractor and low temperature, high vacuum, two stage concentrator apparatus were constructed. This enabled the quantitative and qualitative determination of trace level aroma components of *Vitis vinifera* grape musts and wines by capillary gas chromatography. The sensitivity of the technique was demonstrated by the detection and identification of 2-methoxy-3-isobutylpyrazine (in the parts per billion range or μg/l) for the first time in Cabernet Sauvignon wine.

This enrichment technique has also been used recently for the identification of some characteristic aroma compounds in noble rotted grape berries and Aszu wines from Tokaj by GC-MS (Miklosy *et al.*, 2004). Freon 11 has also been used recently as extraction solvent for the comparative study of aromatic compounds

in two young white wines subjected to prefermentative cryomaceration (Peinado *et al.*, 2004) and also recently for the comparison of odor active compounds in Sherry wines (Section 2.10.3) processed from ecologically and conventionally grown Pedro Ximenez grapes (Moyano *et al.*, 2009).

Nelson *et al.* (1979) used a similar liquid–liquid extraction technique to study the volatile composition of three Catawba wines. The volatiles were isolated by stirring equal volumes of sample with the solvent freon 113 (1,1,2-trichloro-1,2,2-trifluoroethane), for 1 h before concentrating 13, 500-fold. The Catawba extracts were then analyzed by gas chromatography and combined gas chromatography-mass spectrometry. The authors concluded that the unidentified trace components, which were less than 5% of the total freon extractable volatiles, were of critical importance to the aroma.

Freon 113 has recently been adopted as extraction solvent, to characterize the aroma of Brazilian Cachaça and Puerto Rican rum (Section 3.5.4) (de Souza *et al.*, 2006). Beverage samples (50 ml) were extracted with 15 ml of solvent and then sonicated at 40 kHz and 20 °C for 10 min. The liquid phases were then separated in a 250 ml separation funnel and the lower freon layer collected. The remaining upper phase was rinsed with an additional 5 ml of solvent and sonicated as previously. This procedure was then repeated for a third and final time with 15 ml of freon. The extracts were then dried with anhydrous magnesium sulfate and concentrated to 1 ml under reduced pressure using a rotary evaporator, prior to being analyzed by gas chromatography-olfactometry (GC-O) and GC-MS.

Ferreira and coworkers (1993) developed a fast and quantitative determination of wine flavor compounds using microextraction. Typically, 10 ml of wine was extracted with only 100 μl of freon 113 as extracting agent. It was concluded that the proposed method allowed quantification of 26 wine flavor compounds with detection limits in the μg/l range. Precision, linearity and accuracy of the method was tested using different wines and synthetic mixtures. Relative precision was shown to be better than 3%.

This sample preparation technique has also been used for the quantitative determination of trace and ultra trace flavor active compounds in red wines (Ferreira *et al.*, 1998a) and for the classification of Slovak varietal wines (Pet'ka *et al.*, 2001).

Many other solvents and solvent mixtures have been utilized to study the volatile composition of grape juices and wines using the liquid–liquid extraction technique. Examples include: dichloromethane (Moio *et al.*, 1995; Baek *et al.*, 1997; Schneider *et al.*, 1998; Tominaga *et al.*, 1998a; Delfini *et al.*, 2001; Ortega *et al.*, 2001; Rocha *et al.*, 2004; Lee *et al.*, 2006; Perestrelo *et al.*, 2006; Selli *et al.*, 2006a; Selli *et al.*, 2006b; Falcão *et al.*, 2008; Hernanz *et al.*, 2009) pentane/dichloromethane mixtures (Gonzalez-Vinas *et al.*, 1996; Lamikanra *et al.*, 1996; Garcia *et al.*, 2003; Garde Cerdán *et al.*, 2004; Guchu *et al.*, 2006; Sánchez Palomo *et al.*, 2007) and hexane/diethyl ether 1:1, v:v (Rogerson *et al.*, 2002). Diethyl ether/pentane (1:1, v:v) has also been utilized to study the volatile composition of Mencia wines (Calleja and Falque, 2005) and for isolation of aroma compounds for differentiation of white wines by their aromatic index (Falqué *et al.*, 2001).

A comparative study of the ability of different solvents and adsorbents to extract aroma compounds from alcoholic beverages has recently been conducted (Ferreira *et al.*, 2000). Seven liquid solvent systems – dichloromethane, dichloromethane/pentane (1:1, v:v), freon 113, diethyl ether/pentane (1:1 and 1:9, v:v), ethyl acetate/pentane (with and without an additional salting-out effect) (1:3 and 1:20, v:v) were comparatively studied along with seven solid phase extraction (SPE) systems. It was concluded that the best liquid extraction solvents were dichloromethane and freon 113 with salt (ammonium sulfate).

Dichloromethane was the extraction solvent of choice for the characterization of Tequila flavor by instrumental and sensory analysis (Benn and Peppard, 1996). The extract obtained was analyzed by gas chromatography with both flame ionization detection (FID) and sulfur chemiluminescence detection (SCD), as well as combined gas chromatography-mass spectrometry. More than 175 components were identified in the extract and the importance of individual chemical constituents was determined by sensory analysis with the use of a GC equipped with an odor port and aroma extract dilution analysis.

Dichloromethane has also been used recently as extraction solvent to compare the odor active compounds in unhopped beer and those hopped with different hop varieties (Kishimoto *et al.*, 2006) and also for the analysis of heavy sulfur compounds in wines (Moreira *et al.*, 2004).

Tominaga and co-workers (1998a; 1998b) reported a method for the specific analysis and identification of volatile thiols from *Vitis vinifera* L. Cv. Sauvignon Blanc wines. Typically, a 500 ml volume of wine containing 4-methoxy-2-methyl-2-mercaptobutane as internal standard was adjusted to pH 7.0 with a sodium hydroxide solution (10 M) and extracted with dichloromethane (2 × 100 ml) in a 2 l flask with magnetic stirring for 5 min each time. The combined organic phases were then centrifuged for 5 min at 3800G to break the emulsion and separated in a separating funnel. The organic phase obtained was subsequently extracted with 2 × 20 ml *p*-hydroxymercuribenzoate solution (1 mM in sodium hydroxide at 0.01 M) for 5 min each time with a pH maintained >7 by the addition of 10 M sodium hydroxide solution. The two aqueous phases from the extraction were then combined and brought to pH 7 by very slow addition of hydrochloric acid solution (5%) to avoid precipitation of *p*-hydroxymercuribenzoate, then loaded into a strongly basic anion exchanger column (1.5 × 3 cm of Dowex 1) which had been previously reactivated using 50 ml of hydrochloric acid solution (0.1 M) and then rinsed with ultrapure water. Percolation of the aqueous phase took 15 min before the column was washed with 10 ml of potassium phosphate buffer (2 mM, pH 7.2) followed by 50 ml of sodium acetate buffer (0.1 M, pH 6). The volatile thiols were then released from the column by percolating for 30 min using a cysteine solution (640 mg/60 ml adjusted to pH 7, previously purified of any volatile contaminants by repeated extractions with 3 × 5 ml of dichloromethane). The eluate containing the volatile thiols was subsequently collected in a 100 ml flask and extracted using 4, 2.5 and 2.5 ml of dichloromethane for 5 min each with stirring before being combined, dried with sodium sulfate and concentrated under a stream of nitrogen to approximately 500 μl. Finally the concentrate was transferred to a 1 ml vial and concentrated further to 25 μl. The authors reported that the method allowed simultaneous analysis of five important volatile thiols and analysis of 10 typical Sauvignon Blanc wines showed that 4-mercapto-4-methylpentan-2-one, 3-mercaptohexyl acetate and 3-mercaptohexan-1-ol were expected to be involved in varietal aroma, as they were most likely present at concentrations greatly in excess of their respective perception thresholds. This specific extraction method has also been recently used to study the presence of polyfunctional thiols in four different fresh light protected lager beers (Section 2.6.6) (Vermeulen *et al.*, 2006), for the identification and characteristics of new volatile thiols from Nelson Sauvin hops (Takoi *et al.*, 2009) and for the identification, quantification and odor impact of new sulfanyl alcohols on the aroma of young botrytized sweet wines (Section 2.9.1) (Sarrazin *et al.*, 2007).

A similar technique was used by Schneider and coworkers (2003) to report the quantitative determination of sulfur containing wine odorants at sub parts per billion (ppb) levels. Wine samples (500 ml) were initially extracted with dichloromethane and concentrated to 1 ml. The extract was then diluted in 2 ml of pentane and passed through a column produced with 500 μl of Affi-Gel 501 loaded into a Pasteur pipette (glass wool at the bottom and conditioned with 5 ml of isopropyl alcohol followed by 5 ml of pentane/dichloromethane mixture, 2:1, v:v). Thiols were then eluted with 5 ml of a 1,4-dithio-DL-threitol solution (5 mM in pentane/dichloromethane mixture, 2:1, v:v) and the resulting extract washed with water, dried over sodium sulfate and concentrated to *ca.* 100 μl, and analyzed by GC-MS or GC-atomic emission detection (AED).

The volatile profiles of commercial Scotch whisky (Chapter 3.2) samples have recently been studied by comparing a conventional liquid-liquid extraction (LLE) method with solid phase microextraction (SPME – Section 4.2.4) and subsequent analysis by gas chromatography (Caldeira *et al.*, 2007). Seven different extraction solvent systems were evaluated including dichloromethane, *n*-hexane, dichloromethane/diethyl ether (3:1 and 1:3, v:v) and dichloromethane/*n*-hexane (3:1 and 1:3, v:v). The high ethanol concentration of the whiskies required dilution to 12%, (v:v) alcohol for both LLE and SPME methods. This dilution was necessary to minimize emulsion formation during liquid–liquid extraction and loss of sensitivity for most

volatiles determined by SPME. The highest extraction efficiency of whisky volatile compounds was obtained when dichloromethane was used as extraction solvent. The researchers concluded that for a complete and quantitative study of volatile composition in food and beverages, two or more sample preparation techniques are recommended. Headspace SPME was demonstrated to offer greater sensitivity when compared to the traditional method of liquid extraction. Similar conclusions have also been reported for the analysis of brandy (Chapter 3.6) aroma by SPME and LLE (Ebeler *et al.*, 2000). Here, headspace solid phase microextraction and continuous liquid–liquid extraction were used to isolate and analyze aroma volatiles in brandy. The authors reported in general, the SPME method using a non-polar poly(dimethylsiloxane) coating was more selective for esters and acids than was LLE which extracted the higher alcohols more efficiently than SPME with freon 11 as extraction solvent.

Andujar-Ortiz and coworkers (2009) evaluated the analytical performance of liquid–liquid extraction using dichloromethane, solid phase extraction (SPE – Section 4.2.4) using a styrene-divinylbenzene copolymer and headspace solid phase microextraction (SPME – Section 4.2.4) using a carboxen-poly(dimethylsiloxane) coated fiber. A comparative analysis of 30 representative wine volatile compounds was carried out and the authors concluded that LLE and SPE showed very good linearity, covering a wide range of concentrations, low limits of detection and high extraction recoveries for most volatiles under investigation. Higher sensitivity was also observed when compared to the headspace SPME procedure and although LLE and SPE required, in general, more tedious sampling treatment and the use of organic solvents, these analytical techniques were reported to be more adequate for the analysis of wine volatiles. The same conclusion was also reported after the comparison of LLE and SPE alongside simultaneous distillation extraction (SDE – Section 4.2.3) for the volatile compounds of Muscat grape juice (Sánchez-Palomo *et al.*, 2009). Results showed that although the three techniques could be recommended for the quantitative analysis of volatile compounds from musts, LLE and SPE were better sample preparation techniques for the determination of polar compounds such as acids or alcohols.

Liquid–liquid extraction continues to be a dominant sample preparation technique for the analysis of alcoholic beverages. Greer *et al.* (2008) utilized this as the core technique to compare a novel distillation method versus a traditional distillation method for preserving volatile aroma chemicals important to the organoleptic attributes of a four botanical model gin (Section 3.4.2). Gin samples (38 ml) were extracted with 2 ml of hexane/dichloromethane (50:50, v:v) and mixed for 30 min before being allowed to rest and separate for subsequent analysis by GC-MS.

Many specific chemical classes of aroma compounds present in alcoholic beverages have also been studied using liquid extraction. Recent examples include the characterization of pyrazines in some Chinese liquors (Fan *et al.*, 2007b), the determination of volatile phenols in red wines (Fariña *et al.*, 2007; Minuti and Pellegrino, 2008) and to study the distribution and organoleptic impact of sotolon (3-hydroxy-4,5-dimethyl-2(5*H*)-furanone) enantiomers in dry white wines (Pons *et al.*, 2008b).

Liquid extraction has also been utilized extensively for the determination of other extremely important alcoholic beverage analytes unrelated to aroma and flavor. A rapid GC method for the determination of residue levels of the insecticide chlorpyrifos and four fungicides (penconazole, fenarimol, vinclozolin and metalaxyl; see Section 5.10.2) in grapes, must and wine was validated by Oliva *et al.* (1999). Samples were extracted with acetone/dichloromethane (1:1, v:v) prior to filtration and concentration before being subjected to analysis by GC with electron capture detection (ECD) for all target analytes other than metalaxyl, which was detected by mass selective detection (MS) in the selected ion monitoring mode (SIM). The proposed method reported analyte recoveries that ranged between 78 and 101% (fortification level 0.1–1 mg/kg) with linear detection rates in the range of 0.02–2 ng/μl, and limits of determination were, in all cases, lower than the maximum residue limits (MRLs) established by different legislations. This method was further adopted for the rapid determination of 17 fungicides widely used by vineyards (Navarro *et al.*, 2000).

Eight commonly used grapevine fungicides (cyprodinil, fludioxonil, metalaxyl, penconazole, pyrimethanil, procymidone, tebuconazole and vinclozolin) have also been extracted from vineyard soil samples by sonication with water followed by extraction (by shaking) with ethyl acetate and subsequent analysis by GC-MS (Rial-Otero *et al.*, 2004). The method was reported to have adequate precision and high sensitivity however, required the use of both internal standard (to control for between sample variation in injection and instrument response) and the standard addition method to control matrix effects.

Other examples of fungicide residues detected in alcoholic beverage samples include spiroxamine extracted from 10 g samples of either grape, must or wine with cyclohexane/dichloromethane (9:1, v:v) and analyzed by GC-MS (Tsiropoulos *et al.*, 2005), famoxadone, trifloxystrobin and fenhexamid residues in tomato, grape and wine samples also extracted with cyclohexane/dichloromethane (9:1, v:v) and analyzed by GC with nitrogen phosphorus (NP) and electron capture (EC) detection with ion trap mass spectrometry (ITMS) for confirmation (Likas *et al.*, 2007), zoxamide residues in grape, must, wine and spirit samples extracted with hexane and analyzed by GC-MS without any sample clean up (Angioni *et al.*, 2005) and hexane extraction of pyrimethanil, metalaxyl, dichlofluanid and penconazol from must, wine and samples undergoing alcoholic fermentation with subsequent analysis by GC with nitrogen-phosphorus detection (Vaquero-Fernández *et al.*, 2009). Famoxadone has also been successfully detected in grapes and wine following extraction with ethyl acetate/hexane (50:50, v:v) and comparison of GC with electron capture and mass spectrometric detectors (de Melo Abreu *et al.*, 2006).

González-Rodríguez *et al.* (2009) recently developed a GC-MS method for the determination of 11 new generation fungicides (benalaxyl, benalaxyl-M, boscalid, cyazofamid, famoxadone, fenamidone, fluquinconazole, iprovalicarb, pyraclostrobin, trifloxystrobin and zoxamide) in grapes and wines. Samples were extracted with ethyl acetate/hexane (1:1, v:v) followed by additional clean up (to remove matrix coextractants) with graphitized carbon black/primary secondary amine (GCB/PSA) solid phase extraction cartridges (SPE – Section 4.2.4). Acetonitrile/toluene (3:1, v:v) was used as elution solvent. The authors concluded that the proposed method demonstrated good linearity, precision and accuracy along with limits of detection (LODs) and limits of quantitation (LOQs) lower than the maximum residue limits (MRLs) established by different European legislations for grapes and wines, and therefore could be a useful tool in routine analysis to ensure product safety.

The determination of multiple pesticides in grapes, musts and wines has been implemented using acetonitrile as extraction solvent and low pressure GC-MS (Cunha *et al.*, 2009). Optimization of the methodology yielded the separation of 27 representative pesticides by applying a quick, easy, cheap, effective, rugged and safe (QuEChERS) approach. Following optimization of several low pressure GC-MS conditions and evaluation of matrix effects, it was concluded that acceptable recoveries for nearly all of the pesticides under investigation were achieved with good repeatability. Limits of quantification were also lower than the maximum residue limits.

Patil *et al.* (2009) described a multiresidue method for the simultaneous estimation of 83 pesticides and 12 dioxin-like polychlorinated biphenyls (PCBs) in red and white wines. Samples (20 ml wine, acidified with HCl) were extracted with 10 ml ethyl acetate (along with 20 g sodium sulfate for drying) and cleaned by dispersive solid phase extraction (DSPE) with anhydrous calcium chloride and Florisil® successively. The final extract (5 ml) was then evaporated to dryness, and the residues remaining reconstituted in 1 ml cyclohexane/ethyl acetate (9:1, v:v) before being further cleaned by DSPE with 25 mg primary secondary amine sorbent, and analyzed by GC-time-of-flight-MS (GC-TOF-MS). The authors concluded that the method described could be adopted to analyze 95 wine compounds within a single GC run of 31 min with high precision, accuracy and low measurement uncertainties.

Liquid extraction has also been utilized in combination with derivatization agents as a means to selectively extract and detect analytes from alcoholic beverages. Examples include GC-MS determination

of diamines (1,3-diaminopropane, putrescine and cadaverine), polyamines (spermidine and spermine) and aromatic amines (β-phenylethylamine and tyramine) found in Port wines (Section 2.10.7) and corresponding grape juices following extraction and derivatization with heptafluorobutyric anhydride (Fernandes and Ferreira, 2000), the GC-MS analysis of biogenic primary alkylamines in wines following conversion to corresponding pentafluorobenzylimines using pentafluorobenzaldehyde (Ngim *et al.*, 2000), the GC-atomic emission detection (AED) analysis of arsenic species present in wine and beer following derivatization with methyl thioglycolate (Campillo *et al.*, 2008b) and the enantiomeric GC analysis of amino acids in beer samples derivatized with ethyl chloroformate (Junge *et al.*, 2007).

A simple and rapid method was described for the extraction of wine volatile compounds based on ultrasonic assisted extraction (UAE) using pentane/diethyl ether (1:2, v:v) as extraction solvents (Vila *et al.*, 1999). Factorial experimental designs were used to optimize the sonication process. Factors such as sample volume, extraction time and solvent volume were considered. A statistical approach was used to find suitable conditions for the ultrasound extraction of aroma compounds of wine. A factorial design at two levels revealed that lower sample volume (100 ml instead of 125 ml) and solvent volume of 50 ml instead of 60 ml contributed to improved extraction efficiency. Performance of the method was evaluated, and the procedure applied to the analysis of aroma compounds in white wines from 'Condado de Huelva' (Spain). It was concluded that the method had advantages over other extraction methods, such as higher reproducibility and the possibility of the simultaneous extraction of several samples. The proposed method allowed the quantification of 24 wine flavor compounds.

A similar UAE, using dichloromethane as extraction solvent, has been optimized for the extraction of volatile compounds from wines (Cabredo-Pinillos *et al.*, 2006).

Recently, two ultrasound assisted liquid–liquid extraction methods were compared to a solid phase extraction (SPE – Section 4.2.4) technique to assess their effectiveness for the analysis of up to 44 volatile components extracted from a synthetic and several commercial wines from Spain (Hernanz *et al.*, 2008). One of the liquid–liquid extraction protocols evaluated gave higher recoveries for a greater number of aroma volatiles that could be attributed to the aroma of the wines, when compared with the SPE methodology however, the SPE method was demonstrated to provide higher repeatability and throughput, and lower solvent consumption. UAE has also been compared with direct immersion SPME for the analysis of monoterpenoids in wine. Both UAE and SPME methodologies were demonstrated to be quantitative, precise, sensitive and linear, however application of both techniques to analysis of red wine samples showed that UAE provided higher extraction of monoterpenic compounds than SPME.

In supercritical fluid extraction (SFE) the extraction phase (usually carbon dioxide, CO_2) is above its critical temperature and is therefore in a supercritical state. Supercritical carbon dioxide is known to have solvent polarity properties similar to diethyl ether and to be particularly selective for esters, aldehydes, ketones and alcohols. If water is present, this will be isolated also (Parliment, 1997).

The primary advantage of optimized SFE is that a highly selective extraction can be conducted if necessary. Exploitation of the selectivity aspects allows the preparation of extracts that can be easily analyzed using chromatographic methods. Other benefits of this methodology are short extraction times and lower reactivity of thermally labile and oxygen sensitive compounds.

The SFE technique is most efficient for the extraction of aroma materials from dry solids or viscous liquids (Da Costa and Eri, 2004), for example high pressure CO_2 extraction is already widely used for the dealcoholization of wine and the processing of hops, and also to extract specific target compounds or active ingredients from foodstuffs, beverages, tobacco, chemicals, pharmaceuticals and cosmetics. Its use has also been reported in the fields of leather, textile and paints (Werkhoff *et al.*, 2002).

Supercritical solvents tend to discriminate against polar components, but this can be overcome by using modifiers such as methanol or ethanol. The reasons for adding a modifier to the CO_2 are threefold:

(i) to increase the solubility of the target compounds, (ii) to destroy analyte–matrix interactions and (iii) to enhance diffusion by swelling of the matrix (Baltussen, 2000). The main disadvantage of SFE is the difficulty in balancing pressure, flow rate and temperature for each type of sample. This often generates time consuming method development. Also, the sample capacity is relatively limited (Da Costa and Eri, 2004).

The applicability of SFE and gas chromatography for analyzing wine aroma was evaluated by Blanch *et al.* (1995). Three different experimental approaches were considered including: (i) off line SFE using an original commercial configuration, (ii) SFE – capillary gas chromatography analysis via programmed temperature vaporization (i.e. using the quartz injection liner of a programmable temperature vaporizer (PTV) for trapping the extracted solutes and then the chromatographic analysis carried out by placing the liner in the injector body of a gas chromatograph), and (iii) on line coupling between SFE and capillary gas chromatography by means of a PTV. It was concluded that on line coupling between SFE and GC using a PTV injector as interface, seemed to be a good option for performing the extraction, collection and analysis of wine aroma in a single step. Relative standard deviations ranging from 0.9% to 19.1% were achieved for a series of test mixture compounds, whereas the recoveries obtained varied from 57.6% to 109.2%, detection limits ranging from 0.08 to 0.34 ng. Off line SFE-GC could be used for the analysis of wine samples and provided satisfactory relative standard deviations (0.1–8.8%) and acceptable recoveries (77–103%), although detection limits were higher (0.2–4.5 μg). Off line SFE-GC via PTV seemed to be a good alternative for the analysis of wine samples, though the quantitation of the most volatile compounds required further optimization.

Kárasek *et al.* (2003) compared direct SFE of wines with CO_2 and conventional indirect extraction by SFE of the sorbent used for solid phase extraction of the same wine samples (SPE-SFE). For direct continuous SFE, wine samples (170 ml) were extracted with supercritical CO_2 in a packed column operated in a continuous, single pass, countercurrent mode. In parallel with the direct method, the wine samples were also analyzed by a more conventional SPE-SFE method as a reference. In the SPE step, 3.5 g of Amberlite® XAD-7 sorbent was mixed with a 40 g sample of wine. The mixture was stirred with a magnetic stirrer at 70 rpm for 2 h and then filtered through common filter paper. The sorbent on the filter paper was allowed to predry for 2 h at 30 °C in order to remove residual wine. The moist Amberlite® resin with sorbed analytes was finally loaded into the extraction cell of a supercritical fluid extractor and extracted dynamically for 45 min at 50 °C. The released analytes were trapped by bubbling the effluent into 4 ml of ethanol at 5 °C. It was concluded that, compared to the two step process of SPE-SFE, the direct continuous SFE of wine resulted in a more specific and representative fingerprint of the sample, as revealed by the GC analysis of the extracts. Direct SFE was preferable because it was more straightforward than SPE-SFE. The additional analyte–sorbent interactions and sorption/desorption steps involved in SPE-SFE resulted in unfavorable alteration of the GC fingerprint, reducing both the peak number and the information obtained. In addition, the study showed the feasibility and expediency of direct SFE for the purpose of wine analysis with the goal to classify the samples according to the respective wine varieties. The superior robustness of direct SFE compared to SPE-SFE of wines was also apparent from multivariate statistical processing of the analytical results.

Supercritical extraction with CO_2 has recently been adopted for isolation of aroma compounds from sugar cane spirits (Gracia *et al.*, 2007). The effects of pressure and temperature on extraction yield and aroma recovery were studied. The researchers concluded SFE appeared to be a viable technology for obtaining concentrates of aroma compounds from intermediate and residual streams from the industrial process of rum production (Sections 3.5.3 and 3.5.4) and that the results could be extended to alcoholic beverage production so as to provide a source for the isolation of aroma substances with commercial interests in food, pharmaceuticals and cosmetics.

4.2.3 Distillation Methods

One of the most important, valuable and often cited sample preparation methods for the isolation and subsequent analysis of volatile aroma compounds from foods and beverages is the simultaneous steam distillation-extraction method (SDE) as invented by Likens and Nickerson (1964; Nickerson and Likens, 1966) for the analysis of hop oil (Section 2.6.3) (see Figure 4.2.2).

Typically with this technique, the sample (an aqueous solution or solid aqueous slurry) is boiled in a 500 ml to 5000 ml round bottomed flask (with stirring to prevent localized heating and subsequent bumping) connected to the left arm. The steam which is then generated during the extraction procedure increases

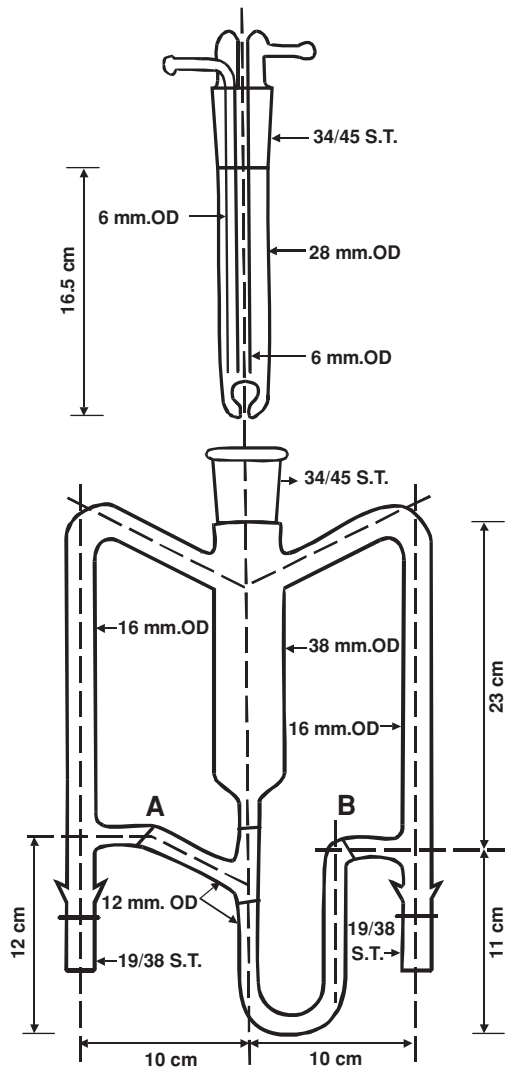

Figure 4.2.2 *Likens' and Nickerson's apparatus for solvent denser than water (Likens and Nickerson, 1964). Reproduced with permission of the American Society of Brewing Chemists, St. Paul, USA*

the vapor pressure of the extraction system. This in turn lowers the boiling point of the water and volatile compounds which are steam distilled through the upper part of the left arm. Simultaneously 10–100 ml of extraction solvent is refluxed and the subsequent solvent vapors distill through the upper part of the right arm of the apparatus. Vapors then condense on the cold finger, and the extraction process begins in the vapor–liquid film that forms. The proposed construction of the apparatus is such that high density extraction solvents (such as dichloromethane) and the low density aqueous layer de-mix and return via the correct return arm back to the appropriate flask to fulfill continuous steam distillation extraction (as shown in Figure 4.2.2). As originally designed by Likens and Nickerson, inverting the apparatus allows the use of low density extraction solvents such as pentane or diethyl ether.

Applied to hop oil components, Likens and Nickerson obtained satisfactory recoveries in the range of 54–99% depending on the compounds used, the extraction time (1–2 h) and the pH. The pentane extract also had to be concentrated prior to GC injection.

Since the original design, various modifications to the SDE apparatus have been made. For example, after increasing the condenser efficiency and insulating the steam distillation arm, yields usually in excess of 80% within 1 h have been reported using dichloromethane as the solvent (Macleod and Cave, 1975). An important and well renowned improvement of atmospheric pressure SDE was reported by Godefroot *et al.* (1981) with a microscale apparatus that allowed the concentration of volatiles into 1 ml of extraction solvent, thus achieving a high concentration factor, reducing problems of artefact build up and allowing direct gas chromatographic injection of the organic extract without the need of a further concentration step (see Figure 4.2.3). Within 1 h, quantitative recoveries were obtained for a wide range of aroma compounds.

Vacuum versions of the SDE technique have also been well discussed in the literature as a way or means of reducing the thermal decomposition of the sample analytes (resulting in artefact formation), i.e. creation of aroma/flavor volatiles in the analytical extract that were not initially present in the original sample. However, it has been reported that operation of SDE under vacuum conditions had a negative effect upon recoveries of analytes when directly compared to atmospheric use (Leahy and Reineccius, 1984). Other researchers have reported that operation under vacuum is complex since the boiling of the two flasks must be balanced, as well as keeping the solvent from evaporating and holding the pressure constant (Parliment, 1997).

An excellent and extensive review of the SDE technique has recently been discussed in the literature (Chaintreau, 2001).

The aroma volatiles of grape juice have been successfully studied with a 1 h distillation time and a further 30 min of solvent extraction with dichloromethane (Blanch *et al.*, 1991; Caven-Quantrill and Buglass, 2006).

Different isolation methods for the volatile components of Muscat grape juice have been compared in terms of the accuracy and precision of the quantitative analysis, and the sensitivity achievable with each analytical procedure (Blanch *et al.*, 1991). The methods included in the study were continuous liquid–liquid extraction, microscale simultaneous distillation-extraction (SDE) with freon-11, dichloromethane and pentane as extracting solvents, as well as a dynamic headspace sampling procedure using both sweeping and an aeration approach. Analysis of the grape juice extracts was carried out by capillary gas chromatography equipped with a programmable temperature vaporizer (PTV) for sample introduction (in the LLE and SDE methods). The injector was also used for trace analysis by enriched trapping in a suitable adsorbent. It was concluded that the use of a PTV injector for the intermediate trapping of Muscat grape juice volatiles, which result from the headspace analysis in the aeration mode, allowed a greater sensitivity than liquid–liquid extraction and steam distillation-extraction procedures, although careful optimization of variables affecting the experimental procedure was required. The headspace method was also less suited for application in quantitative analysis, but could be satisfactorily used for identification purposes. It was recommended that the SDE method with dichloromethane as extraction solvent should be adopted for carrying out an accurate and precise quantitative analysis. Blanch *et al.* (1993) further optimized the SDE method at normal pressure by using the modified sequential simplex method. For this purpose, a new micro steam distillation-extraction

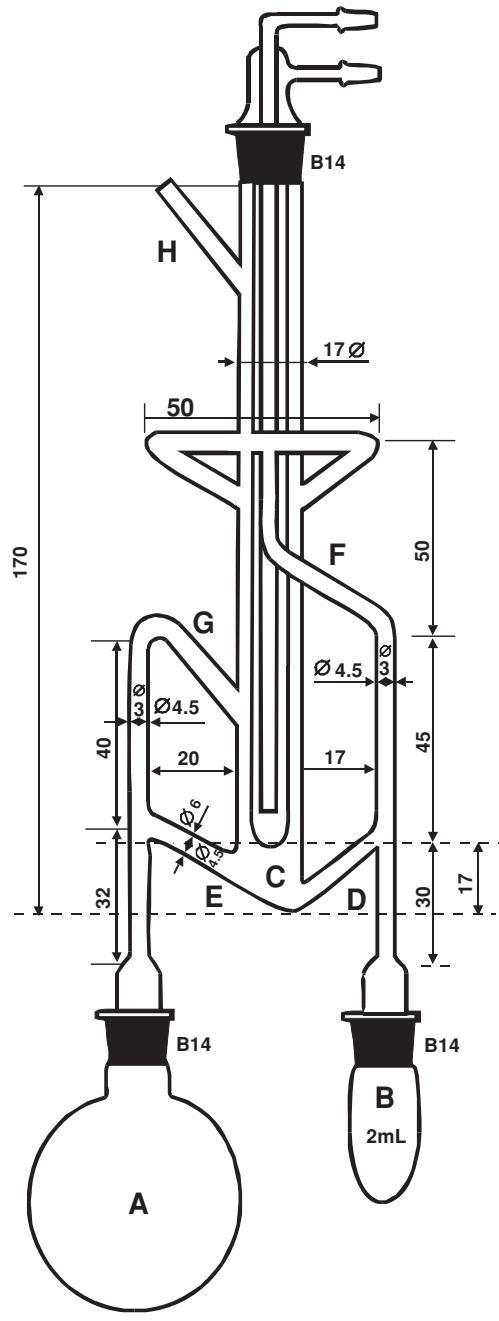

Figure 4.2.3 *Micro-SDE apparatus designed by Godefroot* et al. *(1981). Reproduced with permission of Elsevier Ltd., Oxford, UK*

device was constructed. Determination in the μg/l range of a typical mix of aroma compounds having different polarities and volatilities was tested and accomplished with high recoveries. The optimized method was then used to produce an aroma extract from a Muscat grape juice for determining compounds in the μg/l range and tentative peak identification of several compounds characteristic of the juice was carried out by matching retention times with those of reference compounds. It was concluded that the sequential simplex method allowed the performance of the SDE procedure to be significantly improved. The proposed modified apparatus operated under the optimized conditions rendered, in general, excellent recoveries for the investigated aroma compounds in the mg/l (ppm) or μg/l (ppb) range.

A similar microscale simultaneous distillation-extraction device has very recently been compared with stir bar sorptive extraction (SBSE) for the determination of volatile organic constituents of grape juice (Caven-Quantrill and Buglass, 2005; Caven-Quantrill and Buglass, 2006).

The potential of the micro SDE technique for the rapid enrichment of wine aroma compounds has also been investigated (Blanch *et al.*, 1996). However, the use of dichloromethane as the extracting solvent did not allow performance of the experimental run if a 100 ml volume of wine was placed in the sample flask. This led to ethanol from the sample flask getting into the de-mixing section of the SDE apparatus, thus hindering the equilibrium between the two solvent layers (corresponding to water and dichloromethane) in the separation chamber. This problem was diminished by dilution of the wine sample with water (1:1, v:v). Further dilution of the sample (i.e. 1:3, v:v) made the experimentation more convenient when unattended processing was required, although lower enrichment factors were obtained.

Recently, a compact and versatile distillation unit for the careful isolation of volatiles from complex food matrices has been developed (Engel *et al.*, 1999). The inventors reported that the new technique named solvent assisted flavor evaporation (SAFE) was able to successfully isolate volatiles from either solvent extracts, as well as directly from aqueous foods and beverages (including beer) yielding flavorful aqueous distillates free from co-extracted non-volatile matrix compounds (see Figure 4.2.4). This apparatus (size, 40 × 25 × 7 cm) consists of a dropping funnel (Figure 4.2.4, no.4), a cooling trap (no.6) and a central head (no.2) bearing two 'legs' (nos. 11 and 12), both equipped with ground joints NS 29 (no.17) to fix distillation vessels of various

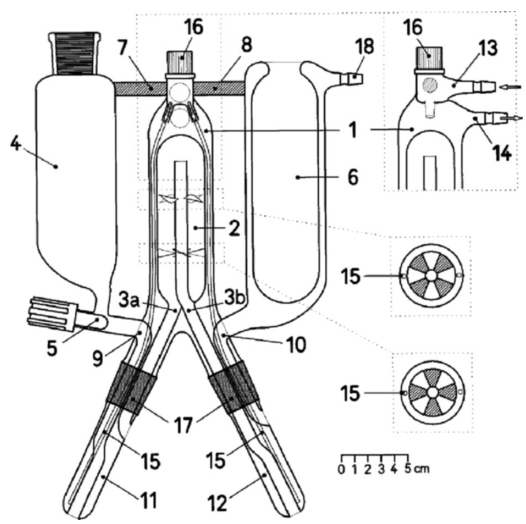

Figure 4.2.4 *Solvent assisted flavor evaporation (SAFE) apparatus designed by Engel* et al. *(1999). Reproduced with permission of Springer-Verlag*

volumes. The outlet of the dropping funnel leads to the bottom of the left 'leg' (no.11). The vapor inlets to the head (no. 3a) and the inlet to the trap are mounted on the sides of each 'leg.' To ensure a constant temperature during distillation and to prevent condensation of the volatiles, the head and the two 'legs' are completely thermostated with water. From the water inlet (no. 13), two flexible polyethylene tubes (no.15) guide the water flow to the bottom of both legs to afford effective temperature regulation by avoiding the formation of air bubbles.

Figure 4.2.5 shows the entire SAFE equipment required for performance as outlined by the inventors. Before commencement of distillation, the head and legs of the apparatus are thermostated at 20 to 30 °C via

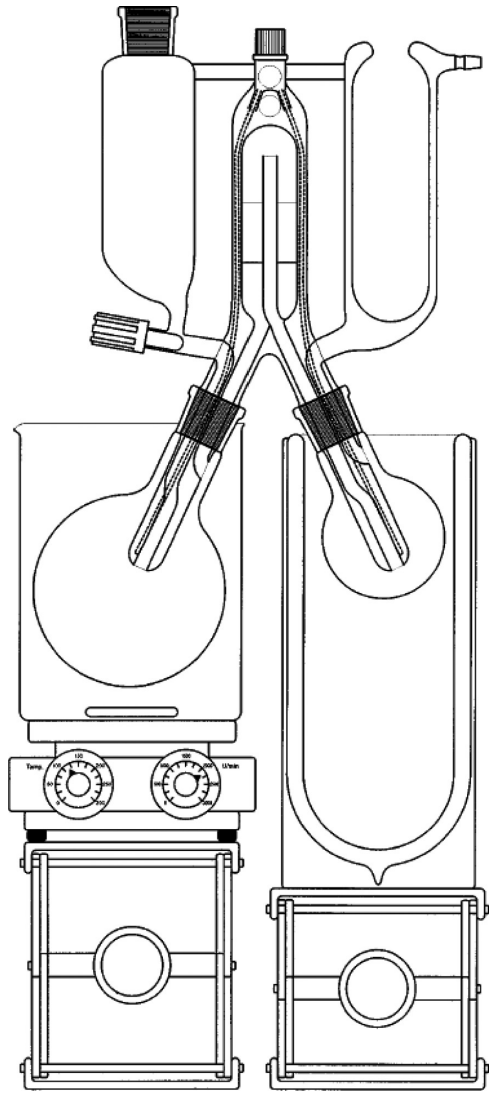

Figure 4.2.5 *Solvent assisted flavor evaporation (SAFE) apparatus assembly. Temperature of water bath, 30 °C; temperature of the trap, −196 °C (Engel* et al.*, 1999). Reproduced with permission of Springer-Verlag*

the outlets 13 and 14 (Figure 4.2.4). The distillation vessel (typically 250 ml to 1000 ml) is heated in a water bath at 20 to 30 °C. A diffusion pump supplying a high vacuum (10^{-3} Pa) is applied to the apparatus via outlet no.18 (Figure 4.2.4) while the high vacuum stopcock of the dropping funnel is carefully closed. Liquid nitrogen is applied to the trap (Figure 4.2.4, no.6) prior to the start of the distillation, which is achieved by dropping aliquots of the sample from the dropping funnel into the left vessel at which point, the volatiles and solvent are transferred via tube 3a (Figure 4.2.4) into the distillation head no.2. Here, two propeller shaped 'barriers' remove non-volatile material from the vapors and the distillate enters a flask, cooled with liquid nitrogen, via tube 3b on the right side of the apparatus. Volatiles, water or other solvents condense along the walls of the vessel.

Since its creation, the SAFE technique has been quickly adopted for the isolation and analysis of aroma compounds from various alcoholic beverages. For example, 500 ml of Bavarian Pilsner type beer (Section 2.6.13) was repeatedly extracted with diethyl ether, before being dried over sodium sulfate and concentrated to 100 ml by distilling off the solvent at 38 °C using a 60 cm × 1 cm i.d. Vigreux column (Fritsch and Schieberle, 2005). Finally, to remove the non-volatile material, the concentrate was distilled using the SAFE method and the resulting distillate treated with aqueous sodium bicarbonate to obtain fractions of the acidic volatiles and of the neutral/basic volatiles. These were subsequently dried with sodium sulfate and concentrated via microdistillation before analysis by high resolution gas chromatography-olfactometry (GC-O) and GC-MS. This approach has also been used to characterize the most odor active compounds in American Bourbon whisky (Section 3.3.4) (Poisson and Schieberle, 2008). Here the researchers extracted the aroma volatiles present in 25 ml of whisky (previously diluted 1:1 with tap water saturated with salt) using diethyl ether (3 × 100 ml). The combined extracts were then dried over sodium sulfate and concentrated to 100 ml using a Vigreux column. SAFE removed the non-volatile compounds and the distillate was finally concentrated to 100 µl and separated into neutral/basic and acidic volatiles as previously.

Similarly, 1000 ml of Oregon Pinot Noir wine was extracted with diethyl ether:pentane (1:1, v:v) three times in a separating funnel (extracts totalled 750 ml) before the aroma volatiles were isolated using SAFE, dried with sodium sulfate and concentrated to 10 ml under a stream of nitrogen. This aroma extract was further separated into acidic/water soluble and neutral/basic fractions by adding 10 ml of distilled water, adjusting the pH of the aqueous phase to pH 11 (with sodium carbonate solution) then separated in a separating funnel and retained. The organic phase was further washed with 10 ml dilute sodium hydroxide solution (pH = 11) three times and the washings combined with the aqueous phase. The organic phase was dried over sodium sulfate, filtered and concentrated to 200 µl for GC-O analysis. The aqueous solution was adjusted to pH 1.7 with sulfuric acid, 10g of salt (NaCl) was added and the solution extracted three times with 50 ml diethyl ether:pentane (1:1, v:v). These extracts were combined, dried with sodium sulfate and concentrated to 500 µl for further GC-O analysis (Fang and Qian, 2005a). An identical approach has also been reported for the characterization of aroma compounds in apple cider (Section 2.8.4) (Xu *et al.*, 2007).

4.2.4 Solid Phase Extraction and Related Methods (Sorption Techniques)

Developed in the late 1970s, solid phase extraction (SPE) quickly became a useful and well cited sample preparation technique and was the forerunner to the present so called 'sorption techniques' solid phase microextraction (SPME) and stir bar sorptive extraction/headspace sorptive extraction (SBSE/HSSE). Solid phase extraction initially evolved from classical liquid solid extraction (LSE) whereby the sorbent was typically placed in a vessel containing the analyte dissolved in a suitable solvent and shaking/mixing for a controlled length of time. The two phases were then separated and the analytes of interest were desorbed using a suitable organic solvent (Handley and McDowall, 1999). This technique was then later modified by packing the sorbent (stationary phase) into glass columns to perform wet column chromatography and then finally the

SPE cartridge was born. An excellent guide to the solid phase technique, including phase types, theory and use is available (Supelco, 1998).

This widely commercially available product contains milligram quantities of sorbent phase which is supported between two fritted disks in a plastic syringe type cartridge. Analytes present in an aqueous/ethanolic matrix can therefore be selectively retained and subsequently eluted by an appropriate solvent.

SPE has several well reported advantages over the previously conventional and dominating liquid–liquid extraction techniques in that it is generally faster, requires less solvent (hence more 'green' and economical), reduces the need for large concentration steps and is easily automated, as well as eliminating the use of expensive speciality glassware.

The 'stationary phases' that are utilized for solid phase extraction are equivalent to those used for high performance liquid chromatography (HPLC) (Section 4.3.3), however, the particle size for conventional HPLC column stationary phases are anywhere between 3 to 10 μm, whereas those for SPE separations are typically in the region of 40–80 μm. There are three main modes of SPE separation. These are reversed phase (non-polar solid stationary phase, polar elution solvent), normal phase (polar solid stationary phase, non-polar elution solvent) and ion exchange (anion or cation) using charged bonded sorbents. In the reverse phase (non-polar extraction), popular commercial sorbent phases include octadecyl (C_{18}), octyl (C_8), phenyl and cyanopropyl. For this type of extraction, the sorbent utilized is generally less polar than the solvent or matrix solution and the non-polar analytes are retained on the SPE bonded phase and eluted with non-polar solvents. In the normal phase (polar extraction), commercial sorbent phases include silica gel (no bonded phase), diol and aminopropyl as examples. Here, polar analytes from the sample matrix are retained and elution is carried out with generally polar solvents. Ion exchange sorbents can contain either a strong or a weak cationic or anionic exchanger and can be silica or resin based. Cationic or anionic analytes in the sample matrix are retained by an oppositely charged group and elution is achieved by altering the pH or increasing the ionic strength of the elution solvent.

The SPE process involves four key steps; conditioning of the column, application of the sample, washing of the column to remove unwanted components and finally elution of the analyte(s) of interest (see Figure 4.2.6).

The SPE cartridge is conditioned by passing through three to four times the sorbent bed volume of a high purity (HPLC grade) organic solvent. Reversed phase sorbents are usually conditioned with a water miscible (polar) solvent such as methanol, followed by water or an aqueous buffer. This conditioning/equilibrium

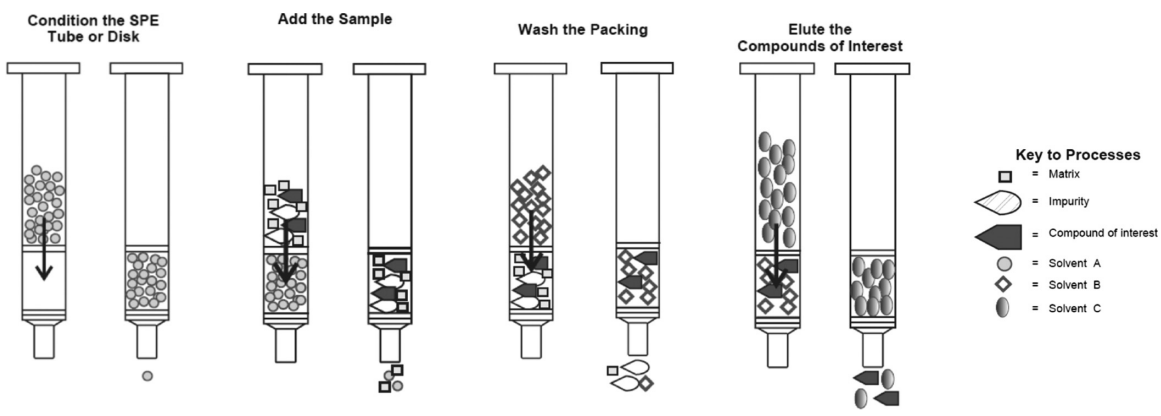

Figure 4.2.6 *Key steps of the SPE process (Supelco, 1998). Reproduced with permission of Sigma Aldrich, St. Louis, USA*

process wets the surface of the sorbent and penetrates the bonded alkyl phases, allowing water to wet the silica surface efficiently and increase the overall surface area available for interaction with subsequent analytes applied to the sorbent phase. This step also aids removal of impurities/residues from the packing material that could interfere with the analysis. Normal phase SPE sorbents are usually conditioned with the organic solvent in which the sample analytes have been dissolved. Ion exchange sorbents that are utilized for analytes in non-polar organic solvents should be conditioned with solvent equivalent to that of the sample, whereas for analytes in polar solvents, a water miscible organic solvent should be adopted, followed by an aqueous solution with a pH, organic solvent content and salt concentration as similar as possible to that of the sample to be extracted.

In the second step of the SPE process (see Figure 4.2.6), the sample is transferred to the column/cartridge and allowed to flow through the sorbent bed. Sample volumes can range from microliters (μl) to liters, however if a large volume aqueous based sample is applied to reversed phase silica packings, the column will gradually lose its 'wetted' effect as the solvent layer added during the conditioning process is lost, and this will result in a reduction in recovery (extraction efficiency) of the analytes of interest. To overcome this problem, for samples above 250 ml, an organic solvent that is water miscible (such as methanol at a concentration of up to 10%) is mixed with the sample prior to transferring to the column. This helps to maintain sufficient wetting of the sorbent phase and hence the equilibrium between the stationary and mobile phases. The sample should be passed slowly through the sorbent phase and many researchers use either vacuum or positive pressure. As the flow rate can affect the retention of the analytes to the stationary sorbent phase, care should be taken to ensure that the sample flow does not exceed 2 ml/min for ion exchange SPE columns/tubes and 5 ml/min for other SPE sorbent phases. Many researchers standardize flow rates to approximately 1 ml/min for most analyzes .

Now that the analytes of interest have been retained on the sorbent phase, unwanted matrix materials or analytes are removed by washing the column with the same solvent in which the sample analytes originated (for example aqueous ethanolic solution for wine). Usually no more than a single column volume of wash solvent is required to remove unwanted matrix or analyte compounds.

The final step of the SPE process is to elute (rinse) the analyte(s) of interest from the sorbent phase and collect the eluent for analysis. This step should only remove the compounds of interest and leave any impurities on the sorbent phase that were not removed previously in the wash step. With SPE cartridges, typically 200 μl of elution solvent is sufficient to remove the analytes of interest. Generally, two 100 μl aliquots are more efficient than a single application of 200 μl and flow rate is critical to ensure high recoveries of analyte(s). Recoveries are highest when each aliquot remains in contact with the sorbent phase for 20 s to 1 min with slow or drop wise flow rates (Supelco, 1998).

Wada and Shibamoto (1997) investigated the recovery efficiencies of the main volatile components of wine from porous polymer Porapak Q, (an ethylvinylbenzene-divinylbenzene copolymer), using dichloromethane, diethyl ether and pentane as solvents. Dichloromethane showed the highest recovery efficiency, followed by diethyl ether and pentane. Volatile components from a commercial wine were trapped on Porapak Q (suspended in a glass column), and subsequently recovered using an organic solvent. This method exhibited satisfactory results on isolation of volatile compounds although relatively low recovery percentages were observed. Overall, this solid-phase extraction method demonstrated the simplicity of the technique with rapid recovery of volatile compounds from aqueous alcohol samples.

In 1985, a method of extraction and determination of free and glycosidically bound grape aroma components was suggested (Gunata *et al.*, 1985). These compounds were adsorbed on the nonionic resin, Amberlite® XAD-2, followed by elution with various selective solvents. Free forms were directly determined by gas chromatography; glycosidically bound forms were first enzymatically hydrolyzed. The method was applied to a number of mature grape varieties and it was found that these could be classified in two groups, those rich in free and bound forms which give aromatic wines (Muscat varieties and Gewürztraminer), and those which contain small amounts of these compounds.

This technique has been used in further studies (Voirin *et al.*, 1992; Wirth *et al.*, 2001; Castro Vazquez *et al.*, 2002; Cabaroglu *et al.*, 2003; Sánchez-Palomo *et al.*, 2006; Botelho *et al.*, 2010) and has also been compared with liquid–liquid extraction for the determination of wine flavor components (Zhou *et al.*, 1996).

Edwards and Beelman (1990) also used a similar SPE method. The relative recoveries of the volatile compounds extracted from a French hybrid wine (Aurore) were found to be quite acceptable, in the range of 90–114%, with the majority being greater than 95%. Diethyl ether was used as the fractionation solvent. This XAD-2 extraction method was shown to be a rapid and simple technique for analysis of volatile compounds in wines. The principle advantage of this method over other commonly used methods (such as liquid–liquid extraction) is the decreased time of extraction. It was concluded that excellent precision and recovery, as well as speed, gave this technique great potential for research and quality control applications.

XAD-2 extraction has also been applied to the recovery of aroma compounds from beer to determine which compounds from hops were most important for beer character (in terms of odor and intensity) via gas chromatography-olfactometry (GC-O) (Lermusieau *et al.*, 2001) and recently it has been used alongside specific liquid–liquid extraction to successfully identify 4-vinylsyringol as a stale beer-like odorant (Callemien *et al.*, 2006).

Other adsorbents have also been adopted to study the volatile aroma compounds of grape juices and wines by solid phase extraction. These include Amberlite® XAD-4 (Ferreira *et al.*, 2002), Merck's Extrelut resin (Gerbi *et al.*, 1992), LiChrolut EN resin (Lopez *et al.*, 2002; Ferreira *et al.*, 2003; Campo *et al.*, 2007, Garde-Cerdán *et al.*, 2008a; Loscos *et al.*, 2010), C-18 (Williams *et al.*, 1982; Mateo *et al.*, 1997; Schneider *et al.*, 2001, Diéguez *et al.*, 2003; Luan *et al.*, 2004; Lukic *et al.*, 2006; Villena *et al.*, 2006), Analytichem's Extube CHEM ELUT (Gelsomini *et al.*, 1990) and IST Isolute ENV+ (Boido *et al.*, 2003). Recently Campo *et al.* (2007) validated an SPE method for the quantification of four powerful aroma compounds (ethyl 2-, 3- and 4-methylpentanoate and ethyl cyclohexanoate) in different samples of wine, whisky and brandy. These compounds were extracted from 100 ml samples on a 200 mg LiChrolut EN bed. Major compounds were eliminated by rinsing with a water:methanol (50:50) solution containing 1% sodium bicarbonate, and the analytes of interest were subsequently eluted with 1.5 ml of dichloromethane. 50 μl of this extract was then analyzed by multidimensional GC-MS. Recoveries were found to be quantitative (99–106%) and the method offered satisfactory repeatability and good linearity.

The notorious off-flavor (cork taint) components 2,4,6-trichloroanisole (TCA) and 2,4,6-tribromoanisole (TBA) have recently been studied using the SPE technique. Insa *et al.* (2006) evaluated the extraction of chlorophenolic compounds from ground cork samples with ethanol/water mixtures combined with SPE or headspace-solid phase microextraction (HS-SPME) and GC-ECD (electron capture detection). Both of the tested sample preparation methodologies were reported to compare well in terms of recovery of the target analytes, and it was demonstrated that chlorophenols could be detected in corks contaminated at the nanogram per gram (ng/g) level, therefore it was suggested that the technique could be successfully applied as a quality control measure in the cork industry.

A miniaturization of the conventional SPE technique has recently been developed as a novel method of sample preparation. This was recently optimized for the quantitative determination of TCA and TBA in wine (Jönsson *et al.*, 2008). Microextraction in packed syringe (commercially known as MEPS) was optimized for the extraction and preconcentration of the analytes using extremely small sample volumes (0.1–1 ml). MEPS was performed using a 100 μl gas-tight syringe filled with 4 mg of C18 phase conditioned with 30 μl of methanol followed by an equivalent volume of water. Extraction was performed by drawing 100 μl or 10×100 μl of the sample through the syringe and the C18 solid phase. The sorbent bed was then subsequently dried and the analytes eluted with 10 μl of toluene into a GC vial for analysis by GC-MS. It was concluded that the optimized and validated technique yielded extremely low limits of detection (LOD) and the target analytes could be detected in wine samples before being sensorially identified as cork tainted.

SPE with a novel porous carbon sorbent CARB GR was reported for the extraction and preconcentration of dicarboxyimide fungicide residues from wines, with subsequent analysis by GC with either flame ionization detection (FID), electron capture detection (ECD) or MS (Matisová *et al.*, 1996). Reliable recovery data was measured at various concentrations of the fungicides vinclozolin and iprodione in standard solutions (0.0005–0.1 mg/ml) and spiked wines (5.90 μg/l–1.96 mg/l) and excellent limits of quantitation (LOQ) were determined.

Recently, Montes *et al.* (2009b) described a novel approach for the determination of seven fungicides (metalaxyl-M, penconazole, folpet, diniconazole, propiconazole, difenoconazole and azoxystrobin) in wine samples. Fungicides were extracted from the samples and then subsequently transferred to a small volume of high density, water insoluble solvent using SPE followed by dispersive liquid–liquid microextraction (DLLME). Following optimization, wine samples (20 ml) were initially concentrated using SPE cartridges packed with 60 mg of Waters Oasis HLB reversed phase sorbent. Target compounds were then eluted with 1 ml of acetone and mixed with 0.1 ml of 1,1,1-trichloroethane before finally being added to 10 ml of ultrapure water. After centrifugation, 1–2 μl of the settled organic phase was analyzed by GC-ECD and GC-MS. The method was reported to provide enrichment factors around 200 times and an improved selectivity in comparison to the use of SPE as a single sample preparation technique. It also offered limits of quantitation low enough for the determination of target analytes in commercial wines.

The existence of pesticide residues in alcoholic beverages offered commercially for public consumption has been extensively studied by SPE and GC. Soleas *et al.* (2000) reported a multiresidue method for the simultaneous quantitation of 17 pesticides in wine: dicloran, dimethoate, diazinon, chlorpyrifos-methyl, vinclozolin, carbaryl, methiocarb, dichlofluanid, parathion-ethyl, triadimefon, procymidone, myclobutanil, iprodione, imidan, dicofol, phosalone and azinphos-methyl. C_{18} bonded porous silica SPE extraction of 0.5 ml of wine sample was initially carried out followed by direct injection of 1 μl and analysis by GC-MS in selective ion monitoring mode (SIM – the operation of the mass spectrometer in which the intensity of one or more specific ion beams are recorded as opposed to the entire mass spectrum). Detection and quantitation limits were around 2 μg/l and 10 μg/l respectively, with linear calibration curves up to 3 mg/l for a majority of the analytes under investigation. Recoveries were reported to be >90% for half of the analytes and >80% for most of the remainder with imprecision (RSD) <10% for most pesticides and <18% in all.

Organochlorine pesticides have recently been determined at low levels in white, red, rosé and sparkling wines by automated SPE and GC-MS-MS detection (Pérez-Serradilla *et al.*, 2010). The efficiency of different commercially available SPE sorbents were initally tested, as well as changes in both the volume flushed through the sorbent and the volume subsequently injected into the GC. This allowed the detection limits of the optimized method to be adapted to the analytes contents in the target wine samples. Preconcentration of 100 ml of wine and injecting 10 μl of extract into the GC, yielded limits of detection obtained for the OCPs in the range of 1.7 and 9.7 ng/l (ppt), depending on the OCP and type of wine. The researchers concluded that the automated method enabled a cheap and fast analysis and provided good sensitivity and precision. The method detection limits (MDLs) were reported to be low enough to determine the target pesticides in wine samples with concentrations far lower than the maximum residue limits (MRLs) of OCPs in grapes (there is not a current legislation regulating the MRLs in wines).

The fate of residues of seven pesticides (chlorfenapyr, quinoxyfen, tebuconazole, fenarimol, pyridaben, and (*E*)- and (*Z*)-dimethomorph) from the treatment of hops to the brewing of beer was studied recently using SPE by Hengel and Shibamoto (2002). Initially a multiresidue analytical method was developed and validated for the determination of pesticide residues in spent hops, trub, wort and finished beer. Recoveries ranged from 73–136% and it was demonstrated that the use of pesticide treated hops resulted in the carryover of certain pesticides into the wort during beer production. Following fermentation, all analytes except (*E*)- and (*Z*)-dimethomorph were found in concentrations lower than 0.0005 ppm in beer. When all seven pesticides were spiked prior to the pinching of yeast into clean wort, most of the non-polar analytes (chlorfenapyr, quinoxyfen

and pyridaben) partioned into the organic material (trub) which settled to the bottom of the fermentation vessel, whereas more polar compounds (fenarimol, tebuconazole, and (*E*)- and (*Z*)-dimethomorph) were generally distributed evenly between the beer and the trub.

Similarly, a multiresidue determination of 142 pesticides (including isomers) in malt beverages was recently reported by Wong and coworkers (2004) using polymeric SPE cartridges and sample cleanup with a magnesium sulfate-topped aminopropyl cartridge, followed by GC-SIM-MS. GC detection limits for most of the analytes studied were 5–10 ng/ml, and linearity was determined from 50–5000 ng/ml. Recoveries were greater than 70% for 85 of the analytes studied and the data demonstrated that different malt beverages matrices had no significant effect on the recoveries. When applied to the screening and analysis of malt beverages for pesticide residues, the method proved effective in detecting the insecticide carbaryl and the fungicide dimethomorph in real samples at concentrations significantly lower than those set by the US Environmental Protection Agency (EPA).

GC determination of *N*-Nitrosamines (Section 5.11.5) in beverages (including beer, wine, liquor, whisky, cognac, rum, vodka, grape juice, cider, tonic water and soft drinks) using automatic SPE and subsequent GC-NPD was recently proposed (Jurado-Sánchez *et al.*, 2007). Following examination of various SPE sorbents, LiChrolut EN was found to provide quantitative elution and the highest preconcentration factors. The authors concluded that the optimized method offered LODs between 7 and 33 ng/kg and precision (RSD) from 4.3–6.0%. The recoveries of *N*-Nitrosamines from real beverage samples spiked with the chosen analytes ranged from 95–102%.

Phthalate esters in wine have been determined using C_{18} SPE and GC-MS (Del Carlo *et al.*, 2008). Following initial optimization, the method was applied to the screening of 62 samples of white and red wines obtained from the market, winemakers and an experimental pilot plant and it was reported that all samples analyzed were phthalate contaminated.

Solid phase microextraction (SPME) was first described for the analysis of environmental chemicals in water by Berlardi and Pawliszyn (1989). Following its commercial introduction by Supelco (Bellefonte, PA), SPME rapidly gained popularity for the extraction of volatile and semi-volatile organic compounds from a wide variety of sample matrices. Since this technique does not require the use of organic solvent, extractions (in either immersion or headspace modes) can generally be performed at ambient temperature without heating the sample, thus reducing, if not completely eliminating, the formation of artefacts. Furthermore, only small sample volumes are required, hence as with SPE, the process is 'green', economical, and is easily automated using specially modified SPME devices now manufactured by a large number of analytical equipment suppliers such as CTC, Gerstel and Thermo Scientific.

The stages of the SPME process are shown in Figure 4.2.7. A small fiber of fused silica (1 cm length and 0.11 mm internal diameter) coated with a polymeric phase is bonded to a spring loaded stainless steel plunger which allows movement of the silica fiber in and out of a hollow needle. For extraction of volatiles by liquid (immersion) sampling, each sample is placed, along with a small magnetic stirring bar, in a glass vial crimp sealed with a septum and cap. The SPME fiber is then drawn into the needle of the syringe, which in turn pierces the septum and the fiber is extended and brought into contact with the liquid sample. Used in the headspace mode, the fiber of the SPME device is extended into the vapor phase above the sample. In either mode, over time with stirring of the sample, aroma/flavor analytes of interest from alcoholic beverages adsorb to the polymeric phase of the fiber. Adsorption equilibrium is usually achieved using sampling times ranging anywhere between 2 and 30 min after which the fiber is drawn back into the needle, which is then removed from the septum and inserted immediately (whether manually or automatically) into the injection port of a gas chromatograph (GC) for 1–2 min for subsequent thermal desorption and analysis. Prior to analysis of samples, the SPME fiber should be cleaned (preconditioned) by inserting the fiber into the heated injection port of a gas chromatogram or in a fiber bakeout station that are now also commonly supplied as an optional extra on automated samplers (CTC, Gerstel and Thermo Scientific).

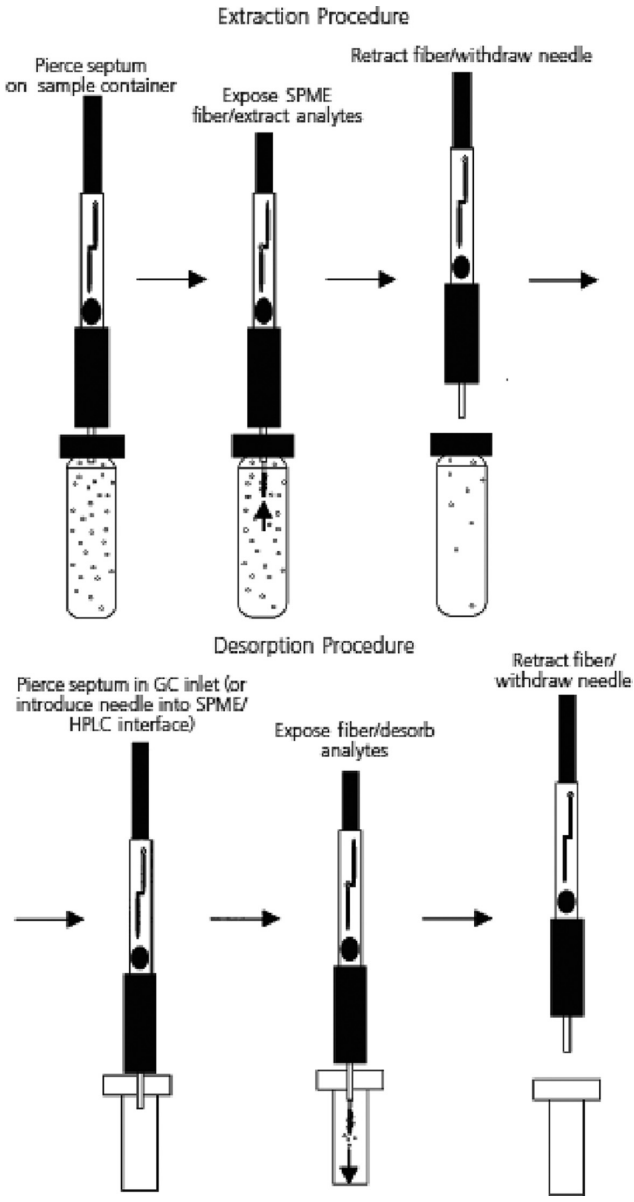

Figure 4.2.7 *Stages of the solid phase microextraction process (Supelco, 2004). Reproduced with permission of Sigma Aldrich, St. Louis, USA*

As with other sample preparation techniques such as liquid–liquid extraction and simultaneous steam distillation-extraction (SDE), extraction efficiency with SPME is also affected by contact time, immersion depth (in liquid sampling), efficiency of mixing, pH, salt concentration, temperature and phase ratio (ratio of sample volume/adsorbent volume). These factors can all affect the partitioning in SPME extractions (Harmon, 1997). Therefore, extraction selectivity can be manipulated by altering the type of polymer coating (adsorbent)

on the fiber, or the coating thickness to match and subsequently extract analytes of interest from aqueous ethanolic matrices (in the case of alcoholic beverages).

For liquid polymeric SPME coatings, the amount of analyte adsorbed by the coating at equilibrium is directly related to the concentration of the analyte in the sample (Zhang and Pawliszyn, 1993; Yang and Peppard; 1994, Supelco, 2004), as expressed in Equation 4.2.2.

$$n = \frac{K_{\mathrm{fs}} V_{\mathrm{f}} C_0 V_{\mathrm{s}}}{K_{\mathrm{fs}} V_{\mathrm{f}} + V_{\mathrm{s}}} \tag{4.2.2}$$

Here, n is the mass of analyte adsorbed by the coating/fiber, C_0 is the initial concentration of the analyte in the sample, K_{fs} is the partition coefficient for the analyte between the coating and sample matrix and V_f and V_s are the respective volumes of the sample and coating (fiber phase). This equation demonstrates a linear relationship between the initial concentration of the analyte in the sample and the amount of analyte adsorbed by the SPME fiber.

Because the adsorbent coatings utilized in SPME are selected to have strong affinities for the analytes they are intended to extract, the partition coefficient for the analyte between the coating and sample matrix is large, hence complete (>90% of the analyte adsorbed by the SPME coating) extractions are rarely required to obtain quantitative results. All extraction conditions should, however, be carefully controlled and repeated for high accuracy and precision from SPME.

As headspace SPME relies on the equilibrium partitioning of an analyte between the fiber coating and the vapor phase above a liquid sample, the sample headspace should be kept as small as possible. The diffusion of analytes to the SPME fiber in the vapor phase has been reported to be in the region of four orders of magnitude greater than diffusion in solution (Zhang and Pawliszyn, 1993; Harmon, 1997).

Desorption of analytes from a SPME fiber depends on a number of factors, such as the boiling points of the analytes adsorbed, the film thickness of the fiber coating and the temperature of the GC injection port. Generally, GC inlet liners with narrow internal diameter of around 0.75 mm aid sharpening of the subsequent chromatographic peaks and often eliminate the need for cryogenic cooling of the inlet (trapping analytes with liquid nitrogen or carbon dioxide at subzero temperatures in a GC inlet as an aid to sharpen early eluting aroma volatiles).

Vas *et al.* (1998) used SPME to study volatile aroma compounds of Blaufrankisch (Gamay Noir) wines. Two different SPME extraction fibers were used in either static headspace mode (by inserting the fiber into the headspace of a 130 ml sampling bottle containing 125 ml of wine), or immersed in the liquid for 1 h at ambient temperature with stirring. The extraction fibers chosen were the non-polar poly(dimethylsiloxane) (PDMS) with 100 μm film thickness and polar poly(acrylate) (PA) with 85 μm film thickness. A 'classical' liquid–liquid extraction method was also used for comparison. Semi-quantitation (peak area measurement) was performed using gas chromatography and mass spectrometry. Thirty-three major volatiles were identified and it was concluded that the SPME sample preparation technique coupled with GC analysis is well suited for qualitative and semi-quantitative analysis of the major components in wine and, because of its speed and reproducibility, had good potential for wine aroma characterization and quality control applications.

An identical technique using a PDMS fiber was later used for the determination of volatiles from red wines made by carbonic maceration (Section 2.9.2) (Vas and Lorincz, 1999).

Recently, SPME has been optimized for analysis of wine aroma compounds (Demyttenaere *et al.*, 2003a; Peña *et al.*, 2005; Tat *et al.*, 2005). Also a PDMS) SPME-GC method for the analysis of volatile compounds in wines has been validated (Pozo-Bayon *et al.*, 2001).

Headspace solid phase microextraction and gas chromatography (HS-SPME-GC) has also been used for the rapid determination of volatile compounds in grapes (Sánchez-Palomo *et al.*, 2005) and for the determination of major aroma compounds in sweet wines (Rodriguez-Bencomo *et al.*, 2003). Here, five different SPME fibers

were tested, including PDMS 100 μm, PDMS 7 μm, PDMS-divinylbenzene (DVB) 65 μm, PA 85 μm and carbowax (CW)-DVB 65 μm. The influence of different factors, such as temperature and time of desorption, extraction time, stirring, sample and vial volume, sugar and ethanol content, were studied and optimized using model solutions. The method was validated using a direct injection method. It was concluded that the CW-DVB fiber offered the greatest sensitivity when applying an ambient temperature 20 min extraction time with stirring and saturation of the 4 ml samples with sodium chloride in a 16 ml vial. The ethanol and sugar content was shown not to influence the extraction, which allowed the technique to be applied to sweet wine samples.

The aroma volatiles of many other wine varieties, from different origins, have been studied using the SPME technique. Examples include: the classification of Nebbiolo-based wines from Italy (Marengo *et al.*, 2001), the analysis of two different palm wine species from Cameroon (Jirovetz *et al.*, 2001), Tokaji Aszu wine from Hungary (Toth-Markus *et al.*, 2002), extraction of the volatile flavor components of mandarin wine (Selli *et al.*, 2004), the rapid analysis of flavor volatiles in apple wines (Wang *et al.*, 2004; Satora *et al.*, 2008) the characterization of an Italian Piedmont wine (Bonino *et al.*, 2003), the analysis of volatile compounds for the analytical classification of Chinese red wines from different varieties (Zhang *et al.*, 2010) and for the profiling of free volatile compounds in Cabernet Sauvignon grapes (during ripening) and wines (Canuti *et al.*, 2009). It has also been used in combination with principal component analysis as a rapid tool for distinction of wines based on the global volatile signature (Rocha *et al.*, 2006). The technique has also been investigated extensively for the analysis of specific aroma components such as sulfur compounds (Mestres *et al.*, 1998; 1999; 2000; 2002; Fang and Qian, 2005b; Lopez *et al.*, 2007), pyrazines (Sala *et al.*, 2000; 2002; Chapman *et al.*, 2004; Ryan *et al.*, 2005), esters (Vianna and Ebeler, 2001; Rodriguez-Bencomo *et al.*, 2002), volatile phenols (Martorell *et al.*, 2002; Mejias *et al.*, 2003; Pizarro *et al.*, 2007) 2-aminoacetophenone (Fan *et al.*, 2007a), 'brett' character (ethylphenols) (Romano *et al.*, 2009), stereoselective formation of rose oxide during fermentation (Koslitz *et al.*, 2008), assessment of C_{13} norisoprenoids (Vinholes *et al.*, 2009) and diacetyl (Hayasaka and Bartowsky, 1999).

A serious and highly costly problem related to the off flavor called 'cork taint' has been studied using SPME-GC-ECD by Alzaga *et al*. (2003). A headspace SPME procedure at 30 °C with a 100 μm PDMS fiber was optimized for the determination of 2,4,6-trichloroanisole (TCA) with 2,4,6-tribromoanisole (TBA) as internal standard. The researchers reported a limit of quantitation for TCA in wine within 2.9–18 ng/l (parts per trillion) with a relative standard deviation of 2.5–13.4%, depending on the TCA concentration level and wine characteristics. It was concluded that the proposed method was comparable to existing HS-SPME methodologies, however analysis cost was reduced. A similar method for the analysis of TCA in wine and cork samples has recently been proposed (Vlachos *et al.*, 2007).

A headspace SPME method for the determination of 12 haloanisoles in wines and spirits using GC-atomic emission detection (GC-AED) was developed and optimized by Campillo *et al.* (2008a). A DVB-carboxen (CAR)-PDMS fiber was reported to be the most suitable for preconcentration of the target analytes from aqueous dilutions of samples. Limits of detection ranged from 1.2–18.5 ng/l with a fiber exposure time of 60 min at 75 °C.

The SPME technique has also been compared to other established techniques for the analysis of wine flavor. This includes liquid–liquid extraction with pentane and diethyl ether as solvents (Castro *et al.*, 2004), solid phase extraction with ENV+® cartridges (highly cross linked styrene-divinylbenzene polyhydroxylated polymer) for the determination of 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate, whereby the application of the two proposed methods to 52 wines of different varieties gave similar results (Fedrizzi *et al.*, 2007), and extraction with Amberlite® XAD-2 (Bohlscheid *et al.*, 2006). In the latter it was concluded that the optimized HS-SPME extraction conditions provided comparable results to XAD-2 extraction for the analysis of higher alcohols, esters and medium chain fatty acids present in wine. The HS-SPME method

provided a less complicated and time consuming analysis of wine volatiles to subsensory threshold levels, whilst retaining high efficiency and reproducibility.

SPME has been very recently adopted to study the influence of saliva on aroma release from both white and red wines in a model mouth system (Genovese *et al.*, 2009). Aroma compounds were analyzed in the dynamic headspace of wines using a DVB-CAR-PDMS fiber and GC with flame ionization detection (FID) and mass spectrometry (MS). It was demonstrated that saliva showed a greater influence on aroma release in white wine than red wine. White wine treated with human saliva yielded a reduction in esters and fusel alcohols (32–80%), whereas in contrast, the concentrations of 2-phenylethanol and furfural (responsible for rose and toasted almond notes) were increased by 27% and 155%, respectively. In red wine treated with human saliva, relatively few esters showed a decrease in concentration due to the protein binding ability of polyphenols that were able to inhibit the activity of the saliva.

The volatile flavor/aroma compounds of both wort (Section 2.6.2) and beer have very recently been studied using headspace SPME. Fibers with different stationary phases and various film thicknesses were evaluated to optimize wort volatile analysis (De Schutter *et al.*, 2008). PDMS 100 μm, PDMS-DVB 65 μm, CW-DVB 70 μm, CW-PDMS 85 μm and DVB-CAR-PDMS 50/30 μm were all compared for extraction performance by placing 10 ml of wort into 20 ml vials containing 3.5 g of sodium chloride prior to capping. Samples were then equilibrated for 10 min at 40 °C with a magnetic stirring speed of 500 rpm. The SPME fiber was introduced automatically through the septum of the vial and exposed to the headspace of the sample for 20 min at 40 °C, under continuous stirring at 250 rpm. Extraction temperatures were also evaluated, along with extraction time and salting out effect, and it was concluded that the HS-SPME method, combined with gas chromatography-mass spectrometry seemed to be an easy and promising technique for the analysis of volatiles in wort. The DVB-CAR-PDMS fiber with an extraction time of 30 min at 40 °C and the addition of 3.5 g salt to 10 ml wort samples was proven to offer the most complete extraction profile of a broad range of volatiles. The method was reported to allow detection limits of volatile compounds in concentrations as low as 12 ng/l (12 parts per trillion). Moreover, the optimized analytical procedure showed good linearity and was applied to the analysis of wort samples taken from a wort boiling process in an industrial brewery.

A similar optimization method development approach has also been completed for the quantification of 32 volatiles which represent the typical chemical reactions that can occur during the ageing of beer (Saison *et al.*, 2008). Detection was achieved using on-fiber derivatization using *ortho*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA) and conventional HS-SPME extraction. The two procedures were optimized for fiber selection, PFBHA loading temperature and time, extraction temperature and time, and effect of salt addition. Matrix interference effects were overcome by calibrating according to the standard addition method and by using internal standards. The method was finally validated and applied to study the flavor stability of different beer samples. It was concluded that aldehydes showed optimal extraction following loading of a PDMS-DVB fiber with PFBHA for 10 min at 45 °C and subsequent extraction of 30 min after saturation of the beer through the addition of 3.5 g of sodium chloride. Extraction of all other compounds was optimal after extraction for 30 min at 40 °C with a DVB-CAR-PDMS fiber, after saturation of the beer with sodium chloride.

Sulfur compounds of beer have also been studied using the SPME technique. Scarlata and Ebeler (1999) proposed a headspace SPME method with a CAR-PDMS fiber for the GC-flame photometric detection (FPD) of dimethyl sulfide (DMS). It was reported that the technique was rapid, low cost, solvent free, and required no sample preparation steps (i.e. decarbonation) or modification to the GC. Furthermore, combination of HS-SPME with sensitive and selective GC detectors such as the FPD provided quantitative results comparable to those of existing methods. HS-SPME with a CAR-PDMS fiber has been further used in combination

with GC-FPD for the determination of volatile and semi-volatile sulfur compounds in beer at trace levels (Hill and Smith, 2000). PDMS-HS-SPME has also been compared with headspace and direct single-drop microextraction (Xiao *et al.*, 2006) and a CAR-PDMS-HS-SPME method for the determination of volatile organic sulfur and selenium compounds in beers, wines and spirits using GC and atomic emission detection (AED) has recently been reported (Campillo *et al.*, 2009).

The application of SPME and GC-MS to the detection of flavor volatiles present in Irish whiskeys (Section 3.3.2) and Scotch whiskies (Section 3.2.6) was investigated by Fitzgerald *et al.* (2000). A method was developed to characterize, identify and quantify 17 congeners which included fusel alcohols, acetates and esters. The optimum fiber was reported to be PA 85 μm, with an extraction time of 35 min, sample size of 3 ml and desorption time of 5 min. The impact of salt on the sorption process was also studied prior to validation of the method with determination of calibration ranges (coefficients of linearity and relative standard deviations) and detection limits. Characteristic profiles were determined for each of the whisky samples studied and the flavor congeners were quantified using 4-methyl-2-pentanol as the internal standard. The researchers concluded that the optimized SPME-GC method could be considered complementary to the previously used method of direct injection GC, and that it provided many advantages over other methods that have previously been adopted, such as purge and trap, solvent extraction and distillation/extraction.

Blended Scotch whisky has also been analyzed by both SPME and stir bar sorptive extraction (SBSE) (Demyttenaere *et al.*, 2003c). PDMS 100 μm, PA 85 μm and DVB-CAR-PDMS 50/30 μm were compared to analyze whisky flavor volatiles. The authors reported that the best fibers for providing the highest enrichment of volatiles and the highest reproducibility of peak areas following subsequent GC-MS analysis were PDMS and DVB-CAR-PDMS. Optimal extraction conditions consisted of a 30 min extraction time with liquid immersion of the fiber in 17 ml of whisky sample (in a 22 ml crimped glass vial) at 25 °C without addition of salt.

A dynamic HS-SPME-GC-MS method was developed and applied to the quantitative determination of volatile compounds present in commercial whiskey samples, previously diluted to give an alcohol concentration of 13 % (v:v) (Câmara *et al.*, 2007). Experimental conditions tested included fiber coating, extraction temperature and time and the best results were reported to be obtained using a 75 μm CAR-PDMS fiber during headspace extraction at 40 °C with stirring at 750 rpm for 60 min, after saturation of the sample with sodium chloride. The optimized technique was applied to study the volatile profiles of three Scotch whisky samples. Approximately 70 volatile compounds were identified and the authors concluded that HS-SPME-GC-MS is a simple sample preparation procedure offering great capacity of concentration and combined extraction and concentration in a single step providing rapid, sensitive and solventless methodology highly applicable to the determination of volatiles and semi-volatile components in whisky samples.

Headspace SPME has recently been compared with conventional liquid–liquid extraction for the evaluation of volatile constituent patterns in commercial whiskeys (Caldeira *et al.*, 2007). Seven extraction solvents covering a wide range of polarities and two SPME fibers were evaluated for their effectiveness in extraction and quantification of volatile compounds. Highest recoveries of volatile compounds were achieved using dichloromethane as solvent for liquid–liquid extraction and using a CAR-PDMS fiber for HS-SPME. Both sample preparation methods were used to determine the responses of 25 analytes from whiskey samples and calibration standards, in order to provide sensitivity comparisons between the two methods. Calibration curves were established using a model whiskey and linear correlation coefficients were greater than 0.9929 for liquid–liquid extraction and 0.9935 from SPME for all target compounds. Recoveries greater than 80% were reported to be achieved with good precision. The authors recommended that for a complete and quantitative study of volatile composition of food beverages, two or more sample preparation techniques should be adopted.

Various parameters affecting the extraction efficiency have been tested to find suitable SPME sampling conditions to enable the characterization of the volatiles of different dry gin samples (Section 3.4.2) (Vichi *et al.*, 2005). Headspace-SPME, using a 50/30 μm, 2 cm long DVB-CAR-PDMS fiber coupled to GC-MS was developed and applied for the qualitative and semiquantitative characterization of distilled gin volatiles. To evaluate the effect of ethanol content on extraction efficiency, a gin sample was diluted with deionized water to yield alcohol concentrations of 5, 10, 20 and 40 % v:v. Various sampling temperatures (30, 40 and 50 °C) and sample volumes (1, 1.5, 2.5, and 5 ml) were tested. To determine optimum extraction time the fiber was held in the sample headspace for periods of 15, 30 and 45 min. After comparison of the relative MS detector responses, the optimized SPME conditions were found to be 2.5 ml of gin sample diluted at 10% ethanol (v:v) placed into a 10 ml vial fitted with a silicone septum. Samples were then placed in a silicon oil bath at 50 °C and maintained under magnetic stirring at 700 rpm. After 5 min of sample conditioning, the fiber was exposed to the sample headspace for 30 min and then immediately desorbed in the GC injector at 260 °C. The authors reported that 70 components (mainly mono- and sesquiterpenic compounds) of the gin sample's volatile fraction were isolated, tentatively identified or identified by reference aroma compounds. The comparative study of London dry gins and gins with geographical indication (Section 3.4.2) permitted clear differentiation between these sample sets, and the authors concluded that the results obtained may be useful for further sensory studies and that the terpenic composition of gin may also be used to distinguish samples from different commercial brands and gins with geographical indication and to indicate the type and proportions of botanical ingredients employed for gin aromatization (Section 3.4.2).

Vichi and coworkers (2008) have also recently carried out the assessment of some diterpenoids in commercial distilled gin using direct immersion SPME (DI-SPME). A multilevel factorial experimental design was adopted to optimize the methodology coupled to GC-MS and used to analyze eight commercial brands of gin. The variables tested included sample dilution, extraction temperature, salt concentration and extraction time. For all experimental design testing, 10 ml of gin was placed into a 10 ml vial fitted with a silicone septum and immersed in a silicon oil bath where the sample was magnetically stirred at 700 rpm. After 10 min of sample conditioning, the fiber was directly immersed in the sample for a range of times and immediately desorbed in the GC injector at 265 °C. The optimum conditions were reported to be with a DVB-CAR-PDMS fiber, sample dilution with bidistilled water to 50% of gin, extraction temperature and extraction time of 60 °C and 60 min, respectively, with no salt addition. It was concluded that for the first time a simple and reliable DI-SPME/GC-MS method with high sensitivity had been developed that enabled the detection and quantification of diterpenoids in juniper flavored spirits. As the diterpenic composition of gin is related to the juniper used for aromatization and to the production process, the authors stated that the method may also be useful for the characterization of distinct types and commercial brands of juniper based spirits, thereby guaranteeing the authenticity and hence quality of the product.

Fatty acid ethyl esters are the main components of rum aroma (Section 3.5.4) and play an important sensorial impact in this type of distilled alcoholic beverage (Pino *et al.*, 2002). Various HS-SPME (with PDMS fiber) variables were studied and optimized including effects of ethanol concentration, sample ionic strength, sample volume and extraction temperature and time. The study demonstrated that the high ethanol content in distilled alcoholic beverages was problematic and interference was minimized by dilution of samples to provide 12%, v:v of ethanol. The addition of salt to samples (3 M concentration) was selected, as this was reported to increase ester extraction and not ethanol extraction. Extraction times of 30 min at 25 °C in 20 ml vials provided increased extraction efficiencies compared to those obtained with 50 ml vials, due to the reduction of the equilibrium time when a smaller headspace was used, hence a sample volume of 10 ml in a 20 ml vial was selected. Finally, extraction times of 10, 35 and 60 min were tested at 10, 30 and 50 °C and the optimum results were obtained in 35 min at 30 °C. Validation of the optimized method was reported to provide recoveries greater than 91% for the analytes of interest with limits of detection between 0.007 and 0.027 mg/l (ppm), all of which were lower than the range found in the real rum samples

under investigation. This method has been further adopted for the characterization of rum, leading to a total of 184 constituents being identified in the SPME analyses, 68 of which were reported for the first time in rum (Pino, 2007). The author concluded that semi-quantitative analysis (based on peak area percentage) showed very good reproducibility and with the use of standard calibration curves, the method could be applied to measure absolute concentrations. The use of only 15 volatile compounds permitted discrimination between rum samples of three and seven years old.

Zhao and coworkers (2009) devised a headspace SPME-GC-MS method to study the volatile profile of brandy. Before analysis, samples were diluted to approximately 14% (v:v) ethanol with deionized water, saturated with sodium chloride and a 10 ml portion placed into a 20 ml glass vial spiked with 5 μl of internal standard solution (3-octanol, 10 mg/l in ethanol) along with magnetic stirring bar for agitation before sealing with a Teflon faced silicone septum for analysis. Samples were then placed into an isothermal bath held at 50 °C for 15 min to reach equilibrium before extraction. Three fibers, coated with 100 μm PDMS, 75 μm CAR-PDMS and 50/30 μm DVB-CAR-PDMS were evaluated for the extraction of the brandy aroma compounds. The authors observed that PDMS exhibited greater extraction affinity for non-polar compounds than the mixed fibers, whereas the DVB-CAR-PDMS fiber extracted more polar and mid polar compounds and showed the best results overall for the extraction of the volatile compounds of interest. Brandy samples were then extracted for 20, 30, 40 and 60 min and it was demonstrated that 30 min was required to reach extraction equilibrium. Various extraction temperatures were also studied (30, 40, 50, 60 and 70 °C) and it was found that volatile compounds extracted increased with temperatures up to 50 °C, but the extraction efficiency for lower molecular weight dropped dramatically when the temperature was increased to 70 °C. Using the optimized method to study the volatile profile of the real samples, the authors achieved identification of 144 compounds, of which 57 were common to all 11 samples. The volatiles identified were mainly represented by esters and alcohols, such as 2-methyl propanol (isobutyl alcohol), 3-methyl butanol (isoamyl alcohol), 1-hexanol, ethyl octanoate and ethyl decanoate, which were quantitatively determined. Principle component analysis (PCA) and cluster analysis (CA) were applied to study the relationships between volatile composition and brandy, and this enabled the samples to be differentiated into three groups consistent with age and quality.

Previously both SPME and liquid–liquid extraction with freon 11 had been adopted for the analysis of brandy obtained from a commercial Californian distillery (Ebeler *et al.*, 2000). It was reported that, in general, SPME using a PDMS fiber was more selective for analysis of esters and acids than liquid–liquid extraction (LLX). In contrast, LLX with freon 11 extracted the higher alcohols more efficiently than SPME and it was recommended that for a complete and quantitative picture of the volatile composition of foods and beverages, two or more sample preparation techniques should be used.

Tequila (Section 3.5.5) volatile characterization and ethyl ester determination has recently been carried out with SPME-GC-MS as the method of choice (Vallejo-Cordoba *et al.*, 2004). Several factors were observed to determine qualitative and quantitative differences in tequila volatile profiles, namely sampling mode (headspace or immersion extraction), fiber coating and fiber exposure time. Along with other researchers of alcoholic beverage aroma components utilizing SPME as the extraction technique, fused silica fibers coated with PDMS (100 μm), PDMS-DVB (65 μm), CW-DVB (65 μm), and PA (85 μm) were compared for extraction qualities. For direct liquid (immersion) sampling, the syringe of the SPME device was introduced through a septum into a 60 ml vial where a 40 ml sample was held at 40 °C in an Orbit shaker bath (75 rpm). For headspace sampling, the fiber was exposed to the headspace above a 40 ml sample saturated with 28% sodium chloride and once again maintained at 40 °C in the identical Orbit shaker bath. Sampling times of 30, 60 and 90 min were studied and thermal desorption was carried out in the gas chromatographic inlet for 5 min at 150 °C for all samples. Optimum conditions were with a PDMS fiber immersed in the liquid sample for 60 min and the ethyl esters (hexanoate, octanoate and decanoate) were successfully quantitatively determined following construction of linear calibration curves with highly significant determination coefficients ($R^2 = 0.99$) and extraction coefficients of variation (reproducibility) less than 10%. Limits of quantitation (LoQ)

for ethyl esters was reported to be 0.05 ppm, which was below the concentration range of the tequila samples studied.

An extensive study by Rodrigues *et al.* (2008) utilized headspace SPME-GC-MS for the determination of volatile and semi-volatile compounds from wine, beer and whisky samples. Key experimental factors such as fiber coating, extraction time and temperature, sample stirring and ionic strength were optimized for each beverage type. The data obtained suggested that use of a DVB-CAR-PDMS fiber provided effective sample enrichment and enabled extraction of a wide variety of compounds from beer samples, whereas PDMS was identified to be the most applicable to wine samples and CAR-PDMS for whisky analyses. The optimal extraction conditions were reported to be 30 ml of sample with an extraction time of 60 min at 30 °C for wine and beer matrices and 40 °C for whisky, by headspace mode of a stirred sample saturated with NaCl (30% w:v). More than 64 compounds in beer, 44 in white wines and 104 in whisky were identified, belonging to a wide range of chemical classes, such as ethyl esters, higher alcohol acetates, isoamyl esters, fattyacids, carbonyl compounds, furanic compounds, terpenoids, C_{13}-norisoprenoids and volatile phenols.

Many other alcoholic beverages have been successfully studied using the SPME technique. Examples include cider – with a DVB-CAR-PDMS mixed phase fiber for the determination of brett character (Pizarro *et al.*, 2009), volatiles of the Brazilian distilled spirit cachaça (Section 3.5.4) using a PA fiber (Nonato *et al.*, 2001; de Souza *et al.*, 2009) and the determination of volatile compounds in *orujo* spirits with a CW-DVB fiber (García-Martín *et al.*, 2010).

A PA fiber SPME method combined with GC-MS, GC-NPD and GC-FID was developed and optimized for the analysis of 22 nitrogen containing herbicides (Section 5.10.2) in water (Boyd-Boland and Pawliszyn, 1995). The proposed method was concluded to be linear over several orders of magnitude, with either of the chosen GC detectors and analysis of the target analytes from more complex matrices, such as wine, was also successfully performed by utilization of the standard addition procedure.

SPME analysis with PA polar fiber phase has also been chosen for the rapid screening of triazole (Section 5.10.2) residues in wine and strawberries (Zambonin *et al.*, 2002) and also organophosphorus pesticide residues (Section 5.10.2) in wine and fruit juices (Zambonin *et al.*, 2004).

A multiresidue SPME-GC-ECD method for pesticide screening in wine was proposed by Correia *et al.* (2000). Following optimization, a 100 μm PDMS fiber, with 30 min immersion extraction in 3 ml wine samples at 45 °C was used to study pesticide extraction yield at different concentrations of aqueous ethanol solutions representative of Portuguese wines. It was concluded that the method enabled detection of 19 of the 23 pesticides studied, however ethanol content of samples affected the extraction yield and calibration curves with extracted standards was recommended. Extraction yields did not exceed 30%, however limits of detection (average 5 μg/l) were reported to be considerably lower than the recommended maximum levels for the chosen pesticides in commercial wine products.

PDMS fibers have also successfully been applied to the SPME-GC-MS determination of oxazole fungicides in malt beverages (Viñas *et al.*, 2008), the determination of the herbicide oxadiazon residues in ground water, soil, must, wine and human urine samples (Navalón *et al.*, 2002) and to study the distribution of fenbutatin oxide in grapes and white wine (Montes *et al.*, 2009a).

Recently, Carillo and coworkers (2007) described the development of a headspace SPME-GC-MS method for the accurate determination of phthalates in wine. The HS-SPME conditions were rigorously studied by firstly evaluating the performance of six fibers at three temperature values and two sample volumes, by means of a multifactor categorical experimental design. The most applicable fibers were shown to be PDMS-DVB and CW-DVB. Repeatability values obtained for both fibers were reported to be similar, however the CW-DVB was more efficient for extracting the lower molecular weight phthalates and the PDMS-DVB for the higher molecular weight analytes of the study. It was concluded that the proposed method enabled the quantification of phthalate esters in wine in the low ng/ml range, however quantitation by the standard addition method was

required in order to avoid systematic errors caused by matrix effects. Later work adopted the successful use of deuterated phthalates as internal standards to eliminate the standard addition requirement and thus reduce analysis time (Carrillo *et al.*, 2008).

Recently a simple, low cost and sensitive method for the determination of phthalate acid esters in beer was developed and optimized using SPME-GC-MS with a novel sol-gel calixarene-contained fiber (Ye *et al.*, 2009). Under optimized conditions, LODs of 0.003–3.429 μg/l were obtained and the relative standard deviation values were ≤13.51% for all of the analytes studied. The method was further validated using standard addition methodology and recovery values were between 86.3 and 109.3%. The analysis of three real bottled beer samples demonstrated that dibutyl-phthalate ester and bis(2-ethylhexyl)-phthalate ester (DEHP) were the main phthalates found in beer and total concentrations were between 6.22 and 7.76 μg/l. Following migration tests, it was revealed that the high content of DEHP incorporated in PVC gaskets in the lids of the bottled samples could have been a potential source of contamination during transportation and subsequent storage.

By the nature of its simplicity and performance, SPME has already secured itself a concrete position in sample preparation/analytical history and has created a huge level of interest as a sorptive extraction technique since its introduction. Many advantages, such as predictable enrichment, inertness and rapid thermal desorption at mild temperatures, are well documented in the literature. One major drawback of SPME, however, is the amount of extraction medium coated on the fiber. For example, a typical PDMS 100 μm fiber has an approximate 0.5 μl volume of extraction phase. Consequently, the extraction efficiency (recovery) for solutes that are partially water soluble can be quite low and for very non-polar (hydrophobic) compounds, competition can occur between the aqueous phase, the SPME fiber, the glass wall of the extraction vessel and the surface of the polytetrafluoroethylene (PTFE) stir bar used to stir samples (David *et al.*, 2003).

Following these observations, a novel extraction method was developed whereby standard laboratory magnetic stir bars were coated with a layer of PDMS and then used to stir aqueous samples, thereby extracting and enriching analytes into the PDMS coating. The technique was named stir bar sorptive extraction or SBSE (Baltussen *et al.*, 1999). With SBSE, the extraction phase is identical to that used on PDMS coated SPME fibers, however the coating typically uses 50–250 times greater amounts of extraction phase.

Sorptive extraction is an equilibrium technique and for water samples, the extraction of solutes/analytes from an aqueous matrix into the PDMS extraction phase is controlled by the distribution coefficient of the solutes partitioned between the PDMS phase and the aqueous phase. This is identical in principle to a standard liquid–liquid extraction of an aqueous or aqueous ethanolic sample with an immiscible organic solvent. Recently this partitioning coefficient theory has been correlated with octanol–water distribution coefficients ($K_{o/w}$). Although this principle is not strictly correct, the coefficient gives, in most cases, a successful, rapid and hence practical indication of potentially how well a given analyte can be extracted with SPME or SBSE. It is, however, very important to realise that the sorptive equilibrium is also dependent upon the phase ratio and thus on the amount of PDMS coated to the stir bar. This relationship is shown in Equations 4.2.3 and 4.2.4 (Baltussen *et al.*, 1999; David *et al.*, 2003).

$$\begin{aligned} K_{o/w} \approx K_{PDMS/w} &= \frac{C_{PDMS}}{C_w} \\ &= \left(\frac{m_{PDMS}}{m_w}\right)\left(\frac{V_w}{V_{PDMS}}\right) \\ &= \beta\left(\frac{m_{PDMS}}{m_w}\right) \end{aligned} \tag{4.2.3}$$

The distribution coefficient between PDMS and water ($K_{PDMS/w}$) is defined as the ratio between the concentration of a solute in the PDMS phase (C_{PDMS}) over the concentration in the water (C_w) at equilibrium. This ratio is equal to the ratio of the mass of the solute in the PDMS phase (m_{PDMS}) over the mass of the solute in the aqueous phase (m_w) times the phase ratio (β, with β = volume of water (μl) divided by the volume of PDMS (μl) = V_w/V_{PDMS}).

The recovery, expressed as the ratio of the extracted mass of solute (m_{PDMS}) over the original mass of solute in the water ($m_0 = m_w + m_{PDMS}$), is thus dependent upon the distribution coefficient $K_{PDMS/w}$ and on β, as described in Equation 4.2.4.

$$\frac{m_{PDMS}}{m_0} = \frac{\left(\frac{K_{PDMS/w}}{\beta}\right)}{1 + \left(\frac{K_{PDMS/w}}{\beta}\right)} \tag{4.2.4}$$

Using this equation, the theoretical recovery can be calculated for a solute/analyte with a known partition coefficient and a given phase ratio. For example, the theoretical recovery for limonene (CAS 5989-27-5) extracted from a 10 ml aqueous sample volume with a 10 mm stir bar with a phase thickness of 0.5 mm (24 μl) is as follows:

$$\text{Log}K_{o/w}\text{value of limonene} = 4.83$$
$$\text{Phase ratio } (\beta) = V_w / V_{PDMS} = 10000/24(\mu\text{l}) = 417$$

If:

$$\log K_{o/w} = 4.83, \text{ then} K_{o/w} = \log 10^{4.83} = 67608$$

Therefore:

$$\text{Theoretical recovery } (R) = \frac{(67608/417)}{1 + (67608/417)} = 0.99$$

Thus:

$$\%\text{ recovery} = 0.99 \times 100 = 99\%$$

It is therefore evident that extraction efficiency for any given analyte is increased with increasing $K_{PDMS/w}$. Because $K_{PDMS/w}$ is similar to the octanol–water distribution coefficient ($K_{o/w}$), extraction efficiencies can be predicted. Besides the $K_{PDMS/w}$ factor, the phase ratio (β = volume sample/volume PDMS) is also important. The higher the PDMS amount, the lower the phase ratio and the higher the extraction efficiency (David *et al.*, 2003).

Figure 4.2.8 shows the influence of $K_{o/w}$ and phase ratio on extraction efficiency. For SPME, the volume of PDMS coated to the fiber is approximately 0.5 μl. This results in poor recoveries for solutes with lower $K_{o/w}$ values (less than 10 000 or with a log $K_{o/w}$ value less than 4). In SBSE, greater quantities of PDMS (typically 25–125 μl) coatings are adopted, hence the sensitivity is increased by a factor of 50 to 250 when directly compared to SPME as a sample preparation method. The theoretical extraction efficiency reaches 100% for solutes with $K_{o/w}$ values larger than 500 (log $K_{o/w}$ greater than 2.7). The theoretical recoveries

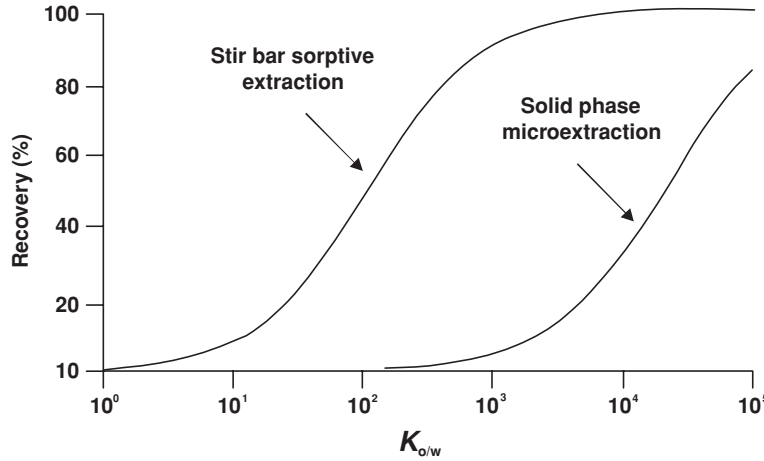

Figure 4.2.8 *Recovery for solutes in function of the octanol-water partitioning coefficient* $K_{o/w}$ *for SPME (10 ml sample, 100 mm PDMS fiber) and for SBSE (10 ml sample, 10 mm x 0.5 mm PDMS-coated stir bar) (David* et al.*, 2003). Reproduced with permission of F. David*

can be calculated for a given sample volume, selected stir bar dimensions and a solute using the $K_{o/w}W$ in a software program (Meylan and Howard, 2000).

Poly(dimethylsiloxane) coated stir bars are manufactured and available from Gerstel GmbH (Mülheim/Ruhr, Germany) under the trade name Twister™. These stir bars have three essential parts (see Figure 4.2.9). The first part at the center of the stir bar is a magnetic stirring rod, which transfers the rotating movement of a stirring plate to the sample liquid. The second part of the stir bar is a thin glass jacket that covers the magnetic stirring rod. The third and outermost part is the sheath or layer of PDMS sorbent into which the analytes are extracted.

To date, only stir bars with PDMS as extraction phase are available commercially (i.e. a non-polar phase), however dual phase bars combining the concentration capabilities of two or more sampling materials operating in different ways (sorption and adsorption) have been described elsewhere and will most likely be available in the future (Bicchi *et al.*, 2005). These new stir bars are reported to consist of a short PDMS tube, the ends of which are closed with two magnetic stoppers, thus creating an inner cavity that can be packed with different types of adsorbents like activated carbons. It was concluded by the authors that the new twister concept was

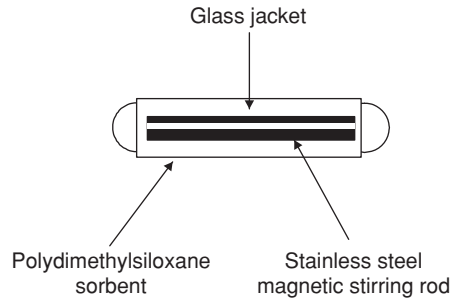

Figure 4.2.9 *Stir bar sorptive extraction device (Twister™) (Lisa Caven-Quantrill)*

shown to improve recovery of volatile and/or polar components when compared to conventional PDMS stir bars.

Interestingly, Gerstel, the commercial manufacturer of the Twister™ device, demonstrated a prototype stir bar with poly(acrylate) (PA) phase at the 2009 Pittsburgh analytical conference (http://www.pittcon.org/) so it is more likely than not that this SBSE option will also soon be available as a standard choice to the practicing analytical chemist.

SBSE of a liquid sample is carried out easily by placing a suitable amount of sample in a headspace vial along with a stir bar. The sample is then typically stirred for 30–240 min. The extraction time is controlled kinetically, determined by sample volume, stirring speed and stir bar dimensions, and must be optimized for a given application (David *et al.*, 2003). This is generally accomplished by measuring the analyte recovery as a function of the extraction time (i.e. optimum conditions are achieved when no additional recovery, or increase in GC-MS peak area counts of analytes of interest, is observed, even if the extraction time is increased further).

On completion of the extraction, the stir bar is removed, rinsed with a small aliquot of analytical grade water (to remove adsorbed co-extracted matrix components) and blotted dry on a clean, lint free tissue to remove water droplets. Finally the stir bar is introduced into a preconditioned empty glass tube for thermal desorption on a GC-MS instrument. Desorption temperatures are application dependent, but typically between 150 °C and 300 °C. Desorption can be accomplished in 5–15 min under a 10–100 ml/min Helium flow (David *et al.*, 2003).

Hoffmann *et al.* (2000) used SBSE and thermal desorption-gas chromatography for the flavor profiling of several alcoholic beverages, including Spumante wine. With this technique, a small magnetic stir bar (10–20 mm length, 1.3 mm outer diameter) coated with PDMS, was placed directly into the sample, which had been previously transferred to a 10 ml headspace vial with minimal headspace, and stirred for either 0.75 to 2 h or overnight (16 h). On completion of the extraction, the stir bar was removed from the sample with forceps, rinsed briefly with distilled water to remove any matrix components such as sugars, blotted dry on a lint free cloth and placed into a clean glass thermal desorption tube for analysis. Analytes were desorbed at 200 °C for 5 min with a 50 ml/min gas flow and cold trapped in a programmable temperature vaporizer with a glass wool liner at –150 °C. Samples were finally transferred to the gas chromatographic column in either split or splitless mode. It was concluded that SBSE was an extremely powerful technique for flavor profiling of different types of beverages since it combined ease of use, ruggedness, precision, speed and sensitivity. In addition, the absence of any organic solvents involved in the sample preparation and analysis made the methodology 'environmentally friendly.' A similar technique has been used recently for the analysis of volatile phenols in wine by GC-MS (Díez *et al.*, 2004). It was concluded that the technique was an easy, fast and reliable analytical method for the quantitative determination of volatile phenols in wines. The procedure was reported to be simple and allowed 15 samples to be extracted simultaneously using a very small sample volume. A low detection limit with good sensitivity was also obtained.

Recently, SBSE coupled with GC-MS was used to analyze wine samples for three applications: flavor and compositional analysis, 2,4,6-trichloroanisole (TCA), a common off-aroma in wine, and agrochemicals (Hayasaka *et al.*, 2003). SBSE was found to be several orders of magnitude more sensitive than modern conventional methodology, allowing for lower detection and quantitation levels and improved confirmation of identity; SBSE often gave better signal to noise in scan mode than other methods in selective ion monitoring (SIM) mode. With the help of their characteristic mass spectra, all agrochemicals could be identified unambiguously at concentrations of 10 μg/l in wine and a further 100 constituents were detected in a Cabernet Sauvignon sample. It was concluded that it was now possible to analyze complex samples such as wine in scan mode, with better confirmation of identity and without sacrificing sensitivity, where previously SIM methodology had to be used.

Caven-Quantrill and Buglass (2006) compared traditional microscale simultaneous steam distillation-extraction (SDE) and SBSE for their effectiveness in the extraction of volatile organic compounds from a

synthetic grape juice and a real grape juice (Huxelrebe) from an English vineyard. The novel immersion mode SBSE method, using stir bars with PDMS sorbent, was optimized using a synthetic grape juice. Although mean percentage relative recoveries and reproducibilities (%CV) of the SBSE method were inferior to SDE (28.4 and 8.5% against 86.9 and 6.3%, respectively), the stir bar method proved to be significantly more sensitive: 126 aroma compounds in Huxelrebe grape juice were identified using SBSE, against 98 using SDE, and five of these were considered as thermal decomposition products of sugars. This allowed the identification of a number of volatile components that had not been reported previously in the juice or wine from the grapes with Muscat ancestry.

The authors concluded that the optimized and validated SBSE method was shown to be a very promising tool for characterizing the aroma compounds of grape juice.

Overall, the microscale SDE method showed excellent recoveries and reproducibility for a majority of the synthetic grape aroma volatiles extracted from a model grape juice, at the concentration studied (400 μg/l of each component).

Although the relative recoveries and reproducibility were generally lower for the SBSE technique, it was emphasized that it was a more sensitive technique than SDE, as the concentrations of aroma compounds in the synthetic grape juice used for SBSE (10 μg/l of each component) were 40 times lower than those used in the SDE study. Moreover, in combination with state of the art analytical instrumentation, the SBSE technique could result in higher sample throughput, as up to 15 samples could be stirred simultaneously. The technique was also solvent free, easy to use, required minimal manpower per sample and the thermal desorption/GC-MSD analysis could be carried out automatically. Furthermore, the whole extract from the stir bar was transferred to the analytical system, which resulted in a higher sensitivity and lower potential analyte loss. Hence, the method could easily be applied to quality control and other related fields. This method was then further adopted for the volatile organic compound analysis of English vineyard Huxelrebe, Ortega, Schönburger and Siegerrebe wine grapes of the 2004 season and Madeleine Angevine 7672 of the 2000 vintage (Caven-Quantrill and Buglass, 2007) and up to four vintages of these varieties to study the seasonal variation of flavor content (Caven-Quantrill and Buglass, 2008).

Similarly, the SBSE technique was used to determine volatile constituents in Monastrell grapes (Salinas *et al.*, 2004). The method proposed involved a maceration step lasting two hours and a sorption time of six hours. The coefficients of variation obtained were below 4% in the case of volatile compounds and below 2% for the internal standard. The behavior of the volatiles during grape ripening was studied, and 34 compounds were determined, some of which were recorded for the first time in this variety. The highest terpene and norisoprenoid contents, together with the lowest values of C6 compounds were observed during the fifth week of ripening, meaning that this would be the optimal time for harvesting in terms of volatile compounds.

SBSE has also been used for the determination of volatile compounds in oak-aged wines (Marín *et al.*, 2005; Garde-Cerdán *et al.*, 2008b), for the enantioselective analysis of monoterpenes in different grape varieties during berry ripening (Luan *et al.*, 2006), for the analysis of wine primary aroma compounds (Zalacain *et al.*, 2007), for off-flavor profiling (Franc *et al.*, 2009), for classification of South African wines according to the volatile composition (Tredoux *et al.*, 2008), to study the impact of deficit irrigation (crop sensitivity to drought stress) during berry development on Merlot wine volatile composition (Qian *et al.*, 2009) and for the characterization of the aroma profile of Madeira wine (Sections 2.10.5 and 2.10.6), where the technique was compared with SPME (Alves *et al.*, 2005). Here the authors concluded that the SBSE methodology allowed better ability for profiling traces and ultra traces of esters, carboxylic acids, alcohols, aldehydes, pyrans, lactones, monoterpenes and C_{13} norisoprenoids.

Recently, SBSE-GC-MS has been used for the analysis of hop derived terpenoids in beer (Kishimoto *et al.*, 2005). Stir bars (20 mm length) coated with 47 μl of PDMS were added to diluted beer samples, and stirred

in a water bath at 40 °C for 2 h prior to removal and subsequent analysis by thermal desorption-GC-MS. The SBSE technique was reported to offer low coefficients of variation, high accuracy and low detection limits.

As an extension of SBSE, headspace sorptive extraction (HSSE) was first developed by Tienpont *et al.* (2000) and Bicchi *et al.* (2002) for the analysis of volatile compounds of solid matrices. In HSSE, the stir bar is suspended in the headspace volume (as with HS-SPME) from where the analytes are sorbed by the PDMS coating. After sampling, the stir bar is placed in a desorption tube and treated identically to SBSE analyses. Applications of this new HSSE technique and its comparison with other extraction methods have been assayed in different matrices (Bicchi *et al.*, 2000; Demyttenaere *et al.*, 2003b; Demyttenaere *et al.*, 2004), however these are not related to the profiling of aroma materials from alcoholic beverages. Only a single research group using HSSE for alcoholic beverage flavor analysis could be identified during the compilation of this chapter (Weldegergis *et al.*, 2007; Weldegergis and Crouch, 2008). The HSSE method was developed for the analysis of alcohols, esters, carbonyls, acids, phenols and lactones in South African wine samples. Extraction conditions were evaluated and it was reported that the optimum conditions consisted of adding 1 ml of wine sample (along with 100 μl of 1.7 mg/l 4-methyl-2-pentanol internal standard solution and 1.5 g of NaCl) and diluting to a total of 6 ml with ultrapure water of 12% ethanol mixture in a 20 ml Gerstel Twister™ headspace vial with glass headspace insert. The pH was adjusted to 3.2 using a formate buffer prior to the addition of a glass coated magnetic stirrer to agitate the sample and a preconditioned 'standard' SBSE stir bar (10 mm length coated with 0.5 mm (25 μl) PDMS layer), which was suspended in the headspace using the glass insert. Following sealing of the vial with a 20 mm aluminum crimp cap and PTFE/silicone molded septa, the spiked sample was stirred for 1 h at 1200 rpm prior to the vial being left standing for 3 h at room temperature (to allow analytes that had migrated into the headspace of the sample to be completely sorbed into the PDMS coating of the stir bar). After sampling, the stir bar was removed, dried with a lint free tissue and placed into a preconditioned thermal desorption (TD) tube ready for analysis by TD GC-MS. The authors concluded that the optimized method was very sensitive, with limits of detection (LODs) and limits of quantitation (LOQs) between 50 pg/l to 299 μg/l and 0.2 ng/l to 0.996 μg/l, respectively, and acceptable repeatability.

A further and most recent spin-off sorptive technique involves SBSE with liquid desorption (Coelho *et al.*, 2008; Coelho *et al.*, 2009; Perestrelo *et al.*, 2009). With this method, SBSE is performed as standard, however on completion the stir bar is removed from the liquid sample extraction vial and back extracted with organic solvent (for example, pentane). The resulting liquid extract is then subjected to large volume injection on the GC instrument. The advantage of this approach is that a dedicated (and often expensive) thermal desorption device is not required, thus lowering the initial financial outlay of this very useful and sensitive sorption technique. Many other examples of this approach are expected to be seen in future flavor research studies.

A method for the determination of stale flavor carbonyl compounds in beer was developed using SBSE with *in situ* derivatization followed by thermal desorption-GC-MS analysis (Ochiai *et al.*, 2003). The derivatization conditions with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine and the sampling mode, salt addition, sample volume, phase ratio (sample volume/PDMS volume) and extraction time for the SBSE conditions were studied. The authors concluded that a sample volume of 30 ml, a PDMS volume of 47 μl and extraction time of 60 min were optimum conditions, and this resulted in the successful application of the methodology to determine the stale flavor carbonyl compounds (*E*)-2-octenal, (*E*,*Z*)-2,6-nonadienal, (*E*)-2-nonenal and (*E*,*E*)-2,4-decadienal in real beer samples. The method was reported to show good linearity over the concentration range studied (0.1–10 ng/ml) for all analytes and the correlation coefficients were higher than 0.9993. The limits of detection ranged from 0.021–0.032 ng/ml for all analytes (which was below the reported odor thresholds (Section 4.7.2) of the extremely important and most feared stale flavor compounds (*E*)-2-octenal and (*E*)-2-nonenal), and the recovery of the method was good (98–101%) with acceptable precision (RSD 2.4–7.3%).

Recently Horák *et al.* (2008) developed a method for the determination of free medium chain fatty acids including caproic, caprylic, capric and lauric acids in beer or wort using SBSE and solvent back extraction. Extraction was performed by placing 10 ml of sample into a 20 ml headspace vial along with a 'standard' 10 mm stir bar with a phase thickness of 0.5 mm (24 μl) PDMS. After crimping of the vial with an aluminum coated septum, the sample was stirred at ambient temperature at 1000 rpm for 60 min, then the stir bar was removed, rinsed briefly with distilled water and dried with a lint free tissue. Solvent back extraction was achieved by placing the stir bar into a 350 μl glass vial insert containing 200 μl of organic solvent mixture (dichloromethane:hexane, 50:50) and this was collectively placed into a 2 ml vial and stirred again at 1000 rpm for 40 min. It was concluded that the proposed method was advantageous in many practical aspects, such as small sample volume, simplicity of extraction and only 200 μl of solvent consumption, and offered high repeatability (RSD <6.7%), good linearity (correlation coefficients higher than 0.9963 for quadratic curves over the concentration range of 0.5–8.0 mg/l) and recoveries (57–89%). This method has also recently been compared with SPE and SPME by the same research group (Horák *et al.*, 2009). Results of beer analyses obtained by using these three methods were reported to be highly correlated, however SPME was suggested as overall a more appropriate technique for medium chain fatty acid analysis.

SBSE has also been compared directly with SPME for extraction of flavor volatiles from malt whisky (Demyttenaere *et al.*, 2003c) (Section 3.2.6). Here it was reported that the best fibers for SPME (resulting in the highest reproducibility of the peak areas after GC-MS analysis) were PDMS and DVB-CAR-PDMS, with a 30 min extraction time by liquid immersion of the fiber in a stirred sample at 25 °C without the addition of salt. SBSE was also demonstrated to be an appropriate method for whisky volatile extraction, however it was stressed that the major drawback with this technique was the requirement of a dedicated thermal desorption device. When SBSE was applied in the split desorption–split injection mode, it did not significantly improve the results obtained by SPME. In contrast, when splitless desorption–split injection was utilized, SBSE yielded a higher enrichment of the whisky volatiles with good chromatography (well resolved sharp peaks). For ultratrace analysis, the use of SBSE in splitless desorption–splitless injection was advised to achieve the highest sensitivity of all.

The dicarboximide fungicides vinclozolin, iprodione and procymidone were analyzed in white wines by SBSE in combination with thermal desorption-capillary GC-MS analysis (Sandra *et al.*, 2001). Limits of quantitation (LOQ) in the full scan MS mode (from method optimization using spiked water samples) were reported to be 0.5 μg/l for vinclozolin and procymidone and 5 μg/l for iprodione. With an MS in selective ion monitoring mode, concentrations 100 times lower could be spiked and analyzed successfully. Because of wine matrix effects on the recoveries of the analytes studied, quantification of the target fungicides in wine had to be carried out using the standard addition method. Procymidone and iprodione were detected in wines in concentrations up to 65 μg/l, whereas the highest concentration of vinclozolin was 3 μg/l.

4.2.5 Headspace Methods

In a static headspace method, the sample is placed into a sealed vial, left to equilibrate and the atmosphere above the sample is drawn into a gas-tight syringe (usually 0.1–2.0 ml) and subsequently injected into a gas chromatograph (Da Costa and Eri, 2004). It is easily automated as an analytical technique; indeed many of the commercial analytical instrument manufacturers offer equipment which has the option of heating sample vials and injecting a known volume of the sample headspace directly into a gas chromatograph (GC). Two main types of sample introduction are commonly available. This can either be by the transfer of a measured sample loop of headspace to the GC, often via a heated transfer line (that connects directly to the GC inlet) or by

simple automation of a headspace syringe. In either case, modern sophisticated autosamplers have the ability to carefully control critical method variables such as the temperature of the sample and the equilibration time.

The static headspace sampling technique has limited applicability to the analysis of alcoholic beverages as these samples contain high quantities of water and ethanol. Since the concentration of these matrix components in the headspace (during sampling) is in equilibrium with the concentration in the liquid beverage sample, the headspace is saturated, and subsequently this results in a lack of analytical sensitivity for the aroma/flavor compounds of interest, that are often at concentrations several orders of magnitude lower than the water and ethanol.

The theory of static headspace, including general considerations, advantages and disadvantages, with particular reference to the analysis of food volatiles has been reported by Wampler (2002) and hence this is not discussed further in this section.

Shimoda *et al.* (1993) evaluated the headspace aroma volatiles of twenty 1986 Cabernet Sauvignon wines from six regions of Napa Valley, California, using a direct static headspace sampling technique. 50 ml of each wine sample was placed into a 300 ml Erlenmeyer flask and 500 μl of an aqueous solution of 3-heptanol (100 μg/ml) was added to serve as an internal standard. After the headspace of the sample flask was purged with nitrogen gas for 10 s to remove oxygen, the flask was sealed with a Teflon plug equipped with a stopcock. The wine samples were then heated in a water bath for 20 min at 25 °C, before a sample of headspace gas (4 ml) was drawn into a 20 ml gas-tight syringe equipped with a fused silica capillary column needle and analyzed via GC-MS with on-column injection. Most of the compounds detected were products of yeast fermentation, although two volatile components had not been detected previously in wine. It was concluded that, although the headspace technique was shown to be reproducible and would be useful for analysis of major grape and fermentation volatiles, it could not detect trace compounds, many of which are significant to the overall aroma of wine.

The static headspace technique has also been used for the chromatographic analysis of volatile sulfur compounds in wines with flame photometric detection (FPD) (Mestres *et al.*, 1997). It was concluded that the method developed appeared to be suitable for the determination of a relatively large number of usual volatile sulfur compounds in wines. The interference from other compounds in the complex wine matrix was eliminated by the use of the specific FPD detector, and low concentrations of sulfur compounds could be detected by concentrating the headspace with a cryogenic trap. The method also seemed to be appropriate for enology laboratory work because of the simplicity of the instrumentation used.

Static headspace has also been recently been used for the determination and quantitative analysis of acetoin in beer (Tian *et al.*, 2009) and also to study a decrease of aged beer aroma by the reducing activity of brewing yeast (Saison *et al.*, 2010).

In dynamic headspace, the volatiles above a sample are swept away by an inert carrier gas, usually helium or nitrogen, onto a trap (glass or glass lined stainless steel tube containing porous polymers such as Tenax™ (poly-2,6-diphenyl-*p*-phenylene oxide), graphitized carbon sorbents (such as Carbotrap, Carbopack), silica gel, poly(dimethylsiloxane) and activated charcoal etc).

With liquid samples such as alcoholic beverages, the sample is purged with a flow of carrier gas and the analytes trapped, hence dynamic headspace analysis of liquid samples is usually referred to as purge and trap (the term dynamic headspace is generally used when the sample undergoing analysis is a solid). Figure 4.2.10 details the basic setup for carrying out a manual purge and trap analysis. Commercial instruments are also available for this technique, offering automated drying of moisture from the trap (see Figure 4.2.10 – dry purge gas), preconditioning and reconditioning of the trap in between a sequence of analyses and most include the ability to cryogenically cool the inlet of the gas chromatograph (for example with liquid nitrogen) to focus the aroma volatiles for sharper peaks during analysis. With careful attention to contaminants and system background, it has been reported that this technique is capable of routinely detecting analytes present in a sample in the parts per trillion (ng/l) range (Wampler, 2002).

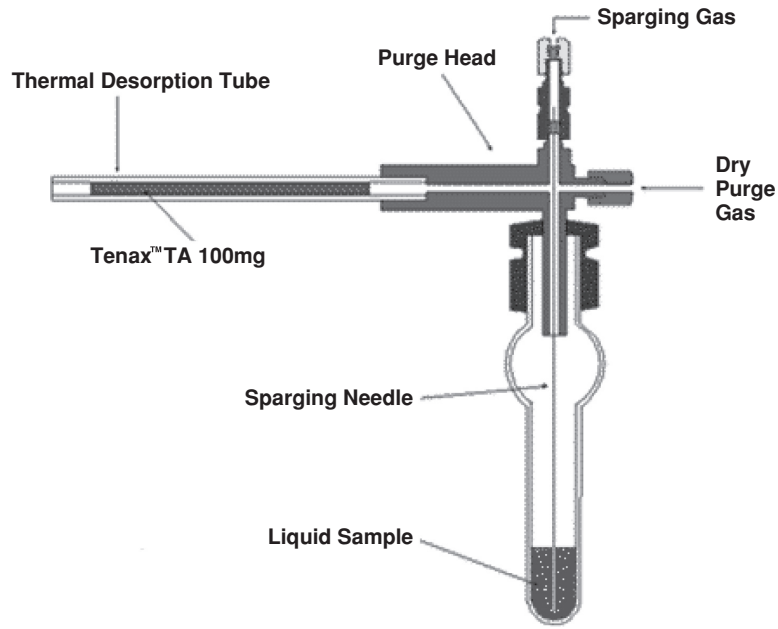

Figure 4.2.10 *Purge and trap system (Scientific Instrument Services). Reproduced with permission of Scientific Instrument Services*

The headspace volatiles of commercially produced kiwifruit wine and wine produced from Müller-Thurgau grapes have been compared by GC-MS (Craig, 1988). The wine (750 ml) was treated with an internal standard, then held in a water bath at 35 °C and purged with a stream of oxygen free nitrogen, at a flow of 60 ml/min for 22 h. The nitrogen, saturated with wine volatiles, was bubbled through a 10% solution of ethanol in distilled water (50 ml), which was continuously extracted with 50 ml of freon 11. After 22 h, the solvent was evaporated through a vacuum jacketed column, using a water bath at 35 °C, until the volume was reduced to approximately 2 μl. Pentane (20 μl) was then added and the resulting solution used for gas chromatography. Forty-seven compounds were identified and all of the major, and most of the minor, components were present in both wines, along with the presence of fenchone, which had not been previously reported in wine, which was confirmed in the kiwifruit wine sample.

The application of adsorption-thermal desorption-gas chromatography to determine the heavy fraction of wine aroma was studied by Salinas *et al.* (1994, 1997). This technique included the processes of isolation and concentration of volatiles performed in three classic stages of purging, concentration and injection. Initially, the volatiles were separated from the wine (50 ml) by purging dry nitrogen at 67 ml/min through the sample at 30 °C. They were then adsorbed in a metal tube trap containing the adsorbent Tenax™ at 25 °C. In the second stage, the temperature of the initial trap was increased to 300 °C and held for 5 min (first thermal desorption) to release the aroma volatiles that were then concentrated in a second Tenax™ trap, which was subjected to low temperature (–30 °C). In the final stage, the temperature of the second trap was raised to 350 °C to desorb the volatiles, which were immediately transferred into a gas chromatograph equipped with flame ionization detection. The reproducibility of the method was demonstrated using standard aroma solutions and in most cases, the variation coefficients were shown to be in the order of 3%. Calibration graphs were also constructed and it was demonstrated that the correlation coefficients were greater than 0.99 for the entire aroma components studied. It was concluded that the proposed method was fast, required no

sample manipulation and was successful for determining the volatiles of wine aroma. This technique was also used to study the volatiles of *Vitis vinifera* grapes (Rosillo *et al.*, 1999) and also Pinot Noir wines from British Columbia (Girard *et al.*, 2001). Ortega-Heras *et al.* (2002) compared liquid–liquid extraction, static headspace and dynamic headspace, for the analysis of volatile aroma components in wine. The advantages and disadvantages of the three methods regarding sample preparation, component losses and artefact formation were evaluated, along with the sensitivity and reproducibility of each of the techniques. It was concluded that the liquid–liquid extraction and static headspace methods showed good reproducibility, but although the static headspace technique was useful for the analysis of highly volatile compounds, it was not able to detect trace compounds due to its lack of sensitivity. On the other hand, the dynamic headspace method provided an extract free of artefacts and foreign substances that were not in the original wine. However, the technique was shown to have very poor reproducibility.

The purge and trap technique has also been utilized in recent studies for the prediction of the wine sensory properties related to grape variety from dynamic headspace gas chromatography-olfactometry data (Campo *et al.*, 2005), for the evaluation of an aroma similar to that of sparkling wine via sensory and GC analysis of fermented grape musts (Mamede *et al.*, 2005), for the analysis of red and white wines from different Spanish regions (Aznar and Arroyo, 2007), for the identification of a previously unreported odorant (2-methyl-3-(methyldithio)furan) in different monovarietal red wines from the Canary islands (Culleré *et al.*, 2008), for modeling quality of premium Spanish red wines from GC-O data (Ferreira *et al.*, 2009) and for the study of methods for the extraction of volatile compounds from fermented grape must (Mamede and Pastore, 2006) where liquid–liquid extraction was compared with purge and trap. It was concluded by the authors that the liquid–liquid extraction method required more handling of the sample and was time consuming. On the other hand, the purge and trap system was automated and practical, leading to minimal volatile loss, but low extraction of the high and medium boiling point compounds was observed. Overall, it was demonstrated that the purge and trap/dynamic headspace system and liquid–liquid extraction method were complementary in the determination of the aroma profiles of fermented grape musts and characterization of the samples.

The purge and trap technique has been recently validated in order to apply it to the analysis of minor esters in ciders (Roberto *et al.*, 2005). Helium at 50 ml/min was used to purge 5 ml cider samples for 30 min at 20 °C and subsequent trapping onto a Tenax™ trap. It was reported that 49 compounds were identified, obtaining recoveries ranging between 93% for ethyl decanoate and 117% for ethyl-3-methylbutyrate and precision (RSD) ranging between 2.2% for hexyl acetate and ethyl decanoate and 10.9% for isoamyl acetate. The researchers concluded that the validated method allowed the determination of esters and styrene in ciders without prior sample treatment, with a good degree of reproducibility and accuracy. Therefore, the technique could be applied to the analysis of commercial ciders to aid the study of the technical processes that influence manufacture.

The aromatic profile as well as the off-odor of cork stoppers has recently been studied via the purge and trap technique. A method based on initial solvent extraction (with pentane) of either cork or wine samples followed by automated purge and trap GC-AED was reported by Campillo *et al.* (2004) for the determination of 2,4,6-trichloroanisole (TCA). Element specific detection and quantification was carried out by monitoring the chlorine (479 nm) emission line. Detection limits of 25 pg/g and 5 ng/l were obtained for corks and wines alongside reported recoveries from spiked samples ranging from 88.5–102.3%.

The sensory properties and aromatic composition of macerates of five synthetic and three natural corks were recently determined (Culleré *et al.*, 2009). Cork samples were initially submerged in a synthetic aqueous solution containing 15% ethanol and 5g/l of tartaric acid (pH adjustment to 3.2) for 10 days at 40 °C. The volatiles of the macerates were then collected using a purge and trap system containing LiChrolut EN resin as sorbent, and eluted with dichloromethane before freezing for 2 h at –30 °C to remove water. Finally, the extract was concentrated to 200 μl under a stream of pure nitrogen for GC-O

analysis. Natural stoppers were reported to impart sweet, toasted, sweet wood and flowery Muscat aroma to the model wine extracts. Conversely, the synthetic stoppers included a sample with a clear rubber aroma and two samples with a cork/mushroom aromatic note. The results of the GC-O analysis confirmed that the natural cork samples had complex aroma profiles of 10–20 aromatic compounds, all well known natural components of healthy wine, whereas, in contrast, the GC-O analysis of the synthetic stoppers were extremely simple and consisted of only a few odorants (the mushroom odor was successfully attributed to 1-hepten-3-one).

4.2.6 Combinations of Methods (With Particular Application to Enological Products)

A young Grenache red wine from the 1995 harvest was continuously extracted with freon 11 and the extract cleaned with sodium bicarbonate in order to remove the fatty acids (Ferreira *et al.*, 1998b). An aroma extract dilution analysis (AEDA) (Section 4.7.3) was carried out with the extract using a carbowax 20M capillary column and simultaneous GC-MS and olfactometric detection. The AEDA analysis showed that there were 43 odor active regions in the chromatogram. To isolate the odorants, the extract was further washed with propylene glycol, concentrated and then fractionated by normal phase HPLC (Section 4.3.3) with UV detection at 220 nm in order to obtain 29 fractions. All the fractions were concentrated and analyzed in the same GC-MS-olfactometric system in which the AEDA experiment was performed. The strategy allowed isolation of most of the odorants, and 30 of them, among which were the most important, could be clearly identified. It was concluded that amongst the most important odorants there were some well known fermentation esters, but surprisingly the role played by some minor esters, such as the esters of isobutyric, isovaleric and 2-methylbutyric acids, seemed to be very important. Equally important could be the role played by some volatile phenols, terpenols, lactones and norisoprenoids. The suitability of reversed phase HPLC for the semi-preparative fractionation of aroma extracts from wine and other alcoholic beverages was also explored by Ferreira *et al.* (1999). Aroma extracts were separated in a 250 × 10 mm Kromasil-C_{18} column using a water–ethanol gradient system as mobile phase. It was demonstrated that the chromatographic separation did not induce any chemical change in the sample components. It was also shown that the maximum volume that could be injected without altering efficiency was as high as 2 ml if ethanolic extracts were injected, and slightly less in the case of less polar extracts. Aroma extracts were injected directly without the need of any pretreatment. Major compounds eluted first, so it was possible to fractionate all the volatiles contained in a 1–1.5 l sample, without peak distortion or mass overload problems. The usefulness of the method was demonstrated by fractionating an extract from a Chardonnay wine to get 15 fractions that showed different aromas. The GC analysis with olfactometric and MS detection of the fractions allowed the authors to identify more than 70 aroma compounds and to signal some of them as potential key aromas of Chardonnay wine.

This technique has also been used to identify and compare the impact odorants of young red wines made with Merlot, Cabernet Sauvignon and Grenache grape varieties (Section 2.9.2) (Lopez *et al.*, 1999), for isolation and identification of odorants generated in wine during its oxidation (Escudero *et al.*, 2000a) as well as studying the role of 3-(methylthio) propanal (methional) as a character impact odorant of some oxidized wines (Escudero *et al.*, 2000b) and more recently to identify the volatile compounds responsible for prune aroma in prematurely aged red wines (Pons *et al.*, 2008a) and to study specific ‘red’ and ‘blackberry’ aromas in red wines (Pineau *et al.*, 2009).

Countercurrent extraction is a liquid–liquid extraction process in which the solvent and the process stream (sample) in contact with each other, flow in opposite directions (Section 4.3.4).

Nearly 200 volatile compounds were separated from a wine obtained from *Vitis vinifera* cultivar Syrah, originating from the southeast of France (Vernin *et al.*, 1988). The aroma extract was obtained in two steps

by an original process. The first step was a liquid–liquid extraction of a batch of 20 l of Syrah wine, with 5 l of freon 11 as extraction solvent, and a York Scheibel column with countercurrent circulation of the two liquids for 4 h. The second step involved condensation of the volatiles under high vacuum in a trap cooled at –175 °C in liquid nitrogen. It was concluded that the qualitative composition of the aroma extract did not significantly differ from other red wines. Stepwise discriminant analysis applied to Syrah wines of various vintages allowed the authors to obtain a good classification of these wines into three groups using chromatographic data obtained by the use of a dynamic headspace (purge and trap) technique.

Volatile constituents of experimental wines from five white and five red vine cultivars were analyzed by Baumes *et al.* (1986), using continuous liquid–liquid extraction for 8 h with a pentane/dichloromethane mixture (2:1, v:v). The aroma extracts were then fractionated in a jacketed chromatography column refrigerated to 15 °C, packed with silica gel. The volatiles were successively eluted with 300 ml of pentane/diethyl ether (9:1, v:v) and 300 ml of diethyl ether and analyzed by GC-MS. One hundred and twenty two substances were positively identified and 15 tentatively identified. Fourteen of them had not been detected previously in wine. Some sensory evaluation of the various separated compounds was also attempted and a total of 131 volatile constituents were quantified in triplicate.

Herraiz *et al.* (1991) investigated the aroma components of wine resulting from fermentation of grape must from the Verdejo cultivar. The volatiles were isolated by continuous liquid–liquid extraction with freon 11 and further fractionation on a silica gel column. Portions of pentane, pentane/diethyl ether (90:10, v:v), pentane/diethyl ether (70:30, v:v) and diethyl ether (60 ml) were used as eluting solvents. As a result, four different fractions were obtained. These fractions were concentrated to 0.75 ml and 3 μl aliquots were analyzed by GC-MS. The relative quantities of different components in the sample were established by comparison of the peak area measurements resulting from GC analysis with the area of the internal standard peak, without taking into account the extraction and fractionation. One hundred and thirty two substances, including alcohols, esters, carbonyl compounds, terpenes, acids and sulfur compounds were identified; some had not been previously detected in wines.

Ferreira *et al.* (1995) used a similar sample preparation method for the identification of volatile constituents in wines from the *Vitis vinifera* variety Vidadillo. Several wine samples of 500 ml were continuously extracted with 150 ml of freon 11 for 24 h. The aroma extract was then concentrated to 1 ml in a three-ball Snyder column. The extract was then further fractionated in a silica gel column with successive portions of hexane/dichloromethane/diethyl ether mixtures following in elutropic order. Ten fractions showing different odors were isolated and analyzed by GC-MS and GC. More than 200 compounds belonging to different biosynthetic pathways were identified. It was concluded that the silica gel prefractionation was a very promising tool for characterizing the flavor of the wine. The fractionation of the aroma extract in an adsorption chromatographic column leads to class compound separation. Consequently, compounds with similar aroma properties were eluted in the same fractions, allowing the study of their additive effects.

Similarly, the organoleptic volatiles of Chardonnay wine were isolated by continuous liquid–liquid extraction with freon 11 and further fractionation on a silica gel column (Caven-Quantrill, 1999). One hundred substances, including alcohols, esters, carboxylic acids, carbonyl compounds, terpenes, lactones, sulfur compounds, acetals, amides, phenols and furans, were identified and quantified by GC-MS.

The reproducibility of the liquid–liquid extraction system was evaluated and shown to be excellent, with coefficients of variation in the range of 0.39–5.09% for a wide range of wine aroma volatiles isolated from a model wine solution.

The relative recoveries of 28 typical white wine volatiles relative to the chosen internal standards were also studied after isolation and separation by the extraction and fractionation techniques. The recovery trial indicated that the concentrations of identified aroma volatiles reported in the wine sample, after equivalent isolation and fractionation treatment, could be considered as reasonable estimates of the actual concentrations in the wine investigated for most compounds, except the most volatile or the most hydrophilic.

The organoleptic characteristics of a majority of the aroma compounds were discussed, as well as the possible sensory contributions of some volatile compounds to the aroma and flavor of the Chardonnay wine studied.

Recently a new microextraction/solid phase extraction method for the GC analysis of wine volatiles was developed (Ferreira *et al.*, 1996). Repeatability of the method was reported to be better than 5% (as relative standard deviation) for more than 50 wine volatiles. Linearity and recovery of volatiles was also studied and was satisfactory in most cases. It was concluded that this approach had a number of advantages over classical techniques, such as the elimination of long and tedious evaporation steps, which improved the precision of measurements and helped to reduce the amount of solvents used. It was also concluded that separation of the wine aroma extract into fractions helped to prevent major volatile compounds from overlapping minor constituents in the gas chromatogram.

This combined sample preparation technique has also been used for the specific determination of nitrogenous heterocyclic compounds in wine (Keim *et al.*, 2002).

Aznar *et al.* (2001) identified and quantified impact odorants of aged red wines from Rioja using GC-Olfactometry, quantitative GC-MS and odor evaluation of HPLC fractions. The alcoholic degree of the wine was adjusted with pure water to 6%, v:v. 300ml of the diluted wine was passed through a glass column filled with a 10 cm long $\times$ 1 cm diameter bed of XAD-4 resin. The elution of aroma components was achieved with 40 ml of diethyl ether/pentane (1:1, v:v). The extract was finally dried over anhydrous sodium sulfate and concentrated in a micro-Kuderna-Danish concentrator to 1 ml and finally under a stream of pure nitrogen. The extract was analyzed by AEDA and most of the odorants quantified by GC-MS. A second extract was fractionated in a HPLC system with a C-18 semi-preparative column. Fifty fractions were recovered, their alcoholic degree and pH were further adjusted to those of the wine, and those fractions that showed strong odor characteristics were further re-extracted and analyzed by GC-O and GC-MS. Reconstitution experiments were carried out to confirm the role of the odorants detected in the fractions. Fifty-eight odorants were found in the Rioja wine, 52 of which could be identified. Comparison among the three techniques showed good agreement and demonstrated that they were complementary.

4.2.7 Summary

Alcoholic beverage chemists utilize many sample preparation techniques for isolating and analyzing aroma/flavor. However, a major complicating factor is the presence of high concentrations of ethanol, which can dominate flavor extracts and subsequent chromatographic data. Other non-volatile components such as sugars and acids can also be co-extracted yielding complicated analyses and contamination of chromatographic instruments. As a result, selection of an appropriate isolation technique is critical. The resulting extract should be reminiscent of the original sample and should not result in biases of flavor profile. These are important prerequisites for any isolation technique applied to the study of aroma.

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4.3

Chromatographic Methods

As in other areas of science, chromatography, particularly gas chromatography (GC) and high performance liquid chromatography (HPLC), has revolutionized the analysis of alcoholic beverages and their precursors (fruit juices, cereal mashes, etc.). With such techniques as two dimensional GC and column switching HPLC along with sophisticated sample preparation techniques (Chapter 4.2) and such materials as monolithic HPLC stationary phases it is now possible to efficiently separate most of these complex samples and to identify many of the components using a variety of detectors and methods.

Detectors include selective types: for HPLC, conductometric (ionic analytes), electrochemical (oxidoreducing analytes) and UV-visible (analytes with electronic chromophores) and for GC, electron capture (for chlorinated analytes) and NP detectors (for analytes containing N or P atoms), amongst others. Nonselective detector types include the all powerful mass spectrometer, flame ionization (for GC), refractive index (for HPLC) and evaporative light scattering (ELS) (for HPLC). Many of these detectors are highly sensitive, and frequently, analytes at the μg/l or ng/l level can be detected and identified by coinjection of known standards and/or by use of Kovacs or other experimental indexes.

Moreover, the combination of any chromatographic method (but most often GC or HPLC) with a mass spectrometer often allows identification of unknown components, from molecular weight or fragmentation data. Modern modes of ionization (particularly ESI and APCI) and tandem mass spectrometry (MS/MS or MS^n) have made it possible to determine the structures of complex oligomeric polyphenols and carbohydrates, and to determine the molecular weights of big peptides and proteins.

The aim of this section is to give an account of the application of chromatographic methods to the analysis of alcoholic beverages in such a way that the reader can grasp an overall picture of the contemporary state of the art. A basic knowledge of the theory of chromatography at undergraduate level is assumed. Although a certain amount of general background theoretical and empirical information is given for each technique, the emphasis is on the types of alcoholic beverage components that can be analyzed by a particular chromatographic method, the experimental conditions required (e.g. stationary phases, mobile phases, detectors etc.) and the kind of information that can be obtained from such analyzes. Thus, it will be seen that the application of chromatographic methods not only leads to quantification and identification of components, but chromatographic profiles can be used as 'fingerprints' in the establishment of beverage authenticity, if suitable chemometric or statistical software is used to analyze the results.

The section begins with planar chromatographic techniques (especially thin layer chromatography), which have long been important methods for the brewer or winemaker, but the emphasis is on GC and HPLC,

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

reflecting the greater role played by these two techniques in alcoholic beverage analysis. The final subsection is dedicated to countercurrent chromatography.

4.3.1 Thin Layer Chromatography and Related Methods

Thin layer chromatography (TLC), together with some of its modifications such as high performance thin layer chromatography (HPTLC) and overpressured layer chromatography (OPLC), and along with paper chromatography and chromatography involving monolithic discs are members of the planar chromatography family. TLC in its simplest form is highly versatile and is the least expensive form of chromatography, whilst at the same time in its more sophisticated forms, it can stand comparison with HPLC. Most TLC is performed in normal phase, with a polar stationary phase (such as alumina, calcium sulfate or silica gel) and nonpolar solvents, such as acetone, ethyl acetate or hexane. Like HPLC, mixed solvent elution and gradient elution are possible, and reversed phase, ion exchange and chiral stationary phases have become more readily available during the past two or three decades. Reversed phase TLC layers, apart from the long established cellulose and alkane impregnated silica gel, now include bonded silica phases, such as C_8, C_{18}, NH_2 and diol bonded phases. The application of TLC to food (including beverages) and agricultural analysis has been reviewed fairly recently (Sherma, 2000).

Unlike HPLC (and GC), planar chromatographic techniques lend themselves very easily to two dimensional chromatography, without any extra equipment or significant extra cost. Colorless analytes can be visualized using any one of a multitude of chromogenic and fluorogenic reagents, which are usually specific to particular analyte types. The big disadvantage, until relatively recently, was the greater difficulty in accurately quantifying spots on TLC plates. This was originally done by visual comparison with standards of varying concentration, but nowadays can be performed using densitometry. Spots on TLC plates can also be examined by mass spectrometry (e.g. TLC-MALDI-TOF) (Zarzycki *et al.*, 2005 and references therein) and Camag has produced an automated TLC-MS interface (connected to an LC-MS system) that uses eluent from the HPLC pump to extract the chosen analyte zone (with its depth profile) on the developed TLC plate, and transfers it to the mass spectrometer.

Many TLC separations are still carried out by gravity flow ascending elution in one dimension with a single mobile phase. On the other hand, HPTLC is an instrumentalized form of TLC in which a special TLC plate is sandwiched between metal plates in a sandwich chamber and subjected to (often gradient) elution (sometimes called development in TLC) by a mobile phase. The stationary phase particles are finer for HPTLC (5 μm, as opposed to 12–20 μm for TLC), are of much narrower size distribution and require application of smaller sample spots (0.5–1 mm, as opposed to 2–6 mm for TLC). Gradient elution can be controlled manually, as in the separation of red wine pigments on reversed phase alkane impregnated silica gel plates, although automated multiple development (AMD) can produce a multistep elution gradient (usually stronger to weaker elution power for normal phase HPTLC) for the separation of complex mixtures with analytes of a wide range of polarities. Overpressured layer chromatography (OPLC) is a forced flow elution technique, which involves pumping under pressure the mobile phase through a layer on a plate sandwiched between a flexible membrane (filled with water) and a rigid stainless steel plate. The analytes are eluted off the plate and are passed through a detector before being collected (Mantovani *et al.*, 1998). In essence, OPLC is a form of planar column chromatography. Both HPTLC and OPLC result in high spot capacities (which aids detection and quantification) and short development times.

Besides being a separation technique in its own right, TLC can be used in a number of ways, as a reaction (e.g. malolactic acid fermentation, MLF) monitor, or as a means of preparing fractions for analysis by other methods, and of course it can be used in conjunction with other sample preparation or chromatographic methods, such as column chromatography, solid phase extraction and/or HPLC. For example, TLC and HPLC

were used to monitor the biogenic amines histamine and tyramine produced by lactic acid bacteria isolated from grape must and wine (Costantini *et al.*, 2006). TLC was used to monitor production of histamine in the broth medium enriched with histidine, whereas the tyramine produced by certain bacteria was quantified by HPLC. TLC has long been useful in checking the qualitative progress of MLF, like paper chromatography (Amerine and Ough, 1980a), but an improved method using a TLC-scanner for quantification has been described as being comparable with HPLC (Boido *et al.*, 1999).

Resolution in TLC can be usually further improved by double development (two elutions in the same direction) or two dimensional development (two elutions at right angles). For example, double development using firstly ethyl acetate:heptane (9:1) and then ethyl acetate:methanol:ammonia (4:3:1) enabled the simultaneous study of resveratrol and biogenic amines in red wine (Sherma, 2000). In an extreme case, multidimensional planar chromatography has been used to fully separate 14 pesticides on a 10 cm × 10 cm TLC plate (Tuzimski, 2007).

Planar chromatography has been applied to analysis of most types of constituents of alcoholic beverages or their raw materials, although probably the greatest focus has been on phenolic compounds, including pigments, and especially in grape musts and wines. Flavan-3-ols, including the dimeric procyanidins have been separated on cellulose TLC plates using water as eluent and dimethylaminocinnamaldehyde (DMACA) as the derivatizing agent, for densitometric determination (Glavnik *et al.*, 2009). Quantification at 655 nm (blue) showed good linearity between 2 and 12 ng of analyte, with a limit of detection of 0.2 ng. DMACA was considered to be superior to vanillin, the established reagent for the visualization of flavanols on TLC plates.

Resveratrols and other stilbene phenolic compounds in wine have been determined by standard TLC with fluorescence scanning (Chen *et al.*, 2005). Good linearity of detector response was found in the range 2.0–27.7 ng, recoveries were 97.1–98.5%, with relative standard deviations of 2.0–3.2%. The mobile phase was benzene:methanol:formic acid (10:5:1). The resveratrol and piceid isomers were separated from other polyphenols by SPE treatment (Section 4.2.4) on a C_{18} phase prior to TLC analysis. OPLC was used to separate stilbene isomers in red wine, with limits of detection 40 ng (for *trans*-resveratrol, *cis*- and *trans*-piceatannol, and 60 ng for *cis*- and *trans*-piceid (Kiraly-Veghely *et al.*, 2004). Resveratrol and biogenic amines in red wine were determined together by silica gel TLC, the resveratrol being determined directly by densitometry, whilst the biogenic amines were determined as their dansyl derivatives (Sherma, 2000). TLC has been much used in the study of red wine pigments, anthocyanins and their derivatives (Amerine and Ough 1980b). More recently, normal phase TLC has been used to produce profiles of Croatian red wines (Rastija *et al.*, 2004) and HPTLC on C_{18} silica gel (i.e. reversed phase) with isocrative elution, using methanol-water-trifluoroacetic acid as the mobile phase has been used to study the anthocyanin composition of red wines (Lambri *et al.*, 2003). The anthocyanins were separated from other components (including polyphenols) C_{18} SPE treatment of the samples.

Previous to these studies, anthocyanins were separated by manual multistep elution reversed phase HPTLC, and by TLC, where characteristic differences in anthocyanin composition allowed differentiation between Pinot Noir red wines and those of other varieties (Holbach and Wilken, 1994).

Other phenolic components of red wine (e.g. catechins, chlorogenic acid, gallic acid and protocatechuic acid) were separated from pigments by cellulose TLC with methanol:water (4:1) eluent, were visualized by 5% aqueous $FeCl_3$ and determined by densitometry at 700 nm (Sherma, 2000). Wine pigments were separated on silica gel TLC plates using 1-butanol:butyl acetate:formic acid:water (13:5:2:3) and quantified by densitometry at 540 nm. Likewise, RPTLC (C_{18}) using water-acetonitrile and water-acetonitrile-trifluoroacetic acid mobile phases has been used to separate anthocyanidins, catechins, flavanones, flavones and flavonols in wines and other sources (Baranowski *et al.*, 2005).

Grape and wine proanthocyanidins were fractionated using C_{18} SPE into monomers, oligomers and polymers by using different SPE eluents (Sun *et al.*, 1998). The fractions were further analyzed by TLC and HPLC, allowing quantification of individual procyanidin dimers and trimers.

More unusual polyphenols, including two new C–C linked biflavonoids in Chardonnay grape pomace were analyzed by a combination of TLC and column chromatography (using Sephadex LH 20 and MCI gel CHP 20P stationary phases) (Yeap Foo *et al.*, 1998). NMR analysis of the isolated biflavonoids showed them to be epicatechin-(6′→8)-epicatechin and epicatechin-(6′→8)-catechin.

Planar chromatography has also been much applied to the analysis of pesticide residues (McLean *et al.*, 1999; Jayarman *et al.*, 2003; Tuzimski, 2007) and mycotoxin residues (Abrunhosa *et al.*, 2001; Dugan, 2003; de Oliveira *et al.*, 2007) in food and beverages. Captan (a contact fungicide) residues in grapes were determined by TLC (Jayarman *et al.*, 2003), and the uptake and metabolism of the systemic fungicide triadimenol by grapevines were investigated using TLC (McLean *et al.*, 1999). Instead of finding degradation product residues of triadimenol in grapes, this group found higher molecular weight conjugates of this molecule with its structure largely intact. Carbon-14 labeled triadimenol was used in this study, with autoradiographic determination.

After solvent extraction from food matrices and sample clean up on a silica SPE cartridge, aflatoxins B1, B2, G1 and G2 (Section 5.11.4) were determined on silica gel G TLC plates using (for confirmation), different mobile phases – toluene:ethyl acetate:acetone (3:2:1), chloroform:acetone:1-propanol (85:10:5) and chloroform:THF (90:10) (Dugan, 2003). Patulin (Section 5.11.4) in grapes extracted into ethyl acetate and ethanol was determined by normal phase TLC, using toluene:ethyl acetate:formic acid (6:3:1) as solvent and 0.5% MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) in 5% aqueous formic acid as visualizing agent (Oliveira *et al.*, 2007). The densitometric quantification limit was 15.87 μg/kg. A TLC method for the simultaneous analysis of several mycotoxins (citrinin, ochratoxin A and patulin) in grapes has been developed, using silica gel 60 plates without indicator, by Abrunhosa *et al.* (2001).

Sugars, polyhydroxy compounds and polyamines have also received considerable attention from TLC analysis. Quantitative monitoring of maltodextroses and monosaccharides in beers on amino bonded HPTLC plates with gradient elution-AMD (Brandolini *et al.*, 1995). The sugars on the developed plates were visualized by thermal *in situ* reaction of the analytes with the amino groups of the HPTLC layer and quantification was performed using a Camag TLC scanner with appropriate computer interface and software. A detailed summary of planar chromatographic methods used to analyze the sugars in beet and cane molasses in the sugar industry has been given by Mantovani *et al.* (1998).

Silica gel 60 HPTLC with isocratic elution was used to separate carbohydrates, glycerol, ethylene glycol and diethylene glycol in alcoholic beverages, such as wines (Klaus and Fischer, 1987). The eluted plates were visualized either by spraying with a vanadium pentoxide reagent or by immersion of the plates in dichlorofluorescine solution. More recently, glycerol in wines has been determined using gradient elution HPTLC (Brandolini *et al.*, 2002). The method was used to compare the glycerol producing ability of different wine yeasts (Chapter 2.2), including *S. cerevisiae*, *S. ludwigii*, *Zygosacharromyces bailii* and *Kloeckera apiculata*.

The most popular stationary phases in planar chromatography are silica gel (for normal phase) and bonded silica phases, such as C_{18}, amino and diol phases. Cellulose layers are also used and sometimes alumina or calcium sulfate layers, these being considered especially effective in the separation of more polar compounds. An example of this can be found in the analysis of polyamines (cadaverine, citrulline, ornithine, putrescine, spermidine and spermine) (Section 5.11.3) (Khan, 2006). Here the polyamines in biological fluids were separated on $CaSO_4$ plates using methanol as the mobile phase. This stationary phase was superior to silica gel in resolution of the polyamines and elution time was one-third shorter compared to silica. The limits of detection and quantification (found by scraping the visualized spots (ninhydrin) off the developed plates and measuring the absorbance at 550 nm) were 0.75 and 1.88 μg, respectively. Although this method has not yet (2009) been applied to the determination of polyamines in alcoholic beverages, it would appear to have potential in this area.

4.3.2 Capillary Gas Chromatography

In 1906, the Russian scientist Mikhail Tswett reported the separation of different natural colored pigments of leaves by passing a percolated extract through a glass column packed with finely divided adsorbents. He was later to use the term chromatography, from the Greek words χρώμα: *chroma* meaning 'color' and γράφει: *graphei* meaning to 'write.'

Today, gas chromatography (GC) is a popular analytical technique that is widely used around the world to achieve the separation, qualification (identification) and quantification (concentration) of the volatile chemical components in complex mixtures (such as alcoholic beverages).

As with other chromatographic techniques, GC uses a *stationary* phase and a *mobile* phase, whereby chemical components of a mixture are carried through the stationary phase (most modern state of the art systems contain a uniform thin film of phase that is affixed to the inner periphery of an open capillary tube) by a stream of gaseous mobile phase (carrier gas). However, in contrast to most other types of chromatography, the mobile phase does not interact with molecules of the analyte; its only function is to transport the analyte through the stationary phase. Separation is based on differences in migration rates among the sample components.

Over 50 years have now passed since GC was first conceived and it remains one of the world's most cited analytical methodologies and a heavyweight analytical tool in many industrial and academic laboratories. The extensive theory of GC has been widely reported by one of its pioneers (Jennings *et al.*, 1997), as well as excellent guidance in the care, maintenance and troubleshooting of instruments (Rood, 1999). Therefore, only the basics are described here for modern instruments using fused silica capillary columns with split/splitless or programmable temperature vaporizer (PTV) injectors, and only recent references of alcoholic beverage analysis using GC are offered – purely as examples. Extensive examples of alcoholic beverage research using GC as the main analytical technique can be found under the extraction and focusing methods in sample preparation section (Chapter 4.2) of this book.

Modern GC systems consist of a carrier gas supply (and associated pressure regulators, gauges, flow controllers and gas filter system to remove impurities such as water, oxygen and hydrocarbons), injector (to introduce the sample into the GC), column, oven, detector and a computer data system.

Carrier Gas

Chemically inert carrier gases used historically for GC include helium, hydrogen and nitrogen. It is generally accepted that helium and hydrogen are the best gases for capillary GC columns, as high linear velocities can be used without sacrificing efficiency when compared to nitrogen, for example. Modern gas chromatographs have the addition of electronic pressure controllers (EPC), which give the analyst a choice of either constant pressure (isobaric) or constant flow (isorheic) for carrier gases with extremely accurate control. Evaluation of column performance in constant pressure and constant flow modes has been carried out by Blumberg *et al.* (1999). The researchers concluded that there was almost no difference in the speed separation performance of a column between constant pressure and constant flow modes of temperature programmed GC and that nearly the same maximum peak capacity at nearly the same analysis time could be achieved in both modes of analysis.

Ultra high purity grade helium and hydrogen are used (99.999+%) which contain less than a few parts per million of oxygen and hydrocarbons as contaminants. Oxygen will rapidly degrade the stationary phase of columns and this will result in a reduction of column life and hence the increased expense of replacement.

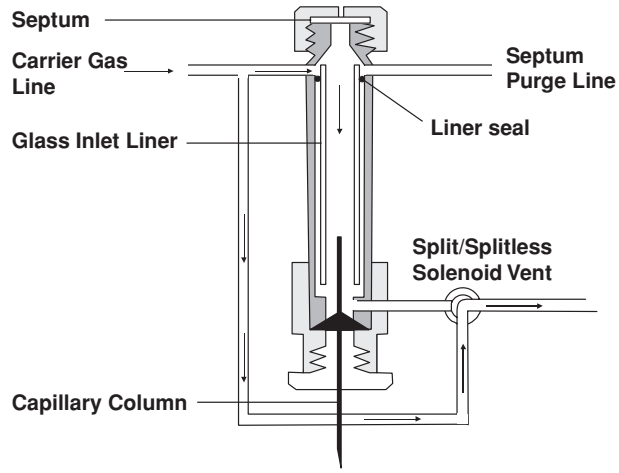


Figure 4.3.1 *A typical GC split/splitless inlet system (splitless mode shown). Reproduced from Gerstel (2002), with permission of Gerstel, GmbH*

Sample Injectors

The most common method of injection involves the use of a microliter syringe to inject a liquid or gaseous sample through a silicone rubber septum into a glass liner that is situated inside a heated metal injector body located at the head of the column. Rapid vaporization of the sample then takes place (the sample port is ideally 50 °C above the boiling point of the least volatile component in the sample or as high as the fused silica stationary phase of the chromatographic column will allow). Figure 4.3.1 is a schematic of a typical split/splitless injection port that is available on modern GC systems from numerous suppliers commercially. Split and splitless injection in capillary GC has also been documented in extensive detail by Grob (2001). Carrier gas enters at the top of the injector and this mixes with the vaporized sample to introduce it to the column for the subsequent separation process to begin. The septum purge is typically 1 to 3 ml/min of carrier gas that flows across the top of the injector immediately below the septum and exits via the septum purge line. This low flow of inert carrier gas is designed to aid the cleanliness of the injection port and to minimize the condensation of nonvolatile and high boiling materials of the sample matrix on the exposed section of the septum.

For samples containing high concentrations of analytes that exceed the capacity of modern columns, the split/splitless injection port is operated in the split mode. In this mode the quantity of sample reaching the column is limited by splitting the sample into two fractions – the size of which can be computer controlled accurately by the analyst. Once split, only a small fraction of the sample enters the chromatographic column and the remainder is discarded down the split line or vent (see split/splitless solenoid vent in Figure 4.3.1). A typical split/splitless injector (Figure 4.3.1) consists of five integral parts, namely the injection port heater, carrier gas line, glass liner, septum purge line and split line/vent. On injection of a sample into the heated injection port, the sample flash vaporizes and is subsequently mixed with the carrier gas and can travel either down the column for separation of volatile components or the split line. The amount of sample entering the column mixed with carrier gas compared to the amount of sample exiting the GC system via the split vent, can be controlled and is measured by what is known as the split ratio. This ratio is generally expressed as the volume of carrier gas entering the column versus the volume leaving via the split line. For example, following injection of a sample with the incoming carrier gas regulated at 100 ml/min, 2 ml/min is lost to the septum

purge vent, then 98 ml/min of carrier gas is sweeping the sample towards the analytical column. If a 1 ml/min column flow is selected for analysis, the remaining 97 ml/min of carrier gas and vaporized sample is sent to the split vent to be discarded. Therefore, the split ratio of this example is 100:1. Because the septum purge flow is low, only a very small volume of sample is lost via the septum purge line and hence is ignored when estimating and calculating split ratios for samples. Many modern split/splitless injectors can deliver 1000 ml/min total gas flow, so high split ratios can be achieved with very high accuracy with computer control.

Many different designs and types of split injection glass liners are available commercially, however the function remains identical, in that it should provide a completely inert environment for the injection and subsequent vaporization of a sample and its volatile constituents. Most modern inlet liners are supplied deactivated (silylated) prior to being vacuum packed ready for use by the analyst. Some also contain a plug of glass wool which is designed to mix the carrier gas and vaporized sample to ensure repeatable split injections. The glass wool also traps nonvolatile materials (such as sugars) and prevents fouling of the column from these contaminants.

Recent examples of research whereby split GC injections have been adopted include analysis of vineyard grape juice for volatile organic compounds with a 20:1 split ratio (Caven-Quantrill and Buglass, 2006), wine volatile profiling with a 60:1 split (Aznar and Arroyo, 2007), analysis of odor active compounds in the spicy fraction of hop essential oil (Section 2.6.3) with a 50:1 split ratio (Eyres *et al.*, 2007), for the direct injection of orujo spirits (Section 3.7.3) (1:1 split) to study the volatile composition (Cortés *et al.*, 2005), for elucidation of the main odor active compounds of whisky (Section 3.2.5) with a 10:1 ratio (Caldeira *et al.*, 2007), amino acid enantiomers in beer with a 30:1 split (Erbe and Brückner, 2000), quantification of major grape polysaccharides with a 20:1 split (Ayestarán *et al.*, 2004) fungicide residues (Section 5.10.2) in wine with a 10:1 split (de Melo Abreu *et al.*, 2006) and free disaccharides and other glycosides in wine with a 40:1 split (Ruiz-Matute *et al.*, 2009).

For trace level analysis, the split/splitless injector can be operated in the splitless mode, whereby a majority of the sample is introduced into the capillary column (see Figure 4.3.1). During a splitless injection, the split/splitless vent remains closed and no carrier gas flows via this line. As the sample is vaporized, it mixes with carrier gas and is introduced to the chromatographic column. As the split vent is closed during this time, the entire vaporized sample is transferred to the column. After a predetermined time, ideally between 40 and 90 s (Rood, 1999), the split/splitless vent is opened and allows carrier gas to flow via the inlet liner to the vent. At this time the inlet purge flow is also switched on to provide an elevated carrier gas flow rate. Any residual sample volatile or nonvolatile material is hence swept away from the injection port and exits via the split vent (identical to a split injection mode). As the vaporized sample to column transfer in the splitless mode occurs over a long period of time, a solvent effect is generally relied upon to focus the sample analytes at the head of the capillary column (by maintaining the start temperature of the column lower than the boiling point of the sample solvent). Other less volatile compounds will be cold trapped at the head of the column regardless of a solvent being present. In contrast to split injection liners, splitless liners rarely contain glass wool as this causes mixing and dilution of the vaporized sample with the carrier gas and this in turn can result in broader and smaller analyte peaks during the resulting analysis.

Recently, splitless injection-GC has been used to analyze the volatile compounds of Muscat grape juice (Section 2.9.1) (Sánchez-Palomo *et al.*, 2009), for the speciation of arsenic in wine and beer (Campillo *et al.*, 2008b), to determine the odorous impact of volatile thiols on the aroma of young botryized sweet wines (Sarrazin *et al.*, 2007), for the determination of volatile phenols in cider (Section 2.8.4) (Pizarro *et al.*, 2009), for the varietal characterization of hop (Shellie *et al.*, 2009) and new thiols derived from hop (Takoi *et al.*, 2009), flavor stability of beer (Section 2.6.12) (Saison *et al.*, 2008), for the aromatic profile of different kinds of wine cork stoppers (Culleré *et al.*, 2009), off flavors and taints in wines (Alzaga *et al.*, 2003, Insa *et al.*, 2007, Pizarro *et al.*, 2007, Campillo *et al.*, 2008a), for the determination of volatile organic sulfur and selenium compounds in beers, wines and spirits (Campillo *et al.*, 2009), for the volatile aroma profiling

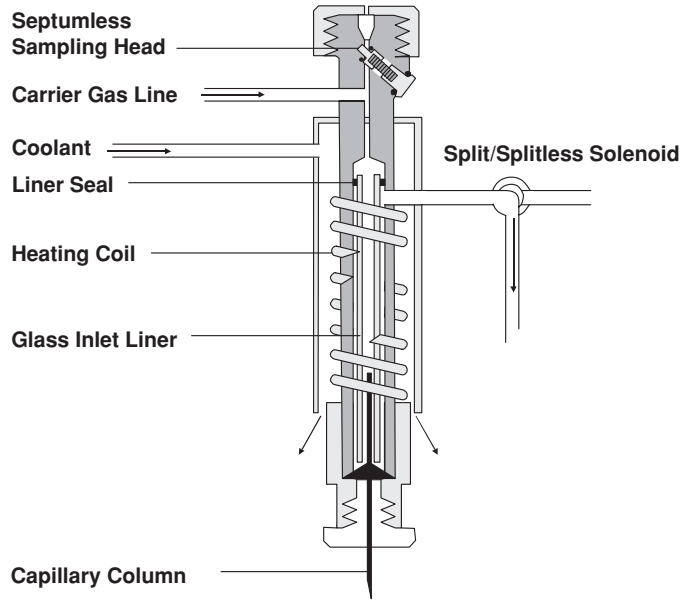


Figure 4.3.2 *A PTV inlet. Reproduced from Gerstel (2002), by permission of Gerstel GmbH*

of whisky (Câmara *et al.*, 2007), brandy (Section 3.6.1) (Zhao *et al.*, 2009), cachaça (Section 3.5.4) (de Souza *et al.*, 2009) and orujo spirits (García-Martín *et al.*, 2010), fungicides in wine (Montes *et al.*, 2009; Vaquero-Fernández *et al.*, 2009), pesticides in grapes, musts and wines (Cunha *et al.*, 2009; Patil *et al.*, 2009) and phthalates in wine (Section 5.10.4) (Carrillo *et al.*, 2008).

Another common design of injection system used for the analysis of volatile alcoholic beverage components is the programmable temperature vaporizer (PTV). This inlet closely resembles a conventional split/splitless inlet (see Figure 4.3.2), however the primary difference is temperature control. The liner of the PTV can be heated or cooled rapidly. Ballistic heating of the inlet body at a rate of 0.5–12 °C/s can easily be achieved, and heating is controlled using a sophisticated electronic controller. Cooling can be performed by using a Peltier element, liquid carbon dioxide or liquid nitrogen (hence the injector can be used as a cold trap). In order to facilitate rapid heating and cooling, the thermal mass of the liner is minimized. For this reason the total internal volume of glass liners for PTV inlets are smaller than those used in conventional split/splitless inlets (150–250 μl versus 1000 μl).

The PTV inlet is a useful sample introduction technique for thermally labile analytes whereby sudden changes in temperature can cause degradation. This can be minimized by injection at a lower temperature and then raised very rapidly to achieve vaporization of the sample. The result is less thermal stress applied to the sample and hence less severe changes in temperature during the analysis.

Rogerson *et al.* (2002) used a PTV injector to analyze liquid extracts (Section 4.2.2) of port wines (Section 2.10.7) and grape berries. Following injection, the PTV was programmed at 70 °C for 0.1 min then heated at 180 °C/min to 250 °C (held 13.9 min) then finally isothermal at 70 °C.

Similarly, Angioni *et al.* (2005) used a PTV to determine residues of the fungicide zoxamide in grapes, grape processing and in the fermentation process. Hexane extracts (8 μl) were injected into the PTV programmed from 60 °C (held 1 min) to 150 °C at 30 °C/min. The injector was operated in the splitless mode (purge valve opened at 2 min). Recently, the residues of 11 new fungicides in grapes and wines were determined via

liquid–liquid extraction (Section 4.2.2) and PTV-GC-MS (González-Rodríguez *et al.*, 2009). Extracts (2 μl) were injected into a PTV operating in solvent split mode which was subsequently programmed from 80 °C (0.1 min), 720 °C/min to 100 °C (0.1 min), 402 °C/min to 275 °C (1.5 min) then finally 402 °C/min to 300 °C (5 min).

Following extraction of wine, whisky and brandy samples by solid phase extraction (SPE, Section 4.2.4) Campo *et al.* (2007) used a programmable injector to perform large volume injections (LVI). Extracts (50 μl) were injected in the solvent split mode and during injection, the PTV was retained at 40 °C and the split valve was opened (split ratio = 30) to promote solvent evaporation. After elimination of solvent (0.25 min), the split valve was closed and the PTV was heated to 250 °C at a rate of 200 °C/min. After 3 min, the split valve was opened again (split ratio = 20) and the PTV was held isothermally at 250 °C. The injection parameters were carefully optimized to ensure a complete transfer of analytes to the GC column with good retention time reproducibility. An identical PTV method has been used recently for the comparative study of the aromatic profile of different kinds of wine cork stoppers (Culleré *et al.*, 2009). Large volume PTV injection has also been reported for the determination of organophosphorus pesticides from spiked samples of red and white wines and apple juice (Schellin *et al.*, 2004), for the low level determination of organochlorine pesticides in wines (Pérez-Serradilla *et al.*, 2010) and for the multiresidue analysis of 50 pesticides in grape and other fruits (Savant *et al.*, 2010).

The PTV injector has also been used successfully with modern techniques such as thermal desorption. In contrast to solid phase microextraction (SPME – Section 4.2.4), in which desorption is performed in a standard GC inlet, stir bar sorptive extraction (SBSE – Section 4.2.4) is used in combination with a thermal desorption system. Because more extraction phase is utilized, the desorption process is slower than that for a SPME fiber, thus desorption combined with cold trapping and reconcentration is required.

The setting of the parameters of thermal desorption will directly affect the desorption efficiency, collection and quantitative analysis of the sample (Grimm *et al.*, 1997). Desorption temperatures must be set high enough to facilitate the stripping of the volatile compounds, yet not alter the sample or analyte. Desorption times should be sufficient to remove the majority of the volatiles. Purge/carrier gas flow rates should be sufficient to purge the analyte from the sample (for example stir bar), but not push the desorbed volatiles through the cold trap.

This whole process has been automated. Two examples of systems that are available commercially are the TDS-2/A, a classic thermal desorption system (see Figure 4.2.3) and a specially designed Twister™ desorption unit (both from Gerstel). These systems can be mounted on Agilent Technologies gas chromatographs equipped with a CIS-4 programmable temperature vaporizing (PTV) inlet (also from Gerstel). The PTV inlet is operated as a cold trap (see Figure 4.3.3) for cryogenic focusing of the thermally desorped analytes. Temperatures as low as –150 °C are achieved using liquid nitrogen cooling (Hoffman *et al.*, 2000; Kreck *et al.*, 2001; Ochiai *et al.*, 2001; Sandra *et al.*, 2001; Wennrich *et al.*, 2001; Kreck *et al.*, 2002; Hayasaka *et al.*, 2003). Both of these systems allow fully automated control of all desorption, trapping and injection conditions, including temperatures, flows and split or splitless modes. Many examples of this technique are discussed in detail under the SBSE section of this book (Section 4.2.4).

A recent addition to the range of GC inlets is a combination of a standard split/splitless with temperature programmable capabilities. This is reported to allow for large volume injection and also support cool injections for improved signal response (Agilent, 2009).

Fused Silica Capillary Columns

Modern GC capillary columns consist of a fused silica tube (a synthetic, quartz like glass) that contains an inner stationary phase and an outer protective polyimide coating. As with glass injection port liners, fused silica tubing is deactivated (silylated) prior to addition of the stationary phase during manufacture. This is

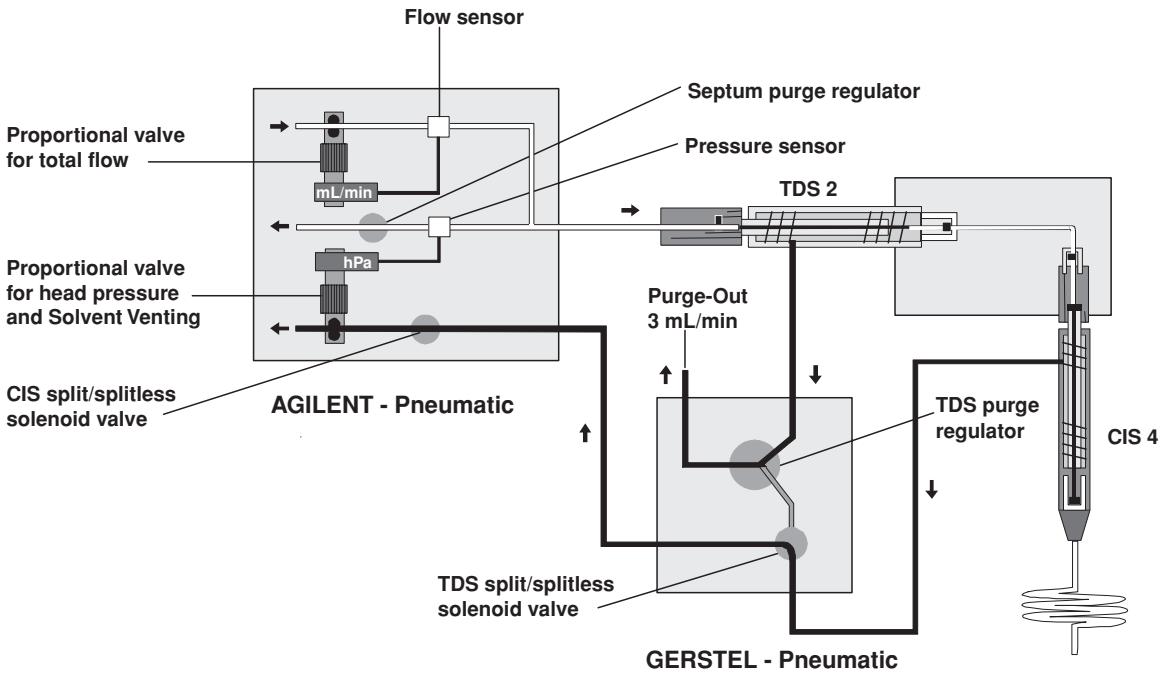


Figure 4.3.3 *TDS-2/CIS-4 flow diagram – TDS splitless desorption. Adapted from Gerstel (1998), with permission of Gerstel GmbH*

designed to limit the surface silanol groups which can hydrogen bond with certain functional groups of volatile compounds during analysis and provide poor chromatographic peak shape. The choice of stationary phase is selected by the analyst to separate the injected volatile organic components of a sample, based on the chemical and physical properties of these analytes. The subsequent retention time of each separated volatile component is a measure of the interaction of the analyte and stationary phase. If one particular stationary phase cannot separate certain target analytes, then it is possible that separation will be achieved with an alternative phase. Stationary phase polarity plays a large role in the ability of a column to separate sample components. Therefore, a nonpolar column is generally chosen for the separation and subsequent analysis of compounds that are composed of only carbon and hydrogen atoms and contain carbon–carbon single bonds (nonpolar compounds) and vice versa, polar columns for target analytes that are composed not only of carbon and hydrogen atoms, but also contain one or more atoms of oxygen, sulfur, nitrogen, chlorine, fluorine, bromine or phosphorus (polar compounds). Compounds that are composed of carbon and hydrogen and also contain double or triple carbon–carbon bonds (for example, aromatic hydrocarbons such as cyclic terpenoids) are classed as polarizable compounds and highly polar stationary phase columns are most commonly employed to separate these compounds of interest. Stationary phase interactions (dispersion, dipole, hydrogen bonding and acid–base) are also important factors of capillary column analysis and have been discussed by Jennings *et al.* (1997) and in some depth by Rood (1999). In basic terms, separations of analytes on a specific stationary phase are determined by differences in the overall effects of these interactions.

Table 4.3.1 lists the most common capillary column stationary phases (in order of increasing polarity) for GC analysis, along with their associated commercial description, stationary phase polarity and examples of typical analytes summarized by many column manufacturers. Low bleed stationary phases (commonly

Table 4.3.1 *Common fused silica capillary column stationary phases (in order of increasing polarity) for GC analysis*

Composition	Commercial Description[a]	Polarity	Typical Analytes
100% Dimethylpolysiloxane	AT-1, BP-1, CP-Sil 5 CB, DB-1, EC-1, HP-1, HP-101, OV-1, PE-1, Rtx-1, SE-30, SPB-1, SPB-Sulfur, SP-2100, Ultra 1, ZB-1, 007-1, AT-1ms, DB-1ms, HP-1ms, Rtx-1ms etc	Nonpolar	Aroma/flavor compounds, sulfur compounds, hydrocarbons, amines, pesticides, polychlorinated biphenyls (PCBs), phenols
5% Phenyl 95% dimethylpolysiloxane or 5% Phenyl 95% dimethyl arylene siloxane	AT-5, BP-5, CP-Sil 8 CB, DB-5, DB-5ht, EC-5, HP-5, OV-5, PAS-5, PE-2, PTE-5, PTE-5QTM, Rtx-5, SAC-5, SE-54, SPB-5, Ultra 2, ZB-5, 007-2, AT-5ms, DB-5ms, HP-5ms etc	Nonpolar	Fatty acid methyl esters (FAMEs), solvents, pesticides, herbicides, aroma/flavor compounds
6% Cyanopropyl-phenyl 94% dimethylpolysiloxane	AT-624, AT-1301, BP-624, CP-624, CP-1301, DB-624, DB-1301, HP-1301, HP-624, Rtx-1301, Rtx-624, SPB-1301, SPB-624, ZB-624, 007-1301, 007-624	Mid polar	Alcohols, pesticides, VOCs, aroclors
35% Phenyl 65% dimethylpolysiloxane or 35% Phenyl 65% dimethyl arylene siloxane	AT-35, BPX-35, DB-35, HP-35, MDN-35, OV-11, PE-35, Rtx-35, SPB-35, SPB-608, SUP-Herb, ZB-35, 007-11, AT-35ms, DB-35ms, HP-35ms etc	Mid polar	Chlorinated pesticides (CLP), aroclors
14% Cyanopropyl-phenyl 86% dimethylpolysiloxane	AT-1701, BP-10, CP-Sil 19 CB, DB-1701, HP-1701, OV-1701, PAS-1701, PE-1701, Rtx-1701, SPB-1701, ZB-1701, 007-1701	Mid polar	Pesticides, herbicides, trimethylsilyl (TMS) sugars, aroclors
50% Phenyl 50% dimethylpolysiloxane or 50% Phenyl 50% dimethyl arylene siloxane	AT-50, BPX-50, CP-Sil 24CB, CP-TAB-CB, DB-17, DB-17ht, HP-17, H5-50+, HP-50+, PE-17, Rtx-50, Rtx-65TG, SP-2250, SPB -17, SPB-50, SP-50, SP-2250, ZB-50, 007-17, DB-17ms etc	Mid polar	Glycols, pesticides
50% Cyanopropyl-phenyl 50% dimethylpolysiloxane	AT-225, BP-225, CP-Sil 43CB, DB-225, HP-225, OV-225, PE-225, Rtx-225, 007-225	Polar	Fatty acid methyl esters (FAMEs)
Polyethylene glycol	AT-Wax, AT-Aquawax, BP-20, Carbowax-20M, CP-Wax 52 CB, DB-Wax, DB-Waxetr, EC-Wax, HP-Wax, HP-20M, HP-INNOWAX, Omegawax, PE-CW, Rtx-Wax, Stabilwax, Supelcowax-10, ZB-Wax, 007-CW, AT-Waxms etc	Polar	Essential oils, aroma/flavor compounds, alcohols, solvents, free organic acids

(*Continued*)

Table 4.3.1 (*Continued*)

Composition	Commercial Description[a]	Polarity	Typical Analytes
Polyethylene glycol – base modified	AT-Cam, Carbowax amine, CP-Wax 51, Stabilwax-DB	Polar	Amines
Polyethylene glycol – acid modified	AT-1000, AT-Aquawax-DA, BP-21, CP-Wax 58CB, DB-FFAP, EC-1000, HP-FFAP, Nukol, OV-351, SP-1000, Stabilwax-DA	Polar	Organic acids, alcohols, aldehydes, ketones, aroma/flavor compounds, essential oils

[a]AT (Grace Davison), BP/BPX (SGE), CP-Sil (Varian now Agilent), DB (Agilent), EC (Grace Davison), HP (Agilent), MDN/Nukol/Omegawax (Supelco), OV (Ohio Valley), PAS (Agilent), PE (Perkin Elmer), PTE (Supelco), Rtx (Restek), SAC (Supelco), SE (Agilent), SPB/SP/Stabilwax/Supelcowax/SUP (Supelco), Ultra (Agilent), ZB (Phenomenex), 007 (Quadrex Corp),

containing an 'arylene') consist of a phenyl group that is added into the backbone of a standard polysiloxane and most are designed as low bleed equivalents to familiar stationary phases (for example, a 5% phenyl 95% dimethylpolysiloxane standard phase has the 5% phenyl 95% dimethyl arylene siloxane low bleed version – see Table 4.3.1). These are particularly useful where analytical sensitivity needs to be considered for certain GC detectors. Column bleed is most commonly observed as a rising baseline during analysis of a sample by GC and is most evident at temperatures around the upper limit of the column stationary phase. This phenomenon is completely normal and continues to increase with the lifetime of the column (and is dependant on the polarity of the stationary phase, the temperature of the column during analysis and the concentration of oxygen in the carrier gas). Therefore, due to the sensitivity of certain GC detectors to the stationary phase degradation products, the analyst may need to take into consideration the possible disappearance of peaks of interest into the column bleed with low level analysis of samples.

It is important to understand that the correct choice of column, to fullfil fit for purpose analysis of alcoholic beverage samples, is not only governed by the chemistry of the stationary phase. The selection of a column to provide the analyst with optimized chromatographic separation also needs to take into account the importance of the internal diameter of the fused silica tubing, the stationary phase film thickness and the overall column length.

The internal diameter (i.d.) of common commercially available capillary columns ranges between 0.10 mm and 0.53 mm. This enables the optimization of two important factors, namely efficiency and capacity. High efficiency is achieved when narrow and well resolved peaks (i.e, baseline separation of closely eluting analytes) are observed and the efficiency of the column (measured in plates (N) or plates per meter (N/m)) increases as the column i.d. decreases. On the other hand, sample capacity, which is the concentration of any one analyte that can be introduced to the column without causing a negative effect on the resulting chromatography (by causing a desired sharp symmetrical peak shape to overload and become a 'shark fin' shape) increases with column i.d. Optimisation of one of these factors is to the detriment to the other opposing factor therefore, 0.25 mm i.d. columns are generally considered standard, as this diameter provides adequate efficiency with acceptable sample capacity. Sharper chromatographic peaks and shorter retention times of separated sample analytes are also achieved generally with thinner film columns. Increasing stationary phase film thickness can reduce the interaction of analytes with the fused silica tubing and increase sample capacity. However this can also lead to an increase in chromatographic peak widths which in turn can lower resolution, increase column bleed (resulting in possible disapearance of peaks of interest into the column bleed with low level analysis of samples – commonly known as having a low signal to noise ratio) and a reduction in the upper temperature limit of the column during use. This can be restrictive to the analyst, as increased film thickness also leads to greater retention of analytes and hence the requirement of higher elution temperatures.

Typical capillary columns are available in lengths of 10 to 60 m. Longer columns will provide increased resolution when directly compared to shorter columns of equivalent stationary phase and film thickness, however the result is an increase in analysis time and head (inlet) pressure required to transport analytes through the column. The most common column length of 30 m for standard GC analysis generally provides the best compromise of resolution, analysis time and column head pressure. For many analyses, the use of a 60 m column to increase resolution should only be considered as a last resort, as a shorter column with a thicker film of stationary phase will most likely achieve an acceptable separation in far less time.

It is a fair statement to make that for a large majority of samples requiring separation of target analytes by GC can be achieved readily by the use of either a highly nonpolar phase (100% dimethyl polysiloxane or 5% phenyl 95% dimethylpolysiloxane and their associated low bleed equivalents) or highly polar phase (polyethylene glycol and modified polyethylene glycol phases). Indeed, a high percentage of the references that use modern capillary column GC analysis discussed under the extraction and focusing methods in sample preparation section (Chapter 4.2) of this book, either used straightforward nonpolar phases (DB-1, HP-1, CP-Sil 5 CB, DB-1ms etc. – see Table 4.3.1) or polar phases (Carbowax, Innowax, DB-Wax, Supelcowax, DB-FFAP etc. – see Table 4.3.1), obviously dependant on the types/chemical classes of volatile compounds that required separation and subsequent analysis.

Examples of alcoholic beverage analytes that have been recently analyzed via GC with a mid polarity phase include sulfur and selenium compounds in beers, wines and spirits using a DB-624 column (6% cyanopropyl-phenyl 94% dimethylpolysiloxane – see Table 4.3.1) (Campillo *et al.*, 2009), aroma compounds from sugar cane spirits, again using a DB-624 (Gracia *et al.*, 2007), odor active compounds from American Bourbon whisky (Section 3.3.4) using a DB-1701 column (14% cyanopropyl-phenyl 86% dimethylpolysiloxane) (Poisson and Schieberle, 2008), hop metabolites with a BPX-35 column (35% phenyl 65% dimethylpolysiloxane) (Shellie *et al.*, 2009), the fungicide famoxadone with a DB-17ms (50% phenyl 50% dimethyl arylene siloxane) (de Melo Abreu *et al.*, 2006) and pesticides with a BPX-50 or low bleed MS equivalent column (50% phenyl 50% dimethylpolysiloxane) (Patil *et al.*, 2009; Ramos *et al.*, 2009).

When a single GC column cannot provide effective resolution of the target analytes of a sample, then multidimensional GC (MDGC) can be applied. With this technique, two columns of different polarity (often housed in separate GC ovens) are connected via a switching mechanism. The sample is introduced onto the first capillary column and as the analyte(s) of interest elute they are switched (also known as heart cut) onto the second column for subsequent separation. For example, Ryan *et al.* (2005) reported an optimized headspace solid phase microextraction (HS-SPME – Section 4.2.4) for the determination of methoxypyrazines in wine. Analysis was performed using comprehensive two dimensional GC with nitrogen phosphorus detection (GC × GC-NPD) and time of flight mass spectrometry (GC × GC-TOFMS). For each system, the first dimension of the GC × GC column set was a BPX-5 (5% phenyl polysilphenylene-siloxane) column with a BP-20 (polyethylene glycol) column in the second dimension. It was concluded that both the multidimensional techniques were highly sensitive, yielding detection limits for 2-methoxy-3-(2-methylpropyl) pyrazine of 0.5 and 1.95 ng/l, respectively.

Following solid phase extraction (SPE – Section 4.2.4) of wine and other alcoholic beverages, Campo *et al.* (2007) used MDGC to determine four novel aroma powerful ethyl esters (ethyl 2-, 3- and 4-methyl pentanoate and ethyl cyclohexanoate). Here, the first dimension of the GC × GC column set was a DB-WAX (polyethylene glycol) and the second a FactorFour-VF5ms (polymethylsiloxane – 5% diphenyl). The authors concluded that the proposed method made it possible to provide (for the first time) an assessment of the levels and potential sensory importance of the novel esters in different beverages.

A further recent example of the use of MDGC for the evaluation of aroma compounds in wines includes the identification of volatile compounds responsible for prune aroma in prematurely aged red wines (Pons *et al.*, 2008), where a SPB-1 (100% dimethylpolysiloxane) and BP-20 (polyethylene glycol) set of columns were used.

Two dimensional GC has also recently been used for the determination of volatile compounds in cachaça (Section 3.5.4) (de Souza *et al.*, 2009). The nonpolar/polar column set consisted of a BPX-5 (5% phenyl 95% dimethyl polysilphenylene-siloxane) in the first dimension and a BP-20 (polyethylene glycol) in the second. The researchers concluded that the GC × GC method proved to be an effective technique to study production processes, due to its high resolution, improved revelation of headspace composition and sensitivity.

MDGC has unsurprisingly also found its use in the field of pesticide analysis. Recently a number of GC × GC–TOFMS methods have been proposed with a nonpolar (5% phenyl 95% dimethyl polysilphenylene-siloxane) and mid polar (50% phenyl 50% dimethylpolysiloxane) set of columns for the determination of pesticide residues in grapes (Banerjee *et al.*, 2008, Ramos *et al.*, 2009).

An extremely useful and analytically important group of GC stationary phases not listed in Table 4.3.1 are the chiral family of capillary columns. These can be used as either the main GC column to separate target analytes into their respective enantiomers or used as one of the dimensions in a GC × GC setup.

Different multidimensional chromatographic approaches applied to the study of wine malolactic fermentation (Chapter 2.3) were reported by Fernandes *et al.* (2003). For enantioselective GC, a 30 m × 0.22 mm i.d. tailor made fused silica capillary column coated with a 0.25 μm film thickness of 15% heptakis (2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin in SE52 (5% phenyl 95% dimethylpolysiloxane – replaced by SE-54) was used. For enantiomultidimensional GC-MS, a dual oven system was used with a SE-52 (30 m × 0.25 mm i.d.) in the first dimension and in the second oven the same DiMe β-CD phase column as used for the enantioselective analysis, but with different i.d. (30 m × 0.25 mm). First and second columns were connected via a live switching T piece. (*R*,*R*), and (*S*,*S*) and meso-butane-2,3-diol and pentane-2,4-diol (reported for the first time in wines) were submitted to untrained sensory panel tests and it was concluded that all stereoisomers revealed different sensory notes; pentane-2,4-diol showed an aromatic impact.

Enantioselective MDGC was used to determine the ratios of various free and glycosidically bound monoterpene polyols in musts of the aromatic grapes *Vitis vinifera* L. cvs. Morio Muscat and Muscat Ottonel (Luan *et al.*, 2004). The dual oven GC system contained a 30 m × 0.25 mm i.d. column coated with 0.25 μm SE52 in the first dimension and in the second oven, a column coated with heptakis (2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin in SE52. It was reported for the first time that the linalool-derived polyol 3,7-dimethylocta-1,7-dien-3,6-diol occurred predominantly as the (3*S*-6*S*)-configured stereoisomer.

The chiral separation of amino acids derivatized with ethyl chloroformate by using comprehensive two dimensional GC has recently been reported (Junge *et al.*, 2007). Analyses were performed on a chiral/polar column set incorporating a 25 m × 0.25 mm i.d. × 0.16 μm film thickness Chiralsil-L-Val (L-valine-*tert*-butylamine linked poly(dimethylsiloxane) ex-Varian – now Agilent) first dimension and a 1 m or 3 m × 0.1 mm i.d. × 0.10 μm BPX50 (50% phenyl 50% dimethylpolysiloxane) in the second dimension. For chiral/low polarity analysis, the first dimension column consisted of either a 1 m or 3 m × 0.10 mm i.d. × 0.10 μm film thickness BP1 (100% dimethylpolysiloxane). It was concluded that the best results were obtained when the enantioselective first dimension column was combined with the nonpolar BP1 phase in the second dimension. A selection of beer samples were analyzed with the optimized method and proline was determined as the major amino acid.

Chiral MDGC has also been utilized for the analysis of many specific volatile alcoholic beverage compounds. Recent examples include wine lactone (Luan *et al.*, 2006) and rose oxide (Koslitz *et al.*, 2008) both using a heptakis (2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin in SE52 chiral stationary phase to separate enantiomers.

The composition of essential oils and their mixtures used to formulate gin is usually too complex to enable separation of all components by standard GC. Minor constituents that possess important organoleptic properties can be masked or hidden by coelution with major sample components. Mac Namara *et al.* (2007) used sequential two dimensional GC-MS (consisting of a 30 m × 0.25 mm × 0.25 μm Supelcowax-10 in the first dimension and a nonpolar HP5-MS column also 30 m × 0.25 mm × 0.25 μm in the

second – see Table 4.3.1) to produce a target compound library, with orthogonal GC × GC providing the separation power required to obtain peak retention times and the corresponding mass spectra needed for the creation of a deconvolution database (once the mass spectrum, retention time window and/or retention index for each component is known, the deconvolution method is constructed). Following this, individual compounds could be identified using standard one dimensional GC and commercially available deconvolution software (Ion Signature Software from Ion Signature Technology, North Smithfield, RI, USA). The authors concluded that 101 unique mass spectra that belonged to compounds from a mixture of essential oils used in the production of gin (Section 3.4.2) (whose resultant ion fingerprints under GC × GC separation produced invariant signals in at least four consecutive scans across the peak) were identified. The identical fingerprints were found in the initial test mixture and a modified mixture where one of the 10 botanical and citrus oils was exchanged with nutmeg oil. In addition to the identification of the constituents of the remaining initial test mix, 17 of the 20 additional components in nutmeg were identified in the model mixture. The peaks for the three common compounds increased in signal count as expected, following fortification of the sample. It was concluded that because the acceptable scan to scan variance in the relative abundance could be set in the deconvolution method, it was easy to visually inspect the peak to determine whether the target compound had been correctly indentified. This made the total product characterization, shelf life studies, routine quality control and consumer flavor/aroma perception studies amenable to low level compound identification in the presence of large concentrations of coeluting matrix components. The deconvolution software allowed the optimization of spectral search matching conditions and hence the method proposed could lead to the identification of low concentration analytes usually difficult to identify without prior sample cleanup procedures.

An ever increasing number of analyses are now moving away from conventional GC analysis with 0.25 mm i.d. columns. This is due to the recent commercialization and hence availability of modern narrow i.d. columns (0.10 to 0.18 mm) which contain thinner films of stationary phase (typically 0.10–0.18 μm) and 10 to 20 m in length. This state of the art GC technique known as fast GC, can provide typically a three- to 10-fold reduction in analysis time, whilst still providing acceptable resolution of target analytes during an analysis. This is achieved by manipulation of the crucial analytical variables, namely column length, column i.d. and film thickness, increased linear velocity of carrier gas, oven temperature and oven ramp rate during analysis. Although historically helium, hydrogen and nitrogen have been used as typical GC carrier gases, hydrogen is considered the optimum choice for fast GC due to its high diffusivity and high optimal linear velocity (Supelco, 2008). One disadvantage of modern narrow i.d. columns is limited sample capacity when compared to conventional 0.25 mm i.d. columns (as less sample can be introduced onto the column for analysis), hence high injection split ratios are adopted to yield narrow, sharp and symetrical peak shapes, which in turn reduces or even eliminates the loss of analytical sensitivity. Many major GC instrument manufacturers now offer instruments and columns that are designed with fast GC in mind, as well as offering free method translation software (or consultancy) that will allow users to translate conventional methods into fast methods very easily, as long as the column phase ratio is maintained (for example, if a conventional method uses a 25 m × 0.25 mm i.d. × 0.25 μm column then a translated fast GC method should adopt the use of a 10 m × 0.10 mm i.d. × 0.10 μm column or 20 m × 0.18 mm i.d. × 0.18 μm to possess the theoretical equivalent resolving power and the order of eluting taget analytes).

Mac Namara *et al.* (2005) reported a fast GC analysis of major volatile compounds in distilled spirits. Initial conditions (split ratio, gas velocity, initial oven temperature and oven ramp rate) were determined from method translation of a similar method using a standard 0.25 mm i.d. column with identical stationary phase. These parameters were then investigated in an experimental design comprising a series of experiments in which the responses were the resolution of two critical peak pairs, the analysis time and the limit of quantitation (LOQ) of eight major compounds. Following chemometric evaluation of the obtained data, a validated model was constructed to allow the prediction of optimum chromatographic conditions. It

was concluded that the use of modern GC hardware made the fast method an ideal candidate for routine analysis.

A recent study focused on the application of 0.18 mm i.d. capillary columns for brewing analysis (Horák *et al.*, 2009). The robustness of the state of the art faster GC, without compromising resolution, was demonstrated with the analysis of beer flavor compounds, and sample run times were reported to be reduced by 60%.

Oven

Column temperature is an important variable that must be controlled very accurately over a wide range of temperatures during a GC analysis. Conventionally, the chromatographic column is housed in an oven which can be either isothermally operated (one in which the oven is maintained at a constant temperature) to elute volatile components of a sample, or by temperature programming (whereby the column temperature is increased either continuously or in steps as the separation proceeds) for complex samples consisting of analytes with a broad boiling range. The optimum column temperature of any method depends upon the boiling point of the sample and the degree of separation required. Roughly, a temperature equal to or slightly above the average boiling point of a sample is generally considered to yield chromatographic results within a reasonable elution time.

With the modern popularity of fast GC and potential decrease of analysis times, the oven temperature ramp rate is also being increased faster than ever previously encountered in conventional analysis. In some cases, a conventional GC oven cannot provide the desired ramp rate for a fast method, therefore new oven inserts have appeared commercially. This type of system enables direct resistive heating of the capillary column using ceramic insulated heating wire with high precision temperature control across the entire length of the chromatographic column, and hence, rapid temperature ramping and cooling rates can be achieved with ease. A recent example of the use of resistive heating gas chromatography (RH-GC) has been reported for the rapid screening of organophosphorus pesticides in fruits (including grape) and vegetables (Patel *et al.*, 2004).

Other devices capitalize on fast oven cooling following completion of an analysis by greatly increasing the mass flow of air through the oven during cool down, resulting in a reduction of the GC cycle time (the time taken to cool the oven from final upper operating temperature to starting temperature for the next analysis to begin – often 15 to 20 min). This is an interesting approach, since it does not interfere with the analysts existing GC methods, only the overall cycle time, hence increasing sample throughput without any change in existing method variables. A commercial version of this approach is available from Anatune (Cambridge, UK), called the GC CoolR+.

Detectors

A number of GC detectors are available commercially; the reason for such a wide and varied choice is because there is currently not one single detector that can satisfy the demands for all GC analyses: Table 4.3.2 details the most common GC detectors. Crucial characteristics and requirements such as dead volume, support gas requirements, detector temperature, linear range, sensitivity and selectivity have been discussed in depth by Rood (1999) and can be found in the literature and websites of the major GC manufacturers. Many extensive examples of alcoholic beverage research using a wide range of GC detectors can be found under the extraction and focusing methods in sample preparation section (Chapter 4.2) of this book.

An ever increasing number of modern GC analyses use only a mass spectrometer as detector for a wide array of analytes. This is because MS detectors can be used as either a universal detector (in scan mode) or a specific detector (in SIM mode). Most modern MS detectors have the ability to be used in dual scan/SIM modes to collect the maximum amount of useful data during each analysis.

Table 4.3.2 *Common detectors for GC analysis*

Detector	Selectivity	Minimal Detectable Level*	Linear Dynamic Range*
Flame Ionization (FID)	Most organic compounds	<1.8 pg C/s (for tridecane)	10^7 (± 10%)
Mass Spectrometer (MS)	Universal (Scan Mode) Specific (SIM Mode)	0.1–1 ng (Full Scan)† 0.1–10 pg (Selective Ion Monitoring)†	10^4–10^5†
Thermal Conductivity (TCD)	Universal	400 pg/mL (for tridecane with He carrier)	10^5 (± 5%)
Flame Photometric (FPD)	Sulfur, Phosphorus	<60 fg P/s, <3.6 pg S/s (with methylparathion)	10^3 (S) 10^4 (P) (with methylparathion)
Pulsed Flame Photometric (PFPD)	Sulfur, Phosphorus	<100 fg P/s, <1 pg S/s‡	Quadratic in response Linear to *ca.* 10^3 Gives 10^5 signal response‡
Sulfur Chemiluminescence (SCD)	Sulfur	<0.5 pg S/s (dimethyl sulfide in toluene)	10^4
Electron Capture (ECD)	Halides, nitrates, nitriles, peroxides, anhydrides, organometallics	<6 fg/ml (for lindane)	5×10^4 (with lindane)
Nitrogen Phosphorus (NPD)	Nitrogen, Phosphorus	<0.4 pg N/s <0.06–0.2 pg P/s	$>10^5$
Nitrogen Chemiluminescence (NCD)	Nitrogen	<3 pg N/s	$>10^4$
Atomic Emission (AED)	Carbon, Sulfur, Nitrogen, Hydrogen, Chlorine, Phosphorus, Oxygen	1 pg C/s (*t*-Butyl disulfide)§ 2 pg S/s (*t*-Butyl disulfide)§ 30 pg N/s (Nitrobenzene)§ 4 pg H/s (*t*-Butyl disulfide)§ 30 pg Cl/s (1,2,4-Trichlorobenzene)§ 2 pg P/s (Triethyl phosphate)§ 150 pg O/s (Nitrobenzene)§	10^4§ 10^4§ 2×10^4§ 5×10^3§ 10^4§ 10^3§ 5×10^3§
Olfactometric (GC-O)	Aroma/flavor compounds	User specific	User specific

*Agilent, 2009
†Rood, 1999
‡O.I. Analytical, 2010
§http://www.jas.de/html/products/AED/aed-specifications.php

Plutowska and Wardencki (2008) recently reported an extensive review of the application of gas chromatography-olfactometry (GC-O) in the analysis and quality assessment of alcoholic beverages. They concluded that despite the fact that odor detectors have been in use for over 40 years, literature indicates that in recent years they have been used more frequently, finding numerous applications in the analysis of food and beverages (including alcoholic beverages). There is, however, still need for improvement of GC-O techniques and the investigations on using them for quantitative analysis of odor compounds. Further studies are also required because past studies on the optimization of working parameters and on the quality and reliability of the obtained results (which is very important considering feasibility of implementing the GC-O technique to industrial practice) have not been well accepted.

4.3.3 Liquid Chromatography

High performance liquid chromatography (HPLC), in its many modes, is probably still the most widely used chromatographic method for the analysis of alcoholic beverages, especially for the determination of less volatile, more polar constituents. A simplified scheme showing a typical HPLC system is shown in Figure 4.3.4.

The mode of HPLC used depends upon the stationary phase in the column (Figure 4.3.4), which in turn depends upon the nature of the analytes (ionic, polar, nonpolar, chiral, polymeric) and influences the mobile phase used. Reversed phase (RP) is the most popular mode, but normal phase (NP), ion exchange (ion chromatography – IC), ion exclusion and size exclusion modes (often known as gel permeation chromatography, GPC or gel filtration chromatography, GFC – for polymers) are also used. Nowadays, many kinds of detectors are available (Figure 4.3.4), but those most widely used in the analysis of alcoholic beverages are mass selective detectors (mass spectrometers) and UV-visible detectors. The former set up (HPLC linked to a mass spectrometer, MS) is usually known as LC-MS. In discussing application of LC-MS to alcoholic beverage analysis, the emphasis in this section will be on the chromatographic aspects; mass spectrometric aspects

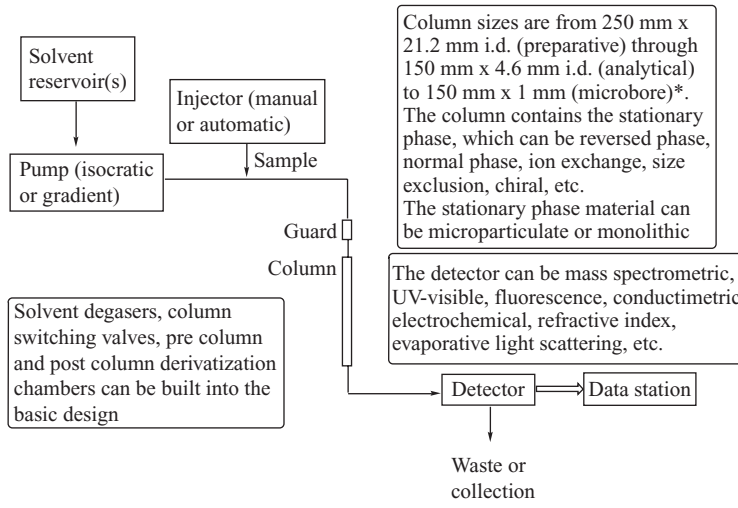


Figure 4.3.4 *Scheme showing arrangement of modules for HPLC analysis. *Nanobore columns are also used, for LC-MS*

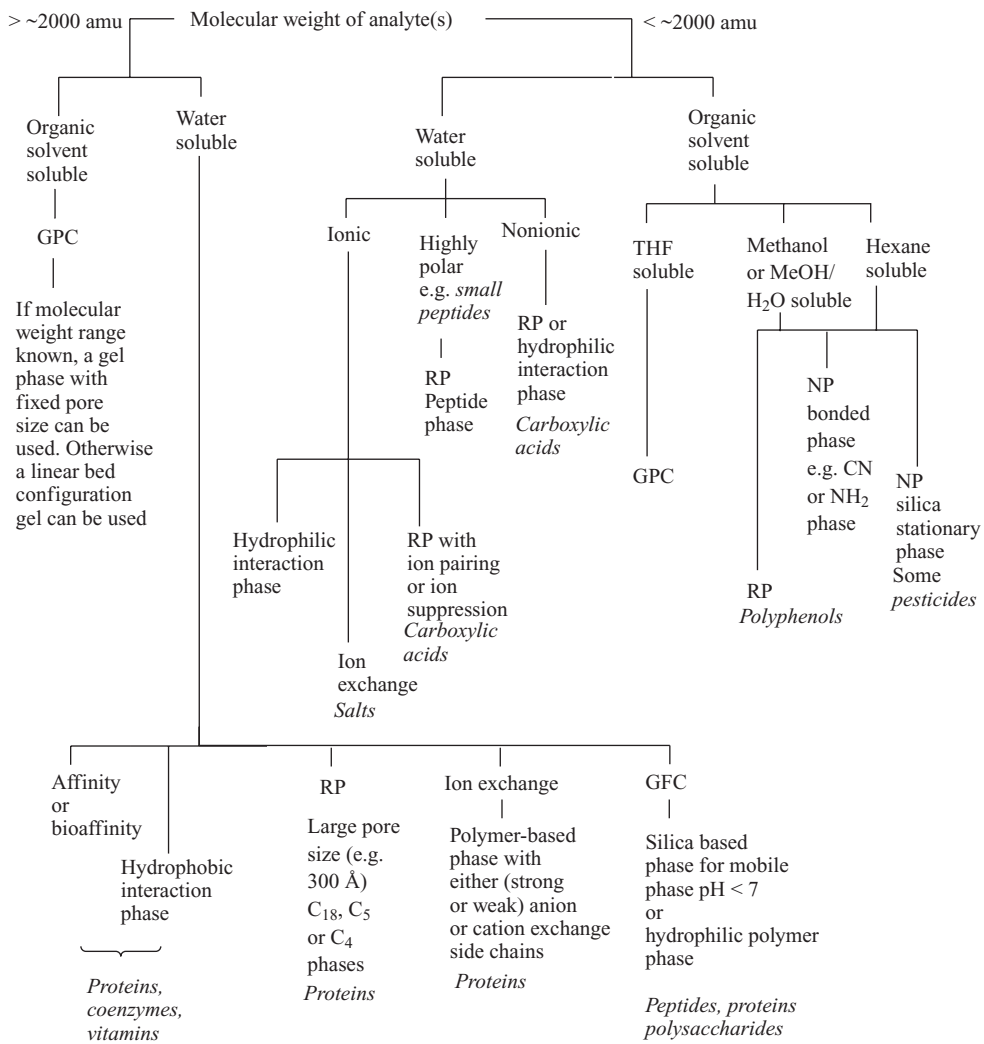


Figure 4.3.5 *Stationary phase selection tree*

(e.g. mode of ionization, fragmentation patterns, etc.) are discussed more fully in Section 4.4.5. In many modern HPLC systems, postcolumn eluent splitting may be used so that analytes can be monitored by two detectors simultaneously, usually a mass spectrometer and a diode array UV-visible detector (LC-MS-DAD).

Deciding on a suitable mode of HPLC for analysis of a particular type of component or range of components in a beverage can be carried out using an HPLC stationary phase tree, as shown in Figure 4.3.5. Once the stationary phase has been decided, initial experimental conditions can be determined by searching the literature and/or by use of software such as ACD Autochrom (a computer assisted method development that combines instrument control and rational method development for Agilent and Waters LC systems). Types of stationary phases that are frequently used to analyze different kinds of compounds in different beverages are summarized in Table 4.3.3. It should be kept in mind that due to the complexity of the

Table 4.3.3 *Summary of applications of HPLC to alcoholic beverage analysis*

Analyte	Beverage	Usual HPLC mode/detector	Selected references* and comments
Polyphenols	All, including must and fruit skins	RP/MS, UV-vis or MS-DAD	Rodríguez *et al.* (2006) RP, cider, UV-vis Masa and Vilanava (2006) RP, grape skin flavonols, MS Hernanz *et al.* (2009), RP, wine aging, UV-vis Jordheim *et al.* (2007), RP, anthocyanins, elderberry
Carboxylic acids	Wine, cider and must	RP, ion exclusion or ion exchange/UV-vis	Masson (2000) must Mato *et al.* (2005) and references therein, must, wine
Sugars	All	RP/various	Benton (2004) RP, amperometric detector Alltech Associates (2002)
Amino acids and biogenic amines	Beer, wine	RP/fluorescence, Usually after derivatization	Berlanga *et al.* (2006) Sherry Chlup *et al.* (2006) beer Dugo *et al.* (2006) wine, all biogenic amines Kabalová *et al.* (2008) amino acids, beer Önal (2007) and references therein
Minerals and ions	All	Ion exchange/ conductometric	Dugo *et al.* (2005) Marsala
Residues, trace substances and miscellaneous components	All	RP, NP/various	Herbert *et al.* (2002) RP, fluorometric, ethyl carbamate Owens *et al.* (2007) RP, fluorometric, folate in beer Pickering Laboratories Inc. (2004) RP, fluorometric, mycotoxins Young (2002) RP (NH_2), glycerol, wine
Colorants	All	RP/UV-vis	Yoshioka and Ichihashi (2007)
Sweeteners	All	RP/RI or ELSD	Wasik *et al.* (2007),
Flavor compounds	All	RP, NP/MS, UV-vis	Carneiro *et al.* (2006) RP, damascenone, brandy, whisky, wine Wood *et al.* (2008) RP, rotundone, wine
Polymers		GPC/various	Visotek (2004) GPC/RI, dextrins, beer

*For more references, see text of Section 4.3.3. Also, see text of Section 4.3.3 and Chapter 4.2 for examples where HPLC is used to purify or isolate substances for analysis by other methods.

analytical matrix (alcoholic beverages contain large numbers of compounds of many different types) sample pretreatment ('clean up'), such as column chromatography or solid phase extraction (SPE) (Section 4.2.4) or a combination of these, will often need to be carried out.

Reversed Phase HPLC

RP chromatography involves interaction between a nonpolar stationary phase and nonpolar sections of solute molecules in the relatively polar mobile phase. The mechanism of RP chromatography involves a partition effect, which is related to the relative solubilities of the solute molecules in the two phases. In general, the greater the nonpolar character of a solute, the greater is the retention (the higher the values of t_R and k') for

a given mobile phase. Reversed phase is still the most widely used mode of HPLC; it probably accounts for ~70% of all HPLC analyses.

Reversed phases fall into two major categories:

- Alkyl bonded silica gels (including polar bonded phases, which sometimes can be used in normal phase). This includes spherical particulate and monolithic materials.
- Cross linked synthetic polymers. This also includes spherical particulate and monolithic materials.

The particulate materials are usually composed of a very uniform distribution of spheres of diameters from lower than 3 μm to10 μm (Meyer, 1998). Within these particles are many channels or pores (mesopores) of diameters ranging from 80 Å to 300 Å (depending on how the silica is made), giving a large surface area. Typically, alkyl bonded particulate phases have surface areas of 150–500 m^2/g. Loading of bonded phase can be calculated as percentage carbon load or bonded phase coverage ($\mu mol/m^2$). Octadecylsilyl phases, probably the most popular RPHPLC phases, have carbon loads from ~5% (Whatman Partisil) through ~11% (Thermo Fisher Hypersil) to 20% (Nacali Tesque Cosmosil), with bonded phase coverages from 0.63 (Whatman Partisil), through 3.47 (Agilent Zorbax) to 5.50 $\mu mol/m^2$ (Phenomenex Jupiter).

Particulate RP stationary phases are usually spherical (Whatman Partisil is an exception, having irregular particles), with narrow size distribution and are available in 3, 5 and 10 μm sizes. A stationary phase made up of smaller particles (lower than 2 μm) is more efficient than a similar phase made of larger particles, but causes high back pressure due to greater restriction of flow of the mobile phase through the particles. Recently, C_8, C_{18} and similar phases based on type B silica 2.7 μm fused core (superficially porous) particles of extremely narrow size distribution have been shown to be highly efficient, with reduced theoretical plates of 1.5 or lower for small molecules (DeStefano *et al.*, 2008). The fused core and HALO® columns of Advanced Materials Technology also exhibited back pressures of only one half or one third of those of composed of 1.9–1.7 μm particles. The fused core particles consist of 1.7 μm solid silica cores with a spherical coating of 0.5 μm thick shells containing 9 nm pores. Although there are no reports yet (2010) of the use of these columns in alcoholic beverages, a remarkable separation of eight pesticides in less than 0.6 min at a flow rate of 4.0 ml/min (pressure 230 bar) at 60 °C was demonstrated.

Alkyl bonded silica gels, the most widely used of all RP materials, can be subdivided into a number of variants, as shown in Figure 4.3.6. Most of the variants are designed to overcome a number operational problems associated with RPHPLC, as outlined below:

- End capping, to prevent polar interactions between residual silanol groups and analytes
- Smothering of residual silanol groups (by steric hindrance, or cross linking) to prevent polar interactions, as above
- Polar end capping or polar embedded alkyl bonding (Rieger and Riering, 2000), to prevent collapse of alkyl chains in the presence of a largely aqueous phase and to stabilize the phase toward low and high pH
- Polyfunctionization, to prevent decomposition of the phase in mobile phases of low or high pH
- Low loading of C_{18} functionality, with no end capping to give the RP phase extended polarity due to residual silanol groups (Chappell, 2002).

Polar bonded phases usually consist of silanol groups linked to short alkyl chains that have a terminal polar group, such as CN, NH_2 or $CH(OH)CH_2OH$. These phases can be used in either reversed phase mode (with polar solvent) or normal phase mode (with nonpolar solvent).

Other categories of RP stationary phases include graphitized carbon (Shandon Hypercarb) (Ross and Majors, 2000), zirconia based (ZirChrom Separations ZirChrom-EZ), perfluorinated alkyl bonded phases (Przybyciel, 2003) and type C silica (MicroSolv) (Majors, 2003). Porous graphitic carbon phases perform in

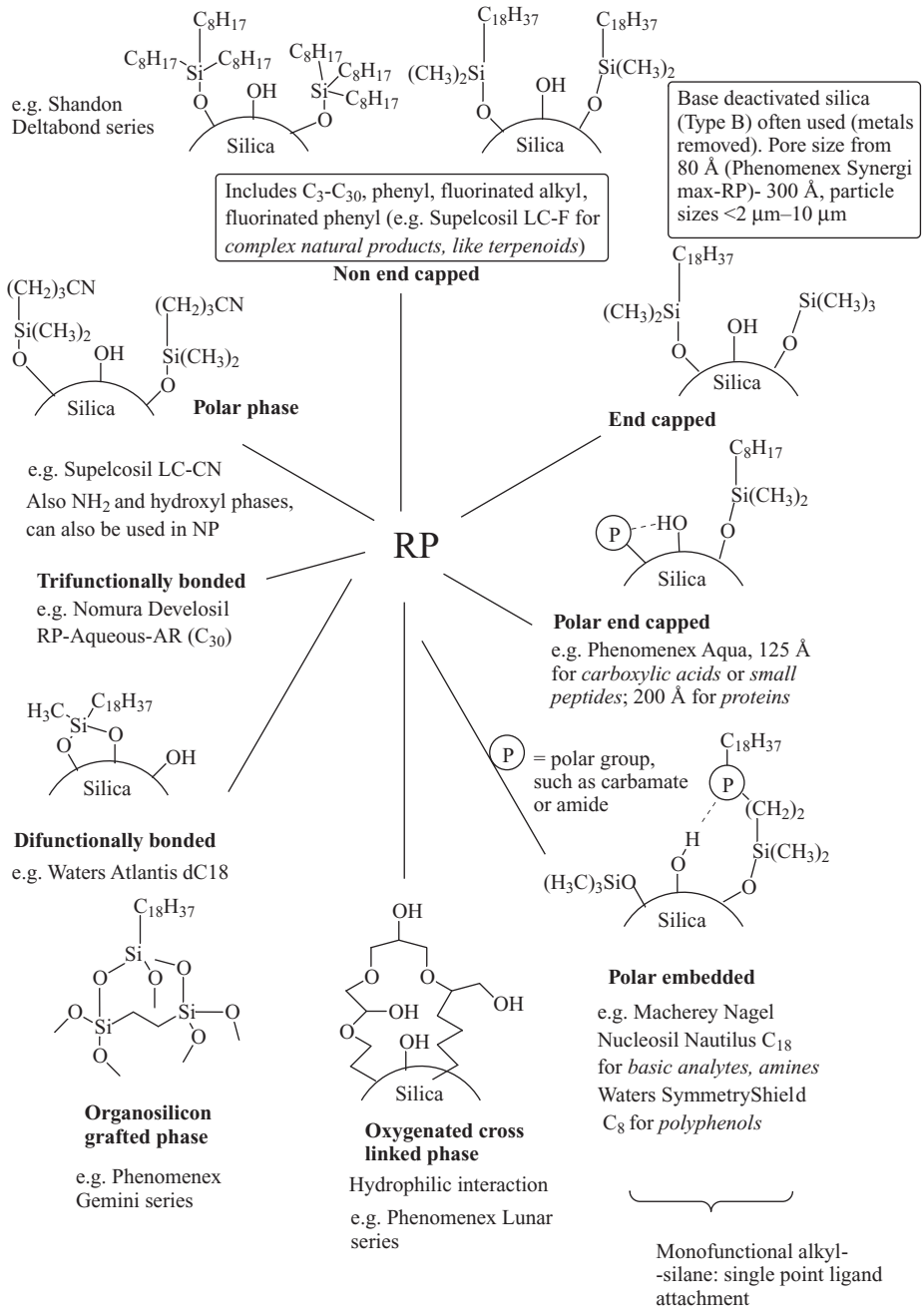


Figure 4.3.6 *Alkyl bonded and related RP stationary phases*

reversed phase mode, but have different selectivities to silica C_{18} phases, in particular they show increased retention of nonpolar analytes and increased selectivity toward structurally related compounds. Zirconia-based RP phases exhibit increased stability at both low and high pH and also at high temperatures.

Many detectors are available for the detection of components separated on an HPLC stationary phase as they emerge from the column (Figure 4.3.5). The most commonly used are UV-visible (including the diode array version, DAD) and mass spectrometric detectors (LC-MS is treated separately, below). Also widely used are fluorometric, refractive index, evaporative light scattering (ELS), electrochemical (amperometric and coulometric), conductometric and chiroptical (polarimetry or circular dichroism) detectors. Less commonly, HPLC systems have also included infrared or nuclear magnetic resonance detectors.

Individual organic acids, important constituents of musts and alcoholic beverages, are often determined by RPHPLC – see Mato *et al.* (2005) for a review of methods for the analysis of grape and wine acids, which includes HPLC. Most methods use UV detection, but photochemically induced luminescence (Peréz-Ruíz *et al.*, 2004) and electrochemical detection (Casella and Gatta, 2002) have also been used. Use of special robust stationary phases, such as radially compressed phases (Billingsley *et al.*, 1996) and acid resistant phases (e.g. Phenomenex Aqua) with largely or wholly aqueous acidic solvents can give good resolution and can be used to monitor malolactic fermentation (Section 2.3.10) (Buglass and Lee, 2003).

The preservative additives benzoic acid and sorbic acid can be determined in alcoholic beverages by RPHPLC with UV-visible detection and often using isocratic mobile phase. Analysis can be simplified by use of a sample pretreatment or clean up procedure, such as reversed phase SPE (Section 4.2.4), as used in the analysis of benzoate and sorbate in Thai rice wines and rice wine distillates (Techakriengkrai and Surakarnkul, 2007). Here, an isocratic method (0.01 M aqueous ammonium acetate buffer:methanol, 60:40) was used with a Waters Spherisorb S100DS2, 250 mm × 4.6 mm (i.d.) stationary phase and detection at 235 nm. Reversed phase SPE (C_{18}) was used for sample pretreatment. Alternatively, acidic preservatives in alcoholic beverages can be determined as their anions (benzoate, sorbate and sulfite) by ion chromatography with conductometric detection (De Borba and Rohrer, 2004).

Sugars in beverages are usually determined by ion exchange or ion exclusion chromatography using cross linked polymer stationary phases (see ion chromatography, below), but the fermentable sugars of beer wort (fructose, glucose, maltose, maltotriose and sucrose) were recently analyzed using a Supelcosil LC-NH_2 phase (an amino bonded silica phase) and acetonitrile:water (70:30) as the mobile phase (Navarro *et al.*, 2007). All five sugars were separated in less than 10 min chromatography run time.

Although terpenoids, norisoprenoids and other flavor compounds in alcoholic beverages are usually determined by GC (often GC-MS) (Section 4.3.2), β-damascenone has been determined in beers, wine and distilled spirits by RPHPLC with UV detection (Carneiro *et al.*, 2006), although a steam distillation/solid phase extraction (Section 4.2.4) preconcentration step was required before HPLC analysis. Carneiro *et al.* (2006) showed that forced ageing of beer, but not wine and spirits, resulted in increased β-damascenone levels (Section 2.6.12). Another flavor compound, rotundone (a sesquiterpene) in Shiraz (Syrah) grapes and wine was determined using normal phase HPLC (Wood *et al.*, 2008). Rotundone is a major contributor to the peppery flavor of certain wine grapes (especially Shiraz) grown in relatively cool regions. Peppery fractions from column chromatography were analyzed on a 5 m silica column (250 mm × 2 mm i.d.) using gradient elution (solvent A was hexane:ethyl acetate (98:2); solvent B was ethyl acetate). Linalool, β-caryophyllene and caryophyllene oxide were used as standard markers and the peppery fractions were combined and studied using GC-MS and GC-MS-O to confirm the presence of (–)-rotundone.

Reversed phase HPLC coupled with liquid–liquid extraction has been suggested as a more specific way than the standard solvent extraction/UV-visible spectrophotometric method (Section 4.4.3) for the determination the *iso*-α-acid bittering agents of hopped beer (Jaskula *et al.*, 2007). The various spectrophotometric methods, although inexpensive and rapid, are rather unspecific, with components other than bittering compounds being measured as well. Although other HPLC methods had been reported before (see references in Jaskula *et al.*,

2007), none were able to resolve all the bittering components. Here, the bittering agents were selectively and quantitatively extracted from beer using *iso*-octane, followed by removal of the solvent and redissolving the residue in ethanol/H_3PO_4 (99.75/0.25 v:v). Analysis was performed on a C_{18} column using isocratic elution at ambient temperature using 48%, eluent A and 52% eleuent B, where eluent A was water adjusted to pH 2.80 with H_3PO_4 and eluent B was acetonitrile. Analysis time was 50 min with a flow rate of 1.8 ml/min. The detector was a diode array detector set at 270 nm (for *iso*-α-acids) and 314 nm (for α-acids). Mean recoveries were very good, ranging from 92.9% to 99.2%, and the bittering agent profile of the extract was identical to profile of the spiked unhopped wort or beer.

The biggest application of RPHPLC to the study of fruit, juices and alcoholic beverages is probably the determination of phenolic compounds, including anthocyanins, flavonoid phenols and their glycosides. Ultraviolet detection is common, with analyte identities generally being obtained by comparison with authentic standards or by combination with LC-MS data. However, the latter technique is the most widely used for the determination of phenols in general, where both quantification from peak areas and identification from mass spectra (often using tandem mass spectrometric techniques) can be achieved, even for more complex polymeric phenols.

Reversed phase HPLC with UV-visible detection has been applied to the study of phenolic compounds in the skins and juice of fruits that can be used to make alcoholic beverages, as well as to cider and wine per se. This technique (aided by NMR and ESI mass spectrometry) was used to identify new anthocyanin glycosides – such as cyanidin 3-*O*-(6″-*O*-arabinosylglucoside) – in the skins of elderberries and other fruits (Jordheim *et al.*, 2007). The changes in phenolic profile of highbush blueberry fruits during ripening were monitored by HPLC-DAD using a Wako Fluofix 120E phase and gradient elution (water:acetonitrile:acetic acid (94.5:5:0.5) (A) and acetonitrile (B)) (Castrejón *et al.*, 2008). Similarly, the anthocyanin content (mainly cyanidin, pelagonidin and peonidin glycosides), flavonol content and nonflavonoid phenol content of blue honeysuckle (*Lonicera Caerulea* L.) fruits (which are similar to bilberries and can be used to make fruit wines and liqueurs) were determined by gradient elution RPHPLC with UV detection (aided by ESI-MS) (Chaovanalikit *et al.*, 2004).

Sixteen flavonoid phenols have been identified by RPHPLC in the skins of the white grape variety Albarín Blanco (Asturias, Spain), where very unusually the most abundant phenols were quercetin dihydroflavonols (Section 5.8.6), thus allowing easy identification of this variety and a useful check of authenticity (Masa and Vilanova, 2008). Also, flavan-3-ols, hydroxycinnamic acids and dihydrochalcones (Section 5.8.6) have been quantified in the juice of crushed Spanish cider apples (Suárez *et al.*, 1998) and the important French cider apple variety Kermerrien using RPHPLC-DAD.

Likewise, the flavonols in Port wine grape varieties (Tinta Amarela, Tinta Barroca, Tinta Cão, Tinta Roriz, Touriga Francesca, Touriga Nacional and Rufete) were determined using a minibore (200 mm × 2.1 mm i.d.) C_{18} RP column and gradient elusion (5% aqueous formic acid (A); methanol (B) (Andrade *et al.*, 2001). Anthocyanins were removed by SPE (C_{18} end capped columns) after the lyophilized grape samples were extracted with methanol. The methanol was then removed after filtration and the residue was redissolved in 0.1 M HCl and applied to the conditioned SPE column. Kaempferol 3-glucoside and isorhamnetin 3-glucoside were significant components of all the varieties, apart from Rufete.

Usually, gradient elution (as in the above examples) is used to analyze fruit skin or fruit extracts, because of the wide range of polarities of the extracted components. However, division of the extraction of *V. vinifera* skins using supercritical CO_2 (with ethanol modifier) (Section 4.2.2) into two fractions allowed the isocratic determination of flavan-3-ols in one fraction and quercetin, rutin and *trans*-resveratrol in the other fraction, using different mobile phases (Chafer *et al.*, 2005).

Reversed phase HPLC with UV detection (often DAD) is also useful in the determination of phenolic and other compounds in wine and other beverages, often the only sample pretreatment being dilution, although solid phase extraction (Section 4.2.4) can be used give analytes in different fractions.

The phenolic profiles of 92 Asturian ciders from two consecutive vintages revealed hydroxycinnamic acids, dihydrochalcones and procyanidins to be the most abundant phenolic compounds, with hydrocaffeic acid, phloridzin, procyanidin B5 and tyrosol being the major of the known components (Rodríguez *et al.*, 2006).

It has long been known that red wines have intrinsically higher phenolic contents than white wines, irrespective of the mode of aging. RPHPLC-DAD analysis of Greek red and white wines from a range of locations and *V. vinifera* varieties supported this generality, with (+)-catechin being the most abundant polyphenol. Retsina white wines (with added pine resin) (Section 2.12.3) had higher levels of phenolic compounds than standard white wines, the extra phenolic content originating from the resin (Proestos *et al.*, 2005). Length of aging in wooden casks, with possible involvement of *Brettanomyces* spp., as well as wine color, can be correlated with concentrations of ethylphenols, which are generally regarded as off flavors if present at levels above their odor threshold values. RPHPLC with fluorescence detection (225/320 nm) showed that 4-ethylphenol and 4-ethylguaiacol were more prevalent in red wines than white wines of the Wurttemberg region, especially if the wine had experienced long storage in wooden casks (Nikfardjam *et al.*, 2009).

The major phenolic compounds in southern Portuguese wines from the *V. vinifera* variety Trincadeira (red) were gallic acid, (+)-catechin, syringic acid, ferulic acid, protocatechuic acid, *trans*-resveratrol and caffeic acid, whereas for the wine of Síria (white), only catechin was abundant (almost as much as in the red wine), with small levels of gallic and protocatechuic acids, and only trace levels of the others (Nave *et al.*, 2007).

The effects of one year's storage on the phenolic contents of wines made from the *V. vinifera* varieties Colombard and Zalema in southwestern Spain have been studied by Hernanz *et al.* (2009). Although the acidity of Colombard wine was higher than that of Zalema wine, the one year's aging had similar effects on the phenolic contents of both types of wine, with concentrations of some (e.g. gallic acid, catechin, caftaric acid and fertaric acid) diminishing, whilst levels of others (e.g. (–)-epicatechin, tyrosol, rutin, *p*-coutaric acid, caffeic acid and ferulic acid) increasing or remaining unchanged.

The free and conjugated flavonol (myricetin and quercetin) content of 65 red wines from many geographical areas were determined by RPHPLC using gradient elution with UV detection (McDonald *et al.*, 1998). The detection limit was *ca.* 0.8 mg/l. Concentrations of total flavonols ranged from 4.6 to 41.6 mg/l, with Chilean wines generally having the highest values. This was attributed to either better exposure of ripening berries to sunlight or to harvesting very ripe grapes, but altitude or other geographic/climatic factors may be important.

Fortified white wines, such as Sherry and Montilla-Moriles are protected from oxidation and browning when undergoing biological aging in the presence of flor (Section 2.10.3). Once removed from the flor, the aging process can be rapid and flor wines, such as fino Sherries are usually best consumed within one year of bottling. In this context, RPHPLC was used to study the effect of various prebottling fining agents on the phenolic content of flor Montilla-Moriles wines (Baron *et al.*, 2000). It was shown that several fining agents; activated charcoal, PVPP and Ridulxhigh, could control browning by removing flavan-3-ols and their dimers from the wine.

On the other hand, some fortified wines, such as Banyuls (Section 2.10.9), Madeira (Section 2.10.5), tawny Ports (Section 2.10.7) are deliberately aged in an oxidative manner. RPHPLC-DAD has been applied to study the phenolic content of a number of these wines (Ho *et al.*, 1999) and it was found that the total phenolic and furan content were greater in the oak aged wines. The chromatograms show clearly that older wines of the same type (e.g. tawny Port) had a higher concentration of total phenols and all the wines studied had significant levels of furans, considered to be the products of nonenzymic browning and the Maillard reaction (Section 2.6.2).

The use of synthetic food colors is often controversial, because different countries have their own regulations regarding permitted food colorants, and falsification of red or white wine or other beverage color by the use of such colorants, although rare, is not unknown. Reversed phase HPLC is very well suited to the identification of such colorants in a complex matrix such as that of wine, vermouth or liqueur. Indeed, Virtanen *et al.* (1999) noted that RPHPLC gave more accurate identification than UV-visible spectroscopy of a number of synthetic

dyes (amaranth, azo ruby, erythrosine, quinoline yellow, ponceau 4R, sunset yellow and tartrazine), as well as natural carmine in wines. In an earlier report, several artificial colorants were separated and quantified by RPHPLC and azorubin, reported to be the main red wine adulterant, could be detected at the 0.8 mg/l level (Flak *et al.*, 1998). More recently, Yoshioka and Ichihashi (2008) have developed an HPLC-DAD method that is able to identify 40 artificial food colorants in beverages in less than 20 min chromatography run time.

Reversed phase HPLC can be used in the analysis of a number of nonphenolic aromatic substances in alcoholic drinks. Coumarin is widely distributed in the plant kingdom and many alcoholic beverages (some liqueurs, Mai Wein, vermouth and some flavored vodkas) are flavored with plant materials that contain coumarin, particularly cinnamon (Section 5.11.2). This substance, once believed to be genotoxic, is presently listed as not being classifiable as a human carcinogen by the International Agency for Research on Cancer (IARC), although there are reports on its hepatotoxicity (Section 5.11.2). With a maximum allowed concentration of 10 mg/kg for some alcoholic beverages in the EU, it is necessary to monitor the coumarin content of beverages that are likely to contain it. Numerous HPLC methods are quoted by Sproll *et al.* (2008), who have developed a fast gradient elution RPHPLC method, with UV detection at 279.8 nm. A Phenomenex Synergi polar-RP column (silica functionalized with $-(CH_2)_3OPh$ and containing polar end capping) was used, as it gives good aromatic selectivity amongst analytes when a methanol containing mobile phase is used.

Folates, such as tetrahydrofolate (THF) and 5-methylene-tetrahydrofolate 5MTHF) are cofactors in the biosynthesis of certain amino acids, but their oxidized form, folates cannot be synthesized by mammals and hence must be obtained from the diet. Cereals are known to be a good source of folic acid. Reversed phase HPLC with fluorescence detection has been applied to the determination of various folates, including folic acid (FA), THF, 5MTHF and others in alcoholic beverages, mostly beers (Owens *et al.*, 2007). The quantity of each folate was calculated in the free acid form using external standard calibration curves. Folates absorbed at 290 nm and emitted at 360 nm. Samples were subjected to a clean up with an SAX (strong acid ion exchange) SPE treatment before analysis. The folate content of beers ranged from 2.2 to 24.2 μg per bottle (355 ml), giving up to 6.1% of the recommended daily allowance.

In HPLC, the universal detectors are mass spectrometric, refractive index and evaporative light scattering; the remaining detectors require the presence of specific groups in the analytes, such as absorption chromophores for UV-visible detection, fluorescent chromophores for fluorescence detection, ionic sites for conductometric detection, chiral groups for polarimetric or circular dichroism detection and oxidizable groups for electrochemical detection. If these groups are not present, then they can be introduced into many specific analyte molecules by the process of derivatization.

Derivatization

Derivatization of analytes is less common nowadays in HPLC than in GC methods. Nevertheless, there are well defined cases where derivatization can help with HPLC analysis. The introduction of detectable groups into analyte molecules can significantly boost detection sensitivity. Groups introduced via derivatization include chromophores (for enhanced UV detection), fluorophores (for fluorescence detection, which is often ultra sensitive) and electrochemically active groups (for electrochemical detection).

The two major types of derivatization processes depend on the timing of the derivatization process, as described below:

- *Precolumn (or exceptionally, on column) derivatization*. Here the previously derivatized analytes are separated as such. Advantages of precolumn derivatization include:
  - Choice: a wide range of derivatization reactions can be used, including slow or high temperature reactions
  - Sample clean up prior to injection can be used to remove by-products or unreacted derivatization reagent, for example
  - Choice of mobile phase is not limited by the derivatization reaction.

- A big disadvantage of the method is that it becomes labor intensive if a large number of samples need to be derivatized; automation is really required here.
- *Postcolumn derivatization*. The analytes are separated normally and then derivatized 'on line' before they reach the detector. Advantages of postcolumn derivatization include:
  - Routine use for the analysis of large numbers of samples; the method lends itself best to automation
  - The separation step is independent of the derivatization reaction; the analytes are separated in their original form.

  Disadvantages of the method include possible long set up times, possible detection interference from unreacted reagent and the necessary compatibility of the derivatization reaction with the mobile phase. Additionally, cost may be an important factor since this method can use large quantities of expensive reagent.

Examples of pre- and postcolumn derivatization schemes and reagents are shown in Tables 4.3.4 and Table 4.3.5, respectively.

Table 4.3.4 *Examples of precolumn derivatization. 3,5-Dinitrobenzoyl chloride can be used to derivatize alcohols; phenacyl halides and 4-Bromomethyl-7-methoxycoumarin can be used to derivatize carboxylic acids*

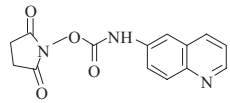
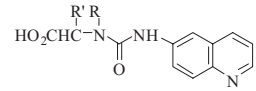
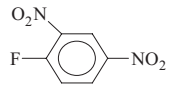
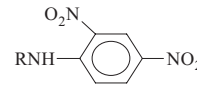
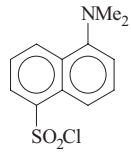
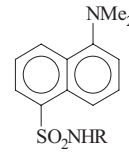
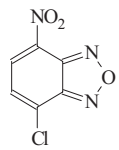
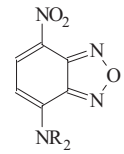
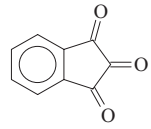
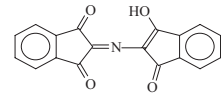
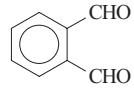
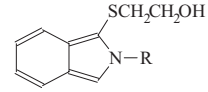
Analyte	Reagent	Product	Detection enhancement
Amino acids $RNH(CHR')CO_2H$	6-Aminoquinolyl-*N*-hydroxy-succinimidyl carbamate (AQC) 		Fluorescence excitation at 250 nm, emission at 395 nm
Amines, RNH_2, etc.	 1-Fluoro-2,4-dinitrobenzene (Sanger's reagent)		UV
Amines and amino acids	 Dansyl chloride		UV/fluorescence UV at 254 nm; fluorescence excitation at 320 nm, emission at 523 nm
Secondary amines, R_2NH	 'NBD chloride'		Fluorescence

Table 4.3.5 *Examples of postcolumn derivatization*

Analyte	Reagent	Product	Detection enhancement
Amines and amino acids	 Ninhydrin	 'Ruhemann's purple'	UV
Amines and amino acids	 *o*-Phthalaldehyde + $CH_2(SH)CH_2OH$		Fluorescence Excitation at 340 nm; emission at 425-450 nm
Carbohydrates	Ce(IV)	C(III)	Fluorescence

The determination of amino acids and biogenic amines (Section 5.11.3) in alcoholic beverages is often carried out by the use of RPHPLC, with detection of the derivatized analytes by UV spectrophotometry, or more often by the more sensitive fluorometry (Önal, 2007). The most popular derivatization agents are dabsyl chloride, dansyl chloride and *o*-phthalaldehyde, but others, such as naphthaquinone-4-sulfonate and 6-aminoquinolyl-*N*-hydroxy-succinimidyl carbamate (AQC) are sometimes used. In most cases, precolumn derivatization is used, with chromatographic separation of the derivatized analytes being carried out by gradient elution. Interest in the biogenic amine content of alcoholic beverages arises largely from concern over their cause of allergenic reactions in sensitive individuals (Section 5.11.3). Beers have generally higher levels of biogenic amines than wines (Gorinstein *et al.*, 1999; Loukou and Zotou, 2003).

The biogenic amine contents of wines of many grape varieties from several countries and also of Chinese rice wines have been determined by RPHPLC with derivatization (Romero *et al.*, 2000; Loukou and Zotou, 2003; Anli *et al.*, 2004; Landete *et al.*, 2005; Dugo *et al.*, 2006; Yongmei *et al.*, 2007; Hernández-Orte *et al.*, 2008; Huang *et al.*, 2009).

Biogenic amines were analyzed in Spanish wines as dabsyl derivatives (Romero *et al.*, 2000), or as *o*-phthalaldehyde derivatives (Landete *et al.*, 2005) or (along with amino acids) as AQC derivatives (Table 4.3.4) (Hernández-Orte *et al.*, 2008). In the second study, histamine and putrescine were found to vary most between different varietal wines of different regions. In the last study, the biogenic amine contents of Cabernet Sauvignonand Tempranillo wines were found to be greater in post malo-lactic fermentation samples, but MLF performed by bacterial cultures resulted in lower amine levels in the wine compared with native MLF samples.

Biogenic amine contents of a wide range of Greek wines (Loukou and Zotou, 2003) and Turkish wines (Anli *et al.*, 2004) as dansyl derivatives revealed rather higher levels of histamine and tyramine in red wines, with the Greek retsina wines generally having the lowest amine levels. The Greek wines were subjected to treatment with poly(vinylpyrrolidone) (PVP) to remove phenolic interferences before derivatization and then to focusing and clean up with SPE after derivatization. The amines generally associated with poor sanitary conditions, putrescine and cadaverine, were present at low concentrations in nearly all the samples.

The gradient elution regimes for some of the above analyses are rather complex (see Önal, 2007, for many examples), often involving an acidified or buffered aqueous solvent and an aqueous organic solvent. However,

a simple gradient elution program involving only acetonitrile and water components has been used to separate the dansyl derivatives of amines in experimental Sicilian wines from a wide range of *V. vinifera* verieties (Dugo *et al.*, 2006).

Precolumn derivatization with dansyl chloride was used to determine biogenic amines in Chinese rice wines (Section 2.7.1) using RPHLC with acetonitrile-water gradient elution and diode array UV detection (Yongmei *et al.*, 2007). The mean total level of biogenic amines in the samples was 107 mg/l (range: 39.30–241 mg/l. These levels are below the levels that are generally thought to cause direct adverse reactions for most consumers. The detection limits were 0.05 mg/l for cadaverine, histamine and spermidine, 0.1 mg/l for tyramine and 0.25 mg/l for spermine.

More recently, the fluorescence probe 2,6-dimethyl-4-quinolinecarboxylic acid *N*-hydroxysuccinimide ester was applied for the precolumn derivatization of biogenic amines in Chinese rice wines (Huang *et al.*, 2009). The derivatives were extracted from the medium using ultrasound assisted liquid–liquid microextraction and were analyzed by standard RPHPLC using methanol:water (60:40, v:v) as mobile phase and fluorescence detection at 326/412 nm. The calibration data of this method were linear in the range of 5–500 μg/ml (for octopamine and tyramine) and 0.025–2.5 μg/ml (for phenethylamine). The relative standard deviations were 2.4–3.2% (n = 6) and the limits of detection were in the range of 0.02–5 ng/ml, typically much lower than those of the above dansyl chloride UV detection method.

Homocysteine (HCy) is nonprotogenic amino acid, whose plasma levels are often used as a marker of oxidative stress from an imbalance of metabolism; for example, high plasma levels have been correlated with smoking, lack of exercise and excessive alcohol intake (Section 5.6.2). However, it is possible that a certain amount of plasma HCy could be obtained directly from the diet, although there are few reports of the HCy content of foodstuffs. Benkova *et al.* (2009) studied the HCy content of 36 Bulgarian wines, using RPHPLC with precolumn derivatization using *N*-(2-acridonyl)maleimide and fluorescence detection. The method was able to quantify down to 68 fmol of HCy and the levels in white wines (0.09–0.64 mg/l) were found to be generally lower than in red wines (0.10–1.37 mg/l).

Reversed phase HPLC has been performed on the dansyl derivatives of amino acids in Montilla-Moriles wines (or simulated wines) undergoing biological aging (Section 2.10.3) in an attempt to determine, amongst other things, which amino acids are best for yeast cell growth (Mauricio and Ortega, 1997; Valero *et al.*, 2003; Berlanga *et al.*, 2006). The main source of nitrogen for yeast growth was found to be L-proline, with L-leucine being released. Beers are rich in amino acids as demonstrated by a study of 35 beers from six different countries using RPHPLC with precolumn derivatization by AQC (Table 4.3.4) and a three solvent system gradient elution programme (Kabelová *et al.*, 2008).

A more specialized application of a gradient elution RPHPLC method is the determination of the dansyl derivative of hydroxyproline in beer (Chlup *et al.*, 2006). Hydroxyproline is a hydrolysis product of isinglass, which contains collagen proteins and is used to fine many beers, especially cask-conditioned ales (Section 2.6.9). This amino acid is not found in the proteins of brewing raw materials or yeast, so its presence in acid hydrolyzed beer is indicative of the presence of isinglass. This is important because isinglass is still (2008) under investigation regarding supposed allergenic properties. This method can detect amino acids at concentrations as low as $\sim 4 \times 10^{-13}$ mg/l.

The derivatization technique can be used in the analysis of a number of other substances in alcoholic beverages. Derivatization of ethyl carbamate (EC) (urethane) by 9-xanthydrol (Figure 4.3.7), followed by RPHPLC separation of fluorescent xanthylurethane on an amine (polar bonded phase) column allowed the estimation of EC in wines, fortified wines and spirits (Herbert *et al.*, 2002). Since EC is a carcinogen, its concentration in alcoholic beverages is constantly under surveillance (Section 5.11.5). The xanthylurethane derivative was excited at 233 nm and the emission was measured at 600 nm. The detection limit using this method was 4.2 μg/l, well below the maximum allowed limits (30–200 μg/l, depending on the beverage) set by the Canadian government (Section 5.11.5). More recently gradient elution HPLC with fluorescence

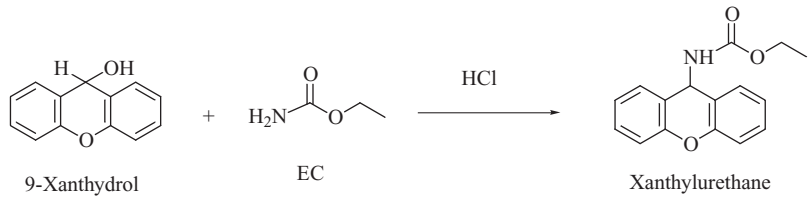


Figure 4.3.7 *Formation of xanthylurethane derivative for HPLC analysis of ethyl carbamate*

detection was used to determine EC, as its 9-xanthhydryl derivative, in cider spirits, with improved detection limit (1.64 μg/l) (Rodríguez Madrera and Suárez Valles, 2009).

Mycotoxin residues (Section 5.11.4) in fungus infected grains used for brewing in the production of beer, whisky and other spirits can be determined by RPHPLC using fluorescence detection and postcolumn derivatization for certain analytes (aflatoxins and fumonisins), whereas others (ochratoxin A and zearalenone) are already fluorescent and DON can be determined by UV (DAD) (Pickering, 2004). Photochemical derivatization was used for aflatoxins, and *o*-phthalaldehyde/thiofluor reagent was used to derivatize fumonisins. The fluorescent analytes could be detected at levels down to 0.01 ppb (for aflatoxin B2) to 9 ppb (μg/l) (for zearelenone).

Ochratoxin A, produced by many species of fungus (e.g. *Penicillium* and *Aspergillus* spp.), is a more serious contaminant of grape must and wines (Section 5.11.4) and needs to be monitored regularly so that levels in wines are below 2 μg/l and weekly intake (from all sources) is less than 100 ng per kilogram of bodyweight, as recommended by JECFA of FAO/WHO (Section 5.11.4). A solid phase microextraction technique (Section 4.2.4) using a PDMS/DVB fiber and interfaced with RPHPLC-fluorescence detection was able to quantify ochratoxin A in wine samples at levels down to a limit of quantification (LOQ) of 0.22 μg/l. The limit of detection was 0.07 μg/l, based on a signal to noise ration of 3:1. Furthermore, the method requires only isocratic conditions and the chromatography run time was under 15 minutes.

The Office International de la Vigne et du Vin (OIV) method for the analysis of ochratoxin A in beverages requires the passage of the pH adjusted, centrifuged and filtered sample (100 ml) through an immunoaffinity column, washing through with water, air drying the column and desorbing the ochratoxin A by passing methanol:acetic acid (98:2 v:v) (1.5 ml) through the column, evaporating to dryness and redissolving in themobile phase (2 ml) (Bellí *et al.*, 2004). Using this method, Bellí *et al.* (2004) were able to determine ochratoxin levels in 240 musts and wines (including Sherry, Malaga and vermouth). The detection limit for this method was 0.05 μg/l, based on a signal to noise ratio of 3:1. Aresta *et al.* (2006) used HPLC with fluorescence detection to determine ochratixin A concentrations in wine samples. Here SPME (Section 4.2.4) was used for sample clean up in place of the usual immunoaffinity column method. The limit of quantification at a signal to noise ratio of 10 was 0.22 ng/ml.

Sugars can be derivatized as their *p*-aminobenzoate esters and analyzed via RPHPLC. Such a method has been used to determine the sugar profiles of ciders, where, with the help of various chemometric techniques, the use of apple juice concentrate (AJC) (Section 2.8.6) can be detected (Blanco Gomis *et al.*, 2004). A C_8 column was chosen for these analyses, with gradient elution and detection at 307 nm. The variables chosen for principal component analysis were arabinose, fucose, D-galactouronic acid, D-glucuronic acid, rhamnose, ribose and xylose, because AJC tends to be richer in these sugars due to the action of hydrolytic enzymes (liquefaction AJC) or due to fast pressing and stabilization (press AJC) that occur in the production of AJC. Using the Bayesean modeling method, it was possible to distinguish between natural ciders, and ciders made from either liquefaction or press concentrate. Moreover natural ciders with more than 10% of

press concentrate added or with more than 5% liquefaction concentrate added could be detected. At the time of writing (2009), the use of AJC in Spanish natural cider is forbidden and so there is a need for analysis methods for the detection of fraud.

In HPLC, an alternative to the process of derivatization, described above, is the use of a universal detector, the most important of which are refractive index (RI) (the original), evaporative light scattering (ELS) and mass spectrometry (MS). The last named is so important (as LC-MS) it is dealt with separately at the end of Section 4.3.3.

Evaporative light scattering detectors measure the scattering of light as components pass through a heated drift tube (70–250 °C) and undergo evaporation. ELSD can be used to quantify and profile the monosaccharide and disaccharide sugars in fruit juices and other media. For example, an Alltech Prevail Carbohydrate ES phase has been used with 75% (v:v) acetonitrile:water mobile phase for the quantification of fructose, glucose and sucrose in orange juice (Alltech Associates, 2002). RPHPLC with ELSD was used to determine the sugar profile (fructose, glucose, lactose, maltose, rhamnose, sucrose and xylose contents) of Marsala wines (Section 2.10.8), the total sugar content of whose categories 'secco,' 'semi-secco' and 'dolce' are fixed by law (La Torre *et al.*, 2008).

Intense sweeteners are used in a wide range of foodstuffs, but are generally not allowed for the sweetening of alcoholic drinks (Section 5.9.4), so it is important to have reliable methods of analysis for the detection of adulteration and fraud. Nine intense sweeteners were simultaneously determined in foodstuffs using RPHPLC and ESLD (Wasik *et al.*, 2007).

Stationary phases used were Agilent Zorbax Extend-C_{18}, Merck Nucleodur C_{18} and Macherey-Nagel C_8 Gravity and C_{18} Pyramid, with gradient elution after SPE clean up of the homogenized sample.

Glycerol is an important constituent of wine (Sections 2.2.4 and 2.2.6), giving an attractive appearance to the wine and contributing to a good mouthfeel, as a result of its high viscosity. When a sample containing glycerol is subjected to HPLC with ELSD the high temperatures experienced in the drift tube result in loss of analyte and glycerol may not be detected. Low temperature (28 °C) evaporative light scattering has been used to successfully separate and quantify glycerol (in the presence of fructose, glucose and sucrose) in Bordeaux white wine, using a bonded amino phase and acetonitrile:0.4 mM aqueous ammonia (80:20), with a Shimadzu ELSD-LT detector (Young, 2002).

Ion Chromatography

This is sometimes the preferred method for the analysis of anions in juices, beverages or potable water, although the analysis of cations is often conducted using atomic spectroscopy (Section 4.4.4), ion selective electrodes (Section 4.5.1) or various voltametric techniques (Section 4.5.2). Nowadays, capillary zone electrophoresis techniques offer a powerful alternative for the analysis of organic anions in juices and alcoholic drinks (Section 4.6.1). Although the stationary phases used in ion methods for the separation of ionic or ionizable solutes are essentially ion exchange materials, the mechanisms of separation are complex. Indeed, some of these stationary phases can be used to separate mixtures of ionizable and polar covalent solutes. The major interactions which contribute toward separation are:

- Ion exchange
- Ion exclusion
- Ligand exchange
- Adsorption
- Partition
- Size exclusion

The mechanisms of some of these processes will be considered later.

The following are the more common types of stationary phases:

(a) *Porous Synthetic Polymers.* These have cationic or anionic exchange functions (e.g. $SO_3^-M^+$, $-CH_2-NR_3^+X^-$). The polymer backbone is typically polystyrene-divinylbenzene copolymer (PSDVB) as in the Bio-Rad Aminex series, Dionex IonPac series, Dionex CarboPac PA1, Supelcogel (H and C-610H) and Phenomenex Rezex series. Alternatively the backbone can be based on poly(methyl methacrylate), as with Waters IC-Pak, or vinyl alcohol copolymers, as with the Asahipak ES series and some Shodex IC columns. The majority of ion chromatography phases are porous synthetic polymers.

(b) *Bonded Silica Phases.* The hydrocarbon chains linked to the silica gel possess ionic (ion exchanger) end groups or with quaternary ammonium groups embedded in C_{18} or similar chains. This gives both RP and IE capabilities.

(c) *Polymer Coated Silica Phases.* Here a polymer, such as butadiene-maleic acid copolymer, is coated onto silica gel and then cross linked.

Advantages and disadvantages of these systems include the following points:

Type (a). Good pH stability (e.g. 0–14), but not so tolerant of high pressure
Type (b). High pressure tolerant, but limited pH stability (e.g. 2–8)
Type (c). High pressure tolerant along with no need for high pH eluents, like type (b), however, pH stability is limited.

The types of separations possible with ion exchange stationary phases are summarized in Table 4.3.6.

Ion chromatography falls into two major categories that depend on the number of ion exchange groups on the stationary phase and hence on the ionic strength of the mobile phase.

- *Low capacity ion exchange columns (unsuppressed ion chromatography).* These are usually rigidly cross linked (typically PS-DVB with 4% to 8% cross linking) polymers with low surface coverage of ion exchanger groups (typically $-NR_3^+X^-$ or $-COO^-H^+$). Relatively weak eluents are used (e.g. 0.005M phosphate buffer or water) and detection is usually by conductometric or spectrophotometric detection.
- *High capacity ion exchange columns (suppressed ion chromatography).* These have a high density of ion exchanger groups at the surface of the spherical polymer beads. They are more widely used than the low capacity type. A typical example of a cation exchanger of this type is the Dionex IonPac CS17

Table 4.3.6 *Summary of analytes determined by ion chromatographic methods*

Solutes	Method	Detection
Inorganic ions	*High capacity ion exchangers	UV, RI/ELSD, (cond)
	*Low capacity ion exchangers	Cond, UV, RI/ELSD
Organic anions	Anion exclusion	UV, RI/ELSD, cond
	Anion exchange	
Organic acids	Cation exchangers (H^+ form)	UV, RI/ELSD
Sugars and polyols	Cation exchangers (Ca^{2+}, Pb^{2+}, Ag^+ forms)	UV, RI/ELSD
Peptides and proteins	PSDVB (15μm)	UV
	PMMA	

Cond = conductometric, RI = refractive index, UV = ultraviolet

phase, consisting of a hydrophobic resin with surface grafted carboxylic groups. It acts as a weak cation exchanger and its main application is in the analysis of biogenic amines, including polyamines. An example of an anion exchanger is the Alltech Novosep A-2 phase, a quaternary ammonium functionalized poly(vinyl alcohol), useful in the determination of anions (including oxyhalide ions) in water that is to used for brewing or dilution of distilled spirits. Another anion exchanger, the Dionex CarboPac PA20 phase, is useful in the separation of mono- and disaccharides. It is an ethylvinylbenzene-divinylbenzene cross linked polymer functionalized with a difunctional latex containing quaternary ammonium groups (Majors, 2003). The carbohydrates are separated in their ionized forms at high pH.

More concentrated ionic eluents must be used with these columns, thus requiring ion suppression methods, if the ideal detection (conductometric) method is to be used. The ion suppression methods involve the conversion of an ionic eluent to a weakly ionized (i.e. weakly conducting) eluent and the removal of counter ions.

Types of ion suppression include packed bed (the original method; see Small *et al.*, 1975, which is really two column ion chromatography), fiber, micromembrane and electrochemical.

The first three methods are chemical methods and work by ion exchange mechanisms as the eluent and solutes leave the column, but before they reach the detector. Nowadays, the chemical methods of ion suppression have given way to self-regeneration suppression (SRS), in which the solution used to suppress ionic background is continually regenerated by electrochemical reactions. The mode of action of a typical SRS system is shown schematically in Figure 4.3.8, for analysis of anions. Some modern ion chromatography systems also have eluent regenerating capabilities, using the regenerator to reconstitute the mobile phase. After passing through the detector, the suppressed eluent is led to a trap column where analyte ions are

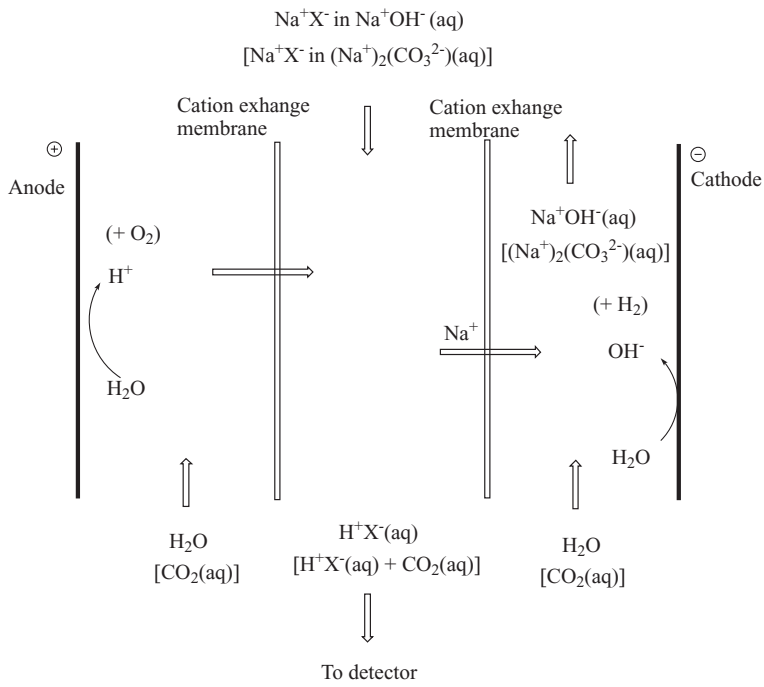


Figure 4.3.8 *Schematic mode of action of an SRS device. X^- is the anion of analytical interest. Schemes for hydroxide and carbonate mobile phases are shown*

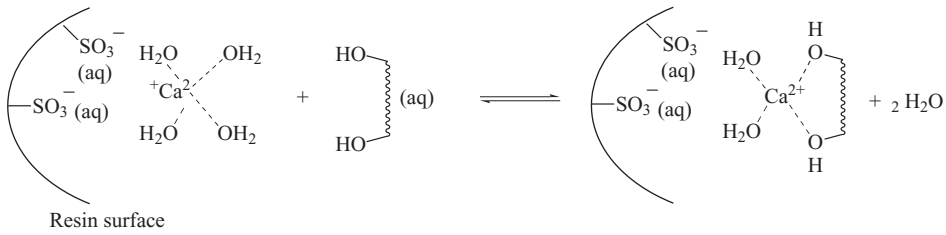


Figure 4.3.9 *Scheme for possible ligand exchange action of some ion exchange stationary phases*

removed. The eluent is then returned to the suppressor to provide the water for electrolytic suppression once more. The suppressor effluent, containing eluent ions, H_2 and O_2, is circulated to a catalytic column (not shown in Figure 4.3.8) where the gases are recombined to give water and is then returned to the eluent reservoir for reuse (Dionex, 2009).

As mentioned earlier, a number of interactions occur in ion chromatography, all of which influence the chromatographic features (retention times, resolution, peak shape etc.) to a greater or lesser extent.

Ligand exchange interaction appears to be dominant in the separation of carbohydrates and polyols, when the metal forms of strongly acidic (sulfonate type) cation exchange resins are used as the stationary phases. Typical metal counter ions include $Ca^{2+,}$ Pb^{2+} and Ag^{+}; examples include the Phenomenex Rezex series and the Bio Rad HPX series, both phases being widely used in the analysis of sugars, polyols and acids in foodstuffs, including alcoholic beverages, particularly the last named.

Separation occurs mainly by selective interaction of solute OH groups with hydrated metal counter ions, via ligand exchange (Figure 4.3.9).

These methods are widely used in the food industry and have the advantages:

- Short retention times
- High resolution
- Use of water as eluent.

Disadvantages are minor, but include:

- Price
- Need for column heating: most experiments need to be carried out at $\sim$50 °C.

Ion exclusion is thought to be the major interaction in the separation of organic acids (e.g. in fruit juice and wines) using strongly acidic cation exchangers in the acid form, as the stationary phase. Examples include Phenomenex Rezex ROA and Bio-Rad HPX-87N. Mobile phases are weakly acidic (e.g. 1% H_3PO_4 or 0.005M-H_2SO_4:MeCN, 90:10).

Presumably, more polar (or more easily ionizable) solutes are excluded from the stationary phase matrix by unfavorable interactions and hence are not retained as well as less easily ionizable solutes.

Size exclusion interaction probably plays some part in all separations involving cross linked polymer stationary phases, especially if there are considerable size differences between solute molecules. The extent of interaction appears to depend on the polymer pore size and the amount of cross linking.

The majority of ion chromatography beverage analysis is used to separate inorganic and low molecular weight organic anions and cations using suppressed conductivity detection and isocratic elution. For anion analysis, common eluents are dilute aqueous sodium or potassium hydroxide (e.g. of a few millimolar

concentration) or dilute aqueous sodium carbonate/sodium bicarbonate are commonly used, and sometimes a small amount of organic component can be included in the eluent (e.g. 13% methanol (v:v)) (Masson, 2000). Many ion chromatography stationary phases are not compatible with organic solvents. Cations are often determined by ion chromatography using aqueous methanesulfonic acid as eluent.

Analysis of inorganic anions in wines by ion chromatography can also be achieved using 8 mM phthalic acid in pH 4 tris buffer in 98:2 (v:v) water:acetonitrile (Dugo *et al.*, 2005), thus allowing determination of sulfate in the presence of tartrate, the ionization of organic acids being suppressed at this low pH.

There are many examples in the literature of analysis of juice or wine organic acids (as anions) (Edelmann *et al.*, 2003; Soyer *et al.*, 2003; Casella and Gatta, 2002; Vonach *et al.*, 1998; Mongay *et al.*, 1996; Hunter *et al.*, 1991) and/or inorganic anions (Dugo *et al.*, 2005; Masson, 2000; Perez-Cerrada *et al.*, 1989) using ion chromatography. Methods for the analysis of organic acids in wines are reviewed by Mato *et al.* (2005). Most chromatographic methods use ion exchange mode with conductometric or spectrophotometric detection, but Fourier transform infrared (FTIR) detection (Edelmann *et al.*, 2003; Vonach *et al.*, 1998) has also been used.

Benzoate (a preservative – see Section 2.5.8) in soft drinks or fruit juices has been determined by reagent free ion chromatography using 35 mM KOH the mobile phase and an IonPac AS18 mobile phase. This allowed the quantification of benzoate in the presence of high concentrations of other anions, such as chloride, phosphate, citrate and others (De Borba and Rohrer, 2004). Although benzoate is little used as a preservative of alcoholic beverages, because of the possibility of its use and because of differing regulatory requirements in different parts of the world, it is important to have a reliable method for its determination.

Carbohydrates and reduced sugars (polyols, such as arabitol, mannitol, sorbitol and xylitol) can be analyzed in the ion exchange mode (i.e. as anions) using special cross linked polymer resin stationary phases and high pH mobile phases. Carbohydrate –OH functions are more acidic than those of reduced sugars, as a result of the absence of the potential carbonyl group in the latter. Hence carbohydrates can be determined on Dionex CarboPac PA type columns (polystyrene/divinylbenzene or ethylvinylbenzene/divinylbenzene substrates agglomerated with a quaternary ammonium functionalized microbead latex) using moderate eluents of moderate pH (e.g. 16 mM aqueous NaOH). Reduced carbohydrates, on the other hand, can be analyzed on a CarboPac MA type of column (macroporous vinylbenzyl chloride/divinylbenzene substrate functionalized with alkyl quaternary ammonium groups – no latex) using a high pH eluent, such as 0.6M aqueous NaOH (Dionex, 2009). In all cases, since carbohydrates and, especially polyols, do not absorb strongly in the UV region, either a universal detection method, such as refractive index or evaporative light scattering, or a highly selective method, such as pulsed amperometry (Dionex, 2009) is preferred.

Similarly, sugars in beer wort (glucose, lactose, maltose, maltotriose and sucrose) have been determined by ion chromatography using a Metrohm Carb-1 phase, gradient elution with 180 mM and 500 mM aqueous NaOH and a pulsed amperometric detector (Benton, 2004). The detection method was considered superior to the traditional refractive index detection, because of poor baseline resolution and because the high temperatures often used with these stationary phases may lead to some decomposition of maltotriose.

Ion exclusion chromatography, usually carried out on cross linked polymer based ion exchange columns at low pH, has been used to determine the organic acids of grape must (Soyer *et al.*, 2003) or wine (Casella and Gatta, 2002), the former using UV spectrophotometric detection, the latter using electrochemical detection.

A similar method can be used to assay carbohydrates (sometimes as well as organic acids) in beverages, using phases such as Alltech Prevail, Bio-Rad Aminex, Phenomenex Rezex, Supelco Supelcogel or Waters Sugar-Pak. Selectivity between carbohydrates and organic acids can be altered for some phases (e.g. Rezex ROA-Organic Acid: an 8% cross linked sulfonated styrene-divinylbenzene copolymer phase in its hydrogen ion form) by change of pH, thus combining ion exchange with ion exclusion. Generally, weakly acidic (e.g. 0.005M H_2SO_4, 1% aqueous phosphoric acid or 0.5% aqueous trifluroacetic acid) mobile phases are used for organic acids or amino sugars, whereas water alone is used for carbohydrates. Refractive index and evaporative light scattering are the preferred methods of detection for carbohydrates.

Size Exclusion Chromatography (SEC): Gel Permeation and Gel Fitration Chromatography (GPC and GFC)

The stationary phases used to effect separation by size exclusion are usually cross linked polymers (either synthetic or natural), which are swellable by certain solvents. Because of their swellability, they are known as gels and contain pores whose sizes lie within a certain, usually quite narrow range. Larger molecules in a mobile phase are unable to enter the pores in the gel as they pass through the stationary phase. They are thus obliged to travel through the stationary phase via the liquid spaces between the particles (the void or interstitial volume). Smaller molecules are able to enter the pores, where they interact with stationary phase in a variety of ways and are hence retained more strongly. The process of size exclusion separation is illustrated in Figure 4.3.10.

Stationary phases for size exclusion chromatography fall into two categories – hydrophobic (organophilic) gels and hydrophilic gels – giving rise to two versions of SEC: gel permeation chromatography (GPC) and gel filtration chromatography (GFC).

Gel permeation chromatography makes use of hydrophobic stationary phases, typically poly(styrene-divinylbenzene) copolymers, cross-linked up to about 8%. They are similar to stationary phases used elsewhere in HPLC, see ion chromatography, for example. They are best used for the separation of synthetic polymer mixtures, the mobile phases being organic solvents, based on acetonitrile or DMF, for example.

Alkyl-bonded silica gels with wide pores (typically 30 nm) are useful hydrophobic phases which are frequently used to separate biopolymers (e.g. proteins), using mainly acetonitrile-water mobile phases. Separation is probably effected by partition (see RPHPLC), as well as size exclusion.

Gel filtration chromatography uses hydrophilic stationary phases, such as Sephadex and related materials, which are essentially cross linked polysaccharides. Also much used are synthetic polymers with hydrophilic side chains, such as poly(acrylamide) and poly(vinyl alcohol), and silica gel based phases. They are of special importance in the separation of larger biomolecules, such as peptides and proteins. Aqueous based mobile phases are used, which minimize loss of biological activity during separation (see hydrophobic example above for contrast). The mode of separation appears to be complex, but size exclusion plays some part.

As with other modes of HPLC, a range of detection methods is possible, but with one method based on molecular size; a reflection of the application to size exclusion chromatography to the separation of polymers and oligomers. The major detection methods used are comparative refractometry, ELSD, UV absorption and molecular size detection.

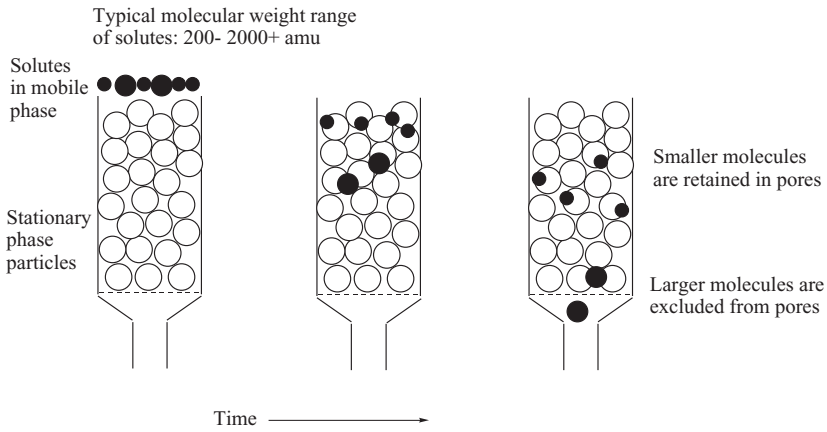


Figure 4.3.10 *Simplified scheme for SEC separation not drawn to scale*

The major application of size exclusion chromatography to alcoholic beverages or materials from which alcoholic drinks are derived, is to protein analysis. Alternative methods of protein analysis include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Section 4.6.1) and RPHPLC-MS (LC/MS) (later in this section). Of particular interest are haze forming proteins, prolamins (Section 5.11.3) and the influence various processing methods (e.g. fining) have on protein/peptide composition.

Thaumatin-like proteins (along with other proteins) are responsible for haze formation in wine and can be costly (in terms of both material costs and loss of quality) to remove by use of bentonite or other fining agents. Grape proteins of Semillon juice have been analyzed on a Phenomenex BioSep SEC S2000 column (a silica gel based phase) using 50 mM aqueous KH_2PO_4 buffer, with UV detection at 210 nm (Marangon *et al.*, 2009). Size exclusion chromatography was carried out on fractions from a hydrophobic interaction chromatography column, one having a protein identified as a VvTL (*Vitis vinifera* thaumatin-like) protein of about 20 000 amu. Chitinases, PR-4 type proteins, vacuolar invertase and lipid transfer protein (LTP) were also found.

The effects of processing (malting, mashing, boiling, fermentation and filtration) on the protein/peptide profile of wort and beer were studied using a Millipore Marex Cellufine GCL-300 Medium phase and 50 mM acetate buffer (pH 5.5) (Osman *et al.*, 2003). The wort and beer samples were centrifuged, concentrated by ultrafiltration and dialyzed prior to gel fitration. The results showed that soluble protein concentrations almost double during malting, especially with regard to proteins of 14,000 amu and lower. The combined GCF and SDS-PAGE results suggested most of the higher molecular weight proteins were formed by the combination of peptides.

Maltodextrins (α-glucans), produced by the hydrolytic degradation of starch during malting and mashing (Section 2.6.2) are important in brewing; many are either slowly fermentable or nonfermentable (depending on the type and extent of branching), but they give texture and viscosity to the beer and may also be flavor carriers. Although the majority of α-glucans in beer are of relatively low molecular weight (typically ∼1000–3600 amu), higher molecular weight species are present in malted barley or can be produced from starch by the action of diastase enzymes. GPC using multiple detectors (e.g. refractive index, low angle light scattering, right angle light scattering differential viscometry and UV) can characterize different maltodextrins according to molecular weight, branching etc. (Clarke, 2004; Viscotek, 2009), particularly with regard to high molecular weight (∼60 000–>800 000) α-glucans. A single Viscotek Viscogel GMPWXL column or two columns in series can be used with 0.1 M or 0.2 M aqueous $NaNO_3$ mobile phase (or similar) to characterize different maltodextrin samples with similar viscosities, in terms of molecular weight, molecular weight distribution, average size and extent of branching.

Chiral HPLC

Although chiral HPLC is of paramount importance in the pharmaceutical industry, it has found relatively few applications in the food and alcoholic beverage industries, although here chiral GC has found greater application, especially in the analysis of aroma compounds (Section 4.3.2). Chiral chromatography involves the separation of enantiomeric analytes. All types of chiral chromatography depend on chiral selectivity, which can be achieved in various ways. These are summarized for HPLC in Figure 4.3.11.

A rare example of method 2 is the determination of amino acid enantiomers in grape must, wines and beers by derivatization of the amino acid analytes using *o*-phthalaldehyde and *N*-isobutyryl-L-cysteine (or its enantiomer) ($(CH_3)_2CHCONHCH(CH_2SH)CO_2H$) and subsequent separation of the diastereoisomeric derivatives on a C_{18} RP phase (Brückner *et al.*, 1995). Fluorescence detection was used, the derivatives absorbing at 230 nm and emitting at 445 nm.

Nowadays, most chiral HPLC is carried out using chiral stationary phases (CSPs), which depend on some chiral moiety in the phase interacting differentially with enantiomers in the mobile phase. For a comprehensive coverage of chiral HPLC, the reader is referred to Aboul-Enein and Ali (2003), although the emphasis here

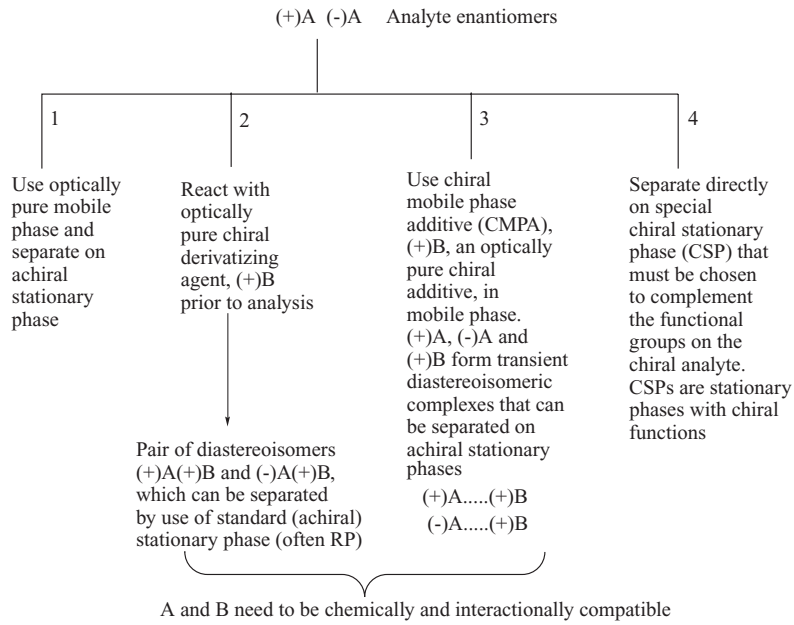


Figure 4.3.11 *Types of chiral chromatography. In recent years, only method 4 has found HPLC application to alcoholic beverages*

is very much on drugs and pharmaceuticals. CSPs are usually composed of silica skeletons bonded or coated with chiral moieties, such as amylose, cellulose, cyclodextrins, macrocyclic glycopeptide antibiotics, aromatic amino acid derivatives (Pirkle phases), proteins, chiral metal complexes, crown ethers and alkaloids.

Most applications of chiral HPLC to alcoholic beverages are based on chiral ligand exchange chromatography (CLEC), where separation involves competition for complexing between the enantiomers (especially amino acids, amino alcohols and hydroxy acids) for the formation complexes with a metal cation (usually Cu^{2+}), which is both in the mobile phase and complexed to a chiral moiety in the stationary phase.

An optically pure chiral ligand such as L-proline or D-penicillamine is bound to silica gel (originally crosslinked polystyrene) and loaded with metal cations, typically Cu^{2+}. The chiral analytes are distributed from an aqueous mobile phase to the immobilized ligand–Cu^{2+} complex, where they form tertiary complexes of different stabilities. Variable interaction between groups on the immobilized ligand and/or the matrix and groups on the analytes give rise to differences in stability and hence to chiral selectivity. A schematic representation of a possible separation mechanism is shown in Figure 4.3.12.

These CSPs are good for the separation of amino acids and various derivatives like amino alcohols and hydroxy acids. Although reversed phase mode is used, ligand exchange CSPs need care in handling; Cu^{2+} needs to be in the mobile phase and the columns require storage in 2 mM $CuSO_4$ solution when not in use. They are also sensitive to low or high pH and to the presence of organic solvents in the mobile phase (15% maximum).

CLEC methods, although useful in the determination of underivatized bifunctional chiral analytes, are in general of low efficiency, giving rather broad peaks.

Lactic acid enantiomers in wines, beers and other foodstuffs have been determined by this method (Buglass and Lee, 2000; 2003), using a D-penicillamine type phase (Phenomenex Chirex 3126 or Daicel Chiralpak MA(+)) and 1 mM aqueous $CuSO_4$. Interferences due to amino acids can be removed by sample treatment with PRS (propanesulfonate ion exchange) SPE (Buglass and Lee, 2000). The detection limit was 3 mg/l.

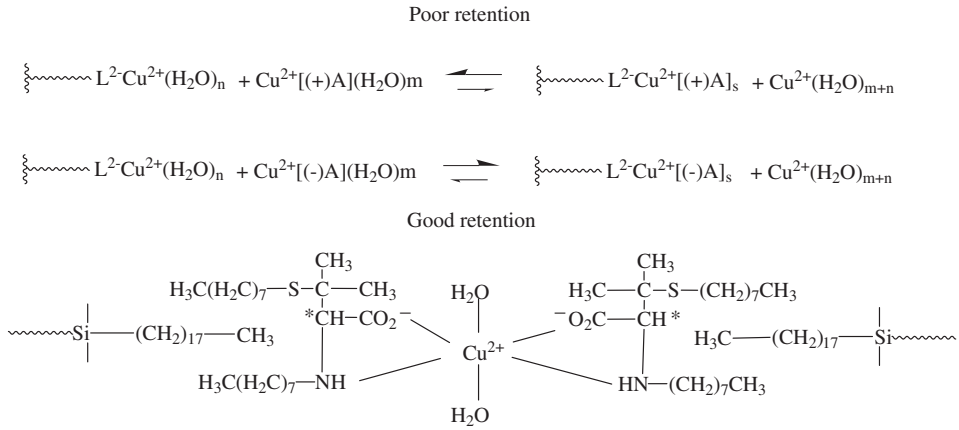


Figure 4.3.12 *Possible mechanism of CLEC and example of D-penicillamine phase. *chiral center*

Changes in concentration of both lactic acid enantiomers during both alcoholic and malolactic fermentation were monitored by this method (Buglass and Lee, 2003).

Zerbinati *et al.* (1994) were able to quantify enantiomers of malic and lactic acids in wines by using an achiral (C_{18}) stationary phase, but with a circular dichroism detector. By comparison of the CD spectra with those of optically pure standards, it was possible to determine the concentrations of major and minor enantiomers of the two acids in wine. The limit of detection for lactic acid was 178 mg/l.

Earlier, Doner and Cavender (1988) used an L-proline phase and 1 mM $CuSO_4$ to separate the enantiomers of malic acid in apple juice. Adulteration of apple juice using inexpensive racemic malic acid could be detected by this method.

More recently, benalaxyl fungicide residues (Section 5.10.2) in grape must were determined using normal phase OD chiral stationary phase, hexane/2-propanol mobile phase and UV detection (Wang *et al.*, 2007). The limit of detection was 0.26 mg/l for both enantiomers.

HPLC Techniques for Difficult Samples and Techniques for the General Improvement of Chromatography

The HPLC analysis of some analytes in some matrices can present certain problems, usually with respect to reasonable retention times or to resolution and accurate quantification, particularly if there are components of widely differing polarities in the sample – which is often the case with alcoholic beverages. Other problems are associated with mobile/stationary phase compatibility with the detector, especially in LC-MS. Yet others are related to high flow rates and back pressure associated with the use of small spherical particulate stationary phases (<3 μm). Other problems are associated with detector sensitivity or with solvent waste. The first of these challenges can be often addressed by the use of some fractionating method or sample preparation method, such as SPE (Section 4.2.4), but the following is a brief account of some techniques applied purely to the chromatographic process itself in order to ease some of these problems.

Column Switching Techniques

Resolution of difficult samples can be improved by using two linked columns, as shown in Figure 4.3.13. The sample is partially resolved on the first column, and at the correct time, the eluent stream containing the

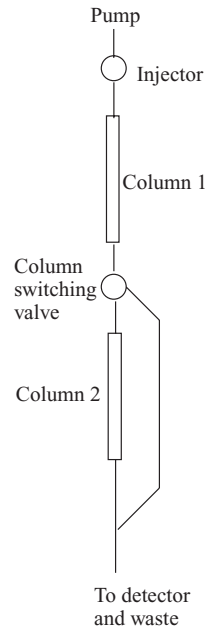


Figure 4.3.13 *Simple 'heart cutting' column switching arrangement*

partially resolved analytes is switched by use of a valve to a second column. Full resolution can then take place on this column without interference from other components. Figure 4.3.13 shows the simplest kind of arrangement – a more complex column switching set up, including a focusing column, is needed if different mobile phases are to be used (Figure 4.3.14).

With the valve in the main switch position (Figure 4.3.14(a)), the sample is pushed by pump A via ports 1 and 2 onto column A, where initial separation takes place. At the same time, pump B is pushing its mobile phase through port 4 and out of port 3 into the ODS retainer column and from here it proceeds back to the valve via port 6, where it emerges from port 5 and goes through column B and detector B.

When the detector indicates that the heart cut part of the mixture coming from column A has almost arrived, pump B is turned off and the valve is switched immediately to position (b) (Figure 4.3.14(b)). Now the components of the heart cut, in a plug of mobile phase A, are diverted to the focusing column via port 6. The mobile phase A, now mixed with some mobile phase B after passing through the focusing column, re-enters the valve at port 3 and leaves via port 2 to the detector.

After a predetermined time (usually 20–30 s), the valve is switched back to the main switch position and pump B is restarted. Now pump B pushes mobile phase B via ports 3 and 4 elutes the components of the heart cut (still unresolved at this point), with their plug of mobile phase A, from the focusing column via valve ports 6 and 5 onto the column B where they are resolved and detected by detector B. In the mean time, pump A pushes mobile phase A via ports 1 and 2 through the detector A, thus washing out the plug of mobile phase B from the previous valve switch position.

An arrangement similar that described above was used to quantify simultaneously malic acid and (*R*)- and (*S*)-lactic acid contents of wines (Buglass and Lee, 2001; 2003). In this case column A contained an RP phase, mobile phase A was aqueous 0.5 mM H_2SO_4 and the heart cut containing the lactic acid enantiomers was resolved on a CLEC stationary phase using 2mM $CuSO_4$ as mobile phase.

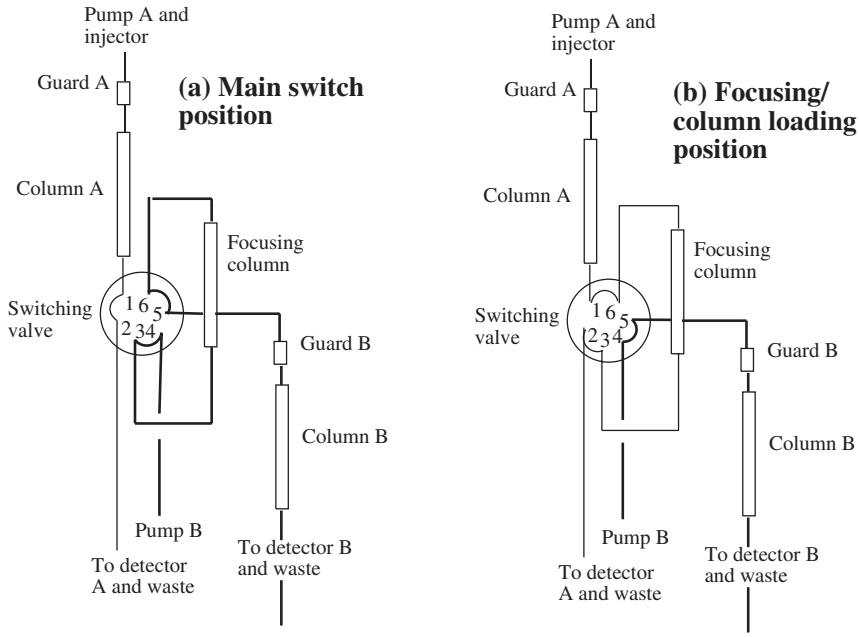


Figure 4.3.14 *Column switching arrangement using different mobile phases and a focusing column. Buglass and Lee (2001). Figure reproduced from the* Journal of Chromatographic Science *by permission of Preston Publications, a division of Preston Industries, Inc*

Narrow Bore Columns

Typical analytical HPLC columns have internal diameters (i.d.) of 4.6–8.0 mm. Smaller bore columns ('midbore,' 3.2 mm i.d.; 'minibore,' 2.1 mm i.d. and 'microbore,' 1.0 mm i.d.) have been shown to both increase detection sensitivity and to reduce solvent consumption (and hence waste).

Special HPLC systems have been devised for the use of microbore columns and such systems have been used to rechromatogram fractions of partially resolved conventionally chromatogrammed complex samples. Also, LC-MS frequently makes use of microbore and even narrower nanobore or capillary columns (e.g. 0.15 mm i.d.) (Marangon *et al*., 2009).

Radial Compression Technology

As the particle diameter of the stationary phase is reduced, particle–particle interactions become more pronounced during the packing process. Thus it becomes increasingly difficult to form a highly efficient packed bed that is stable over a significant period of time. One very useful way round this problem has been patented by Waters as *Radial Compression Technology.* Here the stationary phase is packed into a flexible walled plastic cartridge which is then subjected to a radially compressive force in a compression module prior to HPLC analysis. Radial compression reforms the packed bed and, in particular, removes voids and channels that inevitably form during and after the packing process. Near the rigid wall of a conventional steel column, there is lower resistance to flow as a result of the lower density of the packed bed in these regions. This effect (the 'wall effect') is believed to make a major contribution to band broadening. Radial compression greatly improves flow characteristics near the column wall. The relatively flexible wall of the cartridge molds itself

around the silica particles and significantly reduces voids near the channel wall. Largely because of this, the efficiency of a Radial-Pak radial compression cartridge has been shown to be regularly 25% higher than that of a conventional packed steel column. Furthermore there is good evidence to suggest that the stationary phase life is longer and overall performance better under radial compression: tests at Waters have shown that Radial-Pak cartridges can provide good performance even after 10 500 injections. Billingsley *et al.* (1996) were able to determine the malic and tartaric acid contents of a large number of grape juice samples and the same technique was used to determine the lactic, malic and tartaric acid contents of wine (Buglass and Lee, 2001).

Spherically Ordered Mesoporous Silicas and Monolithic Phases

Many HPLC analyses require stationary phases composed of small spherical particles (say <3 μm) to enable sufficient efficiency and resolution due to more effective mass transfer. However, smaller particles give higher flow resistance, and so to avoid a high column back pressure modest flow rates must be used, thus sometimes resulting in long chromatographic run times. Ordered mesoporous silicas, like those that have been micelle templated (micelle templated silicas; MTS), possess beneficial morphologies, besides having very high surface areas ($\sim$1000 m^2/g) for effective mass transfer. Special morphologies can be acquired by pseudomorphic transformation; amorphous silica particles (like those used in HPLC) are dissolved in alkaline solution and reprecipitated around surfactant micelles into ordered MTS structures. Spherical particles of MTS ($\sim$5 μm) with hexagonal and cubic symmetries, and containing uniform pores with diameters greater than 6 nm have been synthesized and used in fast HPLC separations with low back pressure (Galarneau *et al.*, 2006).

A monolithic phase is a single entity of stationary phase material, without interparticle voids. The more usual particulate stationary phases can be regarded as a large collection of spherical micromonoliths. The first monolithic phases were discs of modified poly(styrene) or poly(methacrylate) copolymers that used ion exchange, hydrophobic interactions or reversed phase in the separation of proteins and other macromolecules (Svec, 2003). In the early 1990s, rigid macroporous polymer columns were developed by reacting the monomers (one of which must be a cross linker), a radical initiator and a porogenic solvent in a sealed tube, which would later act as the chromatography column. After washing out unreacted compounds and porogens from the pores of the columnar monolith, the phase was ready for chromatographic use. Later, tubular and capillary organic polymer monoliths were developed and after those, the first silica based monolithic columns – see Tanaka *et al.* (2002) for a review of monolithic silica columns. Monolithic silica based columns are prepared by hydrolytically initiated polycondensation of tetraalkoxysilane in the presence of poly(ethylene glycol) porogen and are now commercially available (e.g. Merck Chromolith; Phenomenex Onyx) as silica, C_8 and C_{18} phases.

Since monolithic silica based phases are relatively new, there are few applications, but those that exist serve to demonstrate the notable advantages of such phases. A very early application was the analysis of ochratoxin A in red and white wines by LC-MS-MS using a 50 mm Merck Chromolith SpeedRod RP-18e column (Zöllner *et al.*, 2000). The monolithic column and a conventional column gave comparable results (with a limit of detection of 0.5 ng/l), but the former allowed much higher flow rates, reducing analysis time by a factor of three.

Using a second generation 100 mm Chromolith column (C_{18}, end capped), Castellari *et al.* (2002) were able to separate 17 monomeric wine phenolic substances, including hydroxybenzoic acids, hydroxycinnamic acids, hydroxycinnamyltartaric acids, flavanols, flavonols and stilbenes in 36 minutes, including washing and equilibration time. Similarly *cis*- and *trans*-resveratrol and piceid isomers in the must and wine of the *V. vinifera* variety Grenache were separated and quantified by using coupled monolith columns in a single very short run, without any sample preparation (Abert Vian *et al.*, 2005).

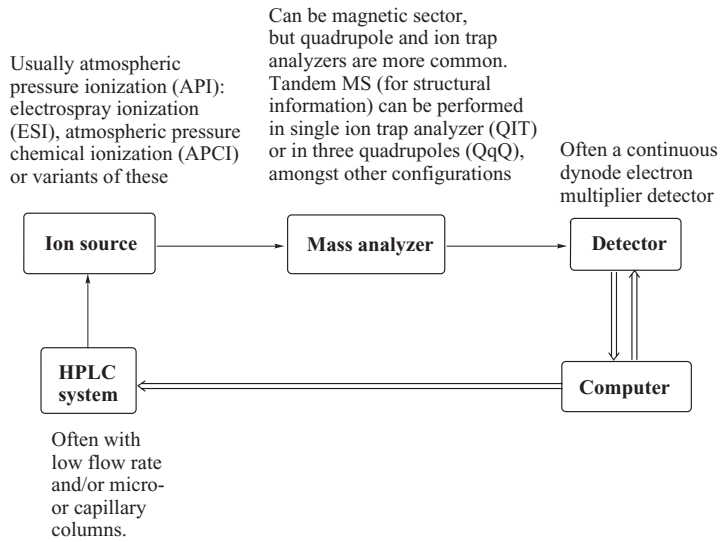


Figure 4.3.15 *Schematic diagram of a basic LC-MS system. Reproduced with permission from the Journal of Chromatographic Science by permission of Preston Publications, A Division of Preston Industries, Inc.*

LC-MS

Nowadays, LC-MS is one of the most powerful of all analytical techniques and is widely used in the analysis of alcoholic beverages, their raw materials and other foodstuffs. In the following paragraphs, the chromatographic aspects of LC-MS will be emphasized, the mass spectrometric aspects being considered largely in Section 4.4.5.

Most modern LC-MS instruments possess interfaces in which the HPLC eluent enters the MS ion source at atmospheric pressure (Figure 4.3.15). Here, ionization occurs, as excess mobile phase is pumped away and the ionized analytes (as cations or anions), now in the gas phase, are passed at very low pressure into the analyzer, where they are sorted or separated (usually according to m/z values) before reaching the detector.

The mode of ionization preferred nowadays is atmospheric pressure ionization (API), of which there are several variants (see Figures 4.4.27 and 4.4.29 in 4.4.5 for details). Electrospray ionization (ESI) (Section 4.4.5) is probably the most widely used API method because, if one includes a variant known as ionspray, it is applicable to the widest range of analyte molecular weights and polarities (Figure 4.3.16). In electrospray ionization, ionized droplets of mobile phase (containing the analyte) are formed as the eluent leaves (at up to 200 μl/min) a heated capillary column (the nebulizer), the end of which is subjected to a high voltage (up to ~5 kV). Regarding the HPLC conditions, typically, minibore (2.1 mm i.d.), microbore 1.0 mm i.d.) or capillary (0.1 mm i.d.) columns are preferred, but larger diameter columns may also be used provided the eluent flow is split before entering the ion source. Eluent flow rates vary from ~1 ml/min down to a few μl/min, depending on the column dimensions and the type of capillary inlet in the ion source.

Alternatively, a high gas flow rate (~ speed of sound), can be used to cause the formation of charged droplets via shear stress as the eluent leaves the capillary tip. Neither heating nor high voltage is required and this ionization technique (sonic spray ionization) may be useful in the study of heat sensitive analytes such as terpenoids, steroids and polyphenols (Volmer, 2000).

Nanospray is a recent development of electrospray, with flow rates of eluent (from a capillary HPLC column or chip based LC system into the ion source) of nl/min (rather than μl/min) via a narrower capillary outlet than in standard ESI. Smaller droplets are produced, leading to more efficient ionization and hence

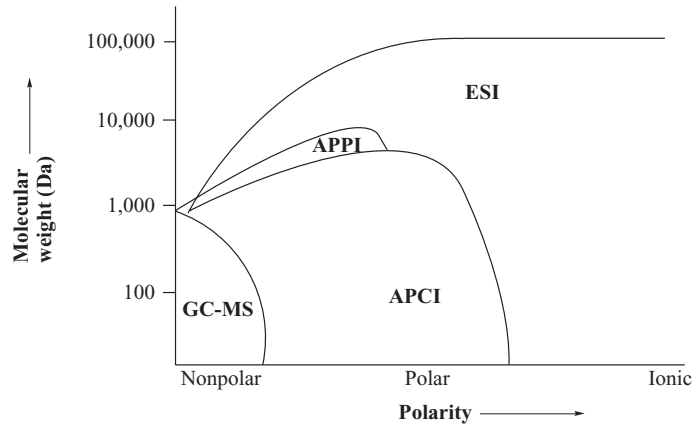


Figure 4.3.16 *LC-MS ionization techniques, analyte polarity and molecular weight*

higher sensitivity. ESI is a 'soft' ionization technique, producing abundant molecular ions, but very little (if any) fragmentation, thus revealing virtually no structural information. However, limited fragmentation can be encouraged by using an increased cone voltage and additionally, collision induced decomposition (CID) and tandem MS (LC-MS-MS) can be used to give the required structural information, if needed (see later and Section 4.4.5).

In APCI (Section 4.4.5), the HPLC eluent is nebulized at high temperature and ionization is caused by the application of a corona discharge in the spray. Solvent molecules are ionized and these ions react with analyte molecules in the gas phase (chemical ionization). A recent variant known as atmospheric pressure photochemical ionization (APPI) uses a photochemically ionized (by UV radiation) 'dopant' substance to initiate a series of ion–molecule reactions to form the ionized analyte. APCI, like conventional chemical ionization, is a 'soft' ionization technique generally producing abundant molecular ions, but usually with more fragmentation than ESI. Once again, tandem MS can be used to gain more structural information via enhanced fragmentation.

Quadrupole and ion trap analyzers are able to scan the m/z range (typically 35–350 units) very rapidly and hence are ideal for LC-MS, despite their other limitations (Section 4.4.5). In practice, the mass spectrometer is programmed to scan continuously (say every 0.5 s) during the course of an HPLC run, over a preselected range of m/z values (e.g. 35–350 units). This means that a typical chromatographic peak of width say 5 or 10 s will be scanned many times during the chromatographic run. In this way, the optimum scanning (close to the peak maximum) can be obtained for each (fully resolved) peak (Figure 4.3.17(a)). For unresolved peaks (coeluting components), continuous rapid scanning can still result in optimum mass spectra for each component (Figure 4.3.17(b)).

The description above is that of a total ion chromatogram (TIC) mode. For the whole of the chromatographic run, the preselected mass range (say, 40–400 m/z) is scanned every (say) 0.5 s. TIC is the most common scanning mode, especially when it is needed to identify unknown components of a mixture. If, however, there is some prior knowledge of the components or if we are searching for specific substances in a mixture, then the selected ion monitoring (SIM) (Section 4.3.2) mode can be used. Here, ions of specific m/z values (often 2–5 characteristic and abundant ions), and no other ions, are monitored during the HPLC separation. Hence, only components whose mass spectra contain ions of the preselected m/z values will be recorded as chromatographic peaks; all other data is not saved. Choice of SIM mode has the effect of greatly simplifying the chromatogram and greatly enhances the sensitivity of the LC-MS experiment (detection limits are often improved from ng/l levels to pg/l levels).

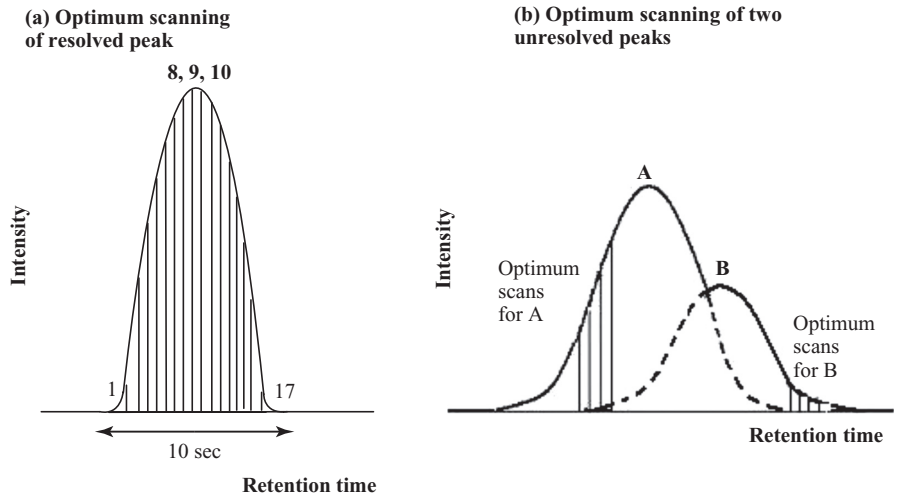


Figure 4.3.17 *Mass spectral scanning of chromatographic peaks*

LC-MS has been used extensively in the quantification and identification of phenolic compounds in both alcoholic drinks and the raw materials from which they are made. Much of the interest focused upon phenolic compounds arises because of their possible *in vivo* antioxidant properties and claimed health benefits (Chapter 5.8). They are present in all raw materials used in the making of fermented beverages, and many survive (but some are modified, transformed or removed, and some are added), during the alcoholic beverage production processes. Thus many alcoholic drinks possess significant concentrations of phenolic compounds. Probably the greater bulk of reports in the literature deal with raw materials, particularly fruit skins and juice. There are many such reports, so only representative examples are discussed here.

LC-MS has been used to study the phenolic content of apple skins and pulp (Kahle *et al.*, 2005), apple extracts (Schieber *et al.*, 2002), pear skins (Lin and Harnly (2008) and of colored fruit skins and must (Dugo *et al.*, 2003; González-Paramas *et al.*, 2006; Pomar *et al.*, 2005; Seeram *et al.*, 2006a; Tian *et al.*, 2005; Vidal *et al.*, 2004). Many of the above references involve a report on some aspect of *in vitro* antioxidant power. Additionally, LC-MS has been used to probe more specific features of fruit juices, such as oxidation of phenols in apple juice (Bernillon *et al.*, 2004), thermal degradation of anthocyanins (Sadilova *et al.*, 2006) and *in vitro* anticancer activity of fruit extracts (Seeram *et al.*, 2006b). Furthermore, more generally, LC-MS methods have been developed for screening and identification of phenols in all plant materials (Lin and Harnly, 2007; Wolfender *et al.*, 1998).

For the analysis of flavonols, flavan-3-ols, hydroxycinnamic acids and other phenols in extracts of apples and pears, either reversed phase C_{18} standard columns with flow rates of 1 ml/min (Schieber *et al.*, 2002; Lin and Harnly, 2007) or a minibore column (100 mm × 2.0 mm i.d.) with a flow rate of 0.2 ml/min (Kahle *et al.*, 2005) were used with gradient elusion, which involved an acidic (formic acid) aqueous solvent as one solvent and either acetonitrile or acidified acetonitrile as the other. The method of ionization was electrospray in both positive and negative mode (Lin and Harnly, 2007), negative mode ESI (Bernillon *et al.*, 2004; Kahle *et al.*, 2005) and negative mode APCI (Schieber *et al.*, 2002).

Analysis of anthocyanins and other polyphenols in colored samples, such as blackberry juice or black grape skins usually involves solvent extraction (González-Paramás *et al.*, 2006; Seeram *et al.*, 2006a; 2006b), freeze drying or other drying and solvent extraction (Lin and Harnly, 2007; Tian *et al.*, 2005; Wolfender *et al.*, 1998), whereas paler fruits, such as oranges, may be simply crushed and filtered (Dugo *et al.*, 2003).

Depending on which groups of polyphenols are under investigation, SPE (González-Paramás *et al.*, 2006) or column chromatographic procedures, such as silica gel (Tian *et al.*, 2005) or XAD-16 and Sephadex (Sadilova *et al.*, 2006) are sometimes used for sample preparation.

Most of the above LC-MS analyses involved normal size analytical reversed phase (C_{18}) columns, although Dugo *et al.* (2003) used a microbore (1 mm i.d.) column. ESI was the most popular LC-MS interface, with positive ionization for anthocyanins (González-Paramás *et al.*, 2006; Seeram *et al.*, 2006a, 2006b; Tian *et al.*, 2004; Sadilova *et al.*, 2006; Dugo *et al.*, 2003, Lin and Harnly, 2007) and negative mode for other phenols (Seeram *et al.*, 2006a, 2006b). FAB and thermospray interfaces, as well as ESI, were used by Wolfender *et al.* (1998).

LC-MS has also been much used in the study of the phenolic components of alcoholic beverages themselves. Reports on the LC-MS analysis of wine and beer phenolic compounds (Callemien and Collin, 2008; Dugo *et al.*, 2004; Morata *et al.*, 2007; Burns *et al.,* 2003; Romero and Bakker, 2000; Salas *et al.*, 2004; Urpí-Sardà *et al.*, 2005; Vivas de Gaulejac *et al.*, 2001) include studies of formation of vitisins from anthocyanins and carbonyl compounds (Morata *et al.*, 2007; Romero and Bakker, 2000), discovery of flavanol-anthocyanin adducts (Salas *et al.*, 2004), identification of resveratrol in LDL and resveratrol metabolites in blood (Urpí-Sardà *et al.*, 2005), identification of monomeric and dimeric phenols in oxidized red wine (Vivas de Gaulejac *et al.*, 2001) and identification of procyanidin dimer and trimer isomers in lager beer (Callemien and Collin, 2008). Examination of the whole anthocyanin profile by HPLC has been shown to be a better indicator of wine authenticity than measurement of certain anthocyanin derivative ratios (Burns *et al.,* 2003).

HPLC conditions were predominantly reversed phase, using either normal size analytical C_{18} columns (Burns *et al.*, 2003; Morata *et al.*, 2007; Vivas de Gaulejac *et al.*, 2001) or minibore columns (Salas *et al.*, 2004; Urpí-Sardà *et al.*, 2005). Gradient elution was again the preferred elution mode and ESI the preferred interface, in the positive ionization mode for anthocyanin analysis (Burns *et al.*, 2003; Morata *et al.*, 2007; Salas *et al.*, 2004) and negative mode for polyphenols, including procyanidins (Callemien and Collin, 2008; Urpí-Sardà *et al.*, 2005; Vivas de Gaulejac *et al.*, 2001).

LC-MS has been applied to the analysis of many other natural components (such as carotenoids, chlorophyll derivatives and terpenoids) of alcoholic drinks and fruits from which they are derived, as well as those obtained from oak maturation. Pentacyclic triterpenoids (Section 5.11.2) and their glycosides are extracted from oakwood during the maturation of wines and spirits. At low concentrations, they often provide a pleasant astringency to the organoleptic properties of the beverage and many of them have been shown to have health benefits (Section 5.11.2); indeed, many are known to be constituents of folk medicines. LC-MS with ESI in the negative ion mode was used to identify and quantify both free and glycosidically bound triterpenoids in wines (red and white) and Armagnac (Arramon *et al.*, 2003). The triterpenoids and their glycosides were isolated from dealcoholized Armagnac samples by extraction with diethyl ether and ethyl acetate, respectively. The detection limits were 1.5–6.0 mg/l and the limits of quantification were 2.5–8.0 mg/l for 2α, 3β, 19α-trihydroxyolean-12-ene-24,28-dioic acid and 2α, 3β, 19α, 23-tetrahydroxyolean-12-ene-24,28-dioic acid and their 28-β-D-glucopyranoside derivatives. The HPLC conditions were reversed phase with gradient elution, the two eluents (95:5 (v:v) water:acetic acid and 95:5 (v:v) methanol:acetic acid, being chosen to facilitate both separation and good electropspray negative ionization. The brandies had much higher levels of triterpenoids than wines, reflecting the superior ability of ~60% ethanol (v;v) over ~12% ethanol (v;v) to extract these somewhat hydrophobic compounds from wood.

Carotenoids, chlorophyll and chlorophyll derived components in grapes and Port wines (Mendes-Pinto *et al.*, 2005), as well as pear skins (Müller *et al.*, 2007) have been investigated using LC-MS. The positive electrospray mode was used to ionize carotenoids, chlorophyllins and pheophytins in grape skins and wine from the Douro region of Portugal, using analytical C_{18} and C_{30} reversed phase columns and gradient elution in the LC part of the instrumentation (Mendes-Pinto *et al.*, 2005). Carotenoids were extracted from homogenized fruit and wine using diethyl ether:hexane (1:1); chlorophyllins a and b and pheophytins a and b were prepared from standard chlorophylls a and b by base and acid hydrolysis, respectively, and

were used with purchased standards to aid identification. Colorless tetrapyrrolic chlorophyll catabolites were extracted from homogenized pears (with skins) after separation of the extracts using preparative HPLC (Müller *et al.*, 2007).

Recently, detailed analysis of proteins in grapes and wine has been achieved by LC-MS/MS, using a variety of analyzers (e.g. ion trap or Q-TOF) (Marangon *et al.*, 2009; Wigand *et al.*, 2009). Proteins from a number of varietal German red and white wines were isolated by lyophilizing (freeze drying) the dialyzed wine (from cellulose membranes with 3.5 kDa molecular weight cutoff (Wigand *et al.*, 2009). Riesling wine (white) and Portugieser wine (red) (both 200 ml) gave 120 mg and 220 mg (respectively) of lyophilized powder. The lyophilized samples were treated with PVP to remove polyphenols and then subjected to SDS-PAGE separation. The relevant protein bands were excised and after various treatments digested with trypsin. The trypsin digests were then analyzed by LC-MS. The HPLC conditions were reversed phase with gradient elution (0.1% formic acid in water; 0.1% formic acid in acetonitrile) using a capillary C_{18} (75 μm × 150 mm) column. The Q-TOF API system was operated in V-mode with 10 000 resolving power and with positive mode ESI using nanospray. Tandem MS for observation of fragmentation sequences was performed using argon atoms for decomposition of molecular ions and peptides/proteins were identified by use of a *V. vinifera* protein and other sequence databases. Twelve grape proteins (pathogenic related, lipid transfer, thaumatin-like, endochitinase and vacuolar invertase proteins) and six yeast proteins, but no fining agent proteins, were found in Portugieser wine.

Using two procedures, proteins from Australian Semillon must and wine were identified by nano LC-MS/MS (Marangon *et al.*, 2009). This time, grape proteins were precipitated by addition of ammonium sulfate, then the protein pellet was redissolved in a Tris-HCl buffer containing 15% glycerol and 1.5% SDS, and subjected to SDS-PAGE separation, excised and, as above, the tryptic digests were subjected to capillary RPHPLC-MS/MS, using nanoelectrospray ionization and a TOF analyzer. Additionally, the protein pellet was subjected to hydrophobic interaction chromatography and five fractions from this were further fractionated by preparative HPLC and all but one of these fractions were subjected to SDS-PAGE in nonreducing conditions and then treated as above, but this time reversed phase capillary gradient elution LC-MS/MS analysis was carried out using a 1.8 kV electrospray voltage and an ion trap analyzer.

LC-MS/MS lends itself well to the analysis of pesticide residues (Section 5.10.2) and mycotoxins (Section 5.11.4) in alcoholic beverages or their raw materials, although in many cases, GC-MS is the preferred method (Section 4.3.2). Generally an extraction/focusing step is required, so that the more concentrated residues can be analyzed in a simplified matrix. Liquid–liquid extraction (Section 4.2.2) and/or sorbent extraction (e.g. SPE, SPME or SBSE) (Section 4.2.4) techniques are the most widely used. Good sample preparation, good chromatography and optimized MS(-MS) operating conditions will minimize matrix effects and maximize sensitivity and selectivity (Bester *et al.*, 2001). The application of LC-tandem mass spectrometry to the analysis of pesticide residues in environmental and food samples has been extensively reviewed (Picó *et al.*, 2004; Budde, 2004). Reversed phase HPLC using minibore columns, with gradient elution, quadrupole (including QqQ) and ion trap (QIT) LC-MS instruments are the most popular, but the more recent hybrid quadrupole-time of flight (Q-TOF) instruments are gaining ground.

LC-MS methods are often developed to analyze a single pesticide residue or residues of specific chemical types in a variety of crops (e.g. cereal, fruit and vegetable matrices), such as the determination of imidazoles and phenylureas in fruit (Bester *et al.*, 2001), fuazipof and imidazolines in cereals, fruit and vegetables (Bolygo and Boseley, 2000), but methods have been developed for the analysis of a wide range of residues, such as amides, benzylimidazoles, carbamates, organophosphates, phenoxy acids, strobilurins and triazoles, often with due consideration of matrix effects (Blasco *et al.*, 2003; Zrotlikova *et al.*, 2003).

Residues of six fungicides (bitertanol, carboxin, flutriafol, pyrimethanil, tebuconazole and triadimefon) in Spanish wine grapes were determined using LC-MS with both APCI and electrospray ionization in positive or negative ion mode, where APCI in positive mode showed the greatest sensitivity (Juan-García *et al.*, 2004). Two extraction/focusing techniques were also compared: SPE and SBSE; the in the latter case, the analytes

being desorbed from the stir bar by sonication in methanol for 15 min. The HPLC conditions were C_{18} reversed phase (150 mm × 4 mm) with methanol-water gradient elution at 0.8 ml/min. The operating conditions for APCI (positive mode) were capillary voltage 4 kV, corona current 10 μA, vaporizer temperature 325 °C and drying gas temperature 250 °C. The limits of quantification were 0.003–0.01 mg/kg (SPE) and 0.01 mg/kg (SBSE).

Cereal crops used for the production of foodstuffs, including alcoholic beverages, are more or less susceptible to a number of fungal infections that give rise to residual mycotoxins in the foodstuff or beverage (Section 5.11.4). These substances usually pose a potential health hazard even at low concentrations and hence require reliable, sensitive and accurate methods for their analysis. LC-MS can provide such methods: 12 mycotoxins (aflatoxins G1, G2, B1 and B2, deoxynivalenol, diacetoxyscripenol, fumonisin B1 and B2, nivalenol, ochratoxin A, T2-toxin and zearalenon) were determined simultaneously in various food products after acetonitrile:water (80:20 v:v) extraction, but with no sample clean up (Huls *et al.*, 2004). The HPLC conditions were reversed phase on a midbore (100 mm × 2.1 mm i.d.) column using gradient elution (0.1% aqueous formic acid; 0.1% formic acid in acetonitrile) and a flow rate of 0.3 ml/min. A heated electrospray was used for ionization and the MS (QqQ) scan mode was highly sensitive reaction monitoring, giving greater sensitivity than standard ESI and single quadrupole instruments. For example, fumonisin B1 and B2 could be quantified at levels well below 0.5 μg/l (0.5 ppb).

A similar reversed phase (C_{18}) minibore LC-MS method, using an MSD with ESI and quadrupole analyzer was able to separate and quantify deoxynivalenol (DON) (vomitoxin) and T-2 toxin in cereal grains (Moncur, 2004). Gradient elution (water; methanol) was used, with a flow rate of 0.2 ml/min at 40 °C column temperature.

Although wine aroma components are volatile and are generally studied by GC-MS (Section 4.3.2), many exist in grape must and wine as glycosides and as such are nonvolatile and hence do not contribute to aroma until hydrolyzed. Terpene glycosides were tentatively determined in must by LC-MS (Prosen *et al.*, 2007). HPLC conditions were reversed phase using gradient elution (acetonitrile:0.5% aqueous acetic acid). The ESI ion source voltage was 5.5 kV and the temperature was 400 °C.

The Maillard reaction (Section 2.6.2) is important in the formation of special aromas and flavors in dark beers and also in certain wines, such as Madeira (Section 2.10.6), that have undergone heat treatments. In reality there are many reactions that constitute the Maillard reaction, involving a large number of intermediates. Maillard intermediates, such as dicarbonyl compounds (e.g. glyoxal, methylglyoxal, diacetyl and pentane-2, 4-dione) and keto acids such as α-keto-γ-(methylthio)butyric acid and β-phenylpyruvic acid were determined by LC-MS (and also as their quinoxaline/quinoxalinol derivatives by HPLC-UV or HPLC-fluorescence) (Silva Ferreira *et al.*, 2007). ESI mass spectra of pure standards were analyzed by LC-MS in both positive and negative ion mode by flow injection.

4.3.4 Counter Current Chromatographic Methods

Counter current chromatography (CCC) can be regarded as a kind of automated liquid–liquid separation procedure or as a kind of partition chromatography in which the liquid stationary phase is not supported in any way. In either view, the analytes, originally in the mobile phase, are partitioned between that phase and an immiscible stationary phase. There are many versions of CCC, summarized in Figure 4.3.18, but probably that which is most popular today is high speed counter current chromatography (HSCCC). This technique uses a rapidly rotating (centrifugal) helical coil to promote mixing and settling cycles, along with their partition chromatographic steps, at a great rate (up to 70 000 cycles per hour). In this way, highly efficient separations can be achieved, from analytical (milligram) levels to full preparative (gram) levels. Many versions of this technique make use of two rotation axes, thus inducing a planetary motion of the coil. In many cases the two

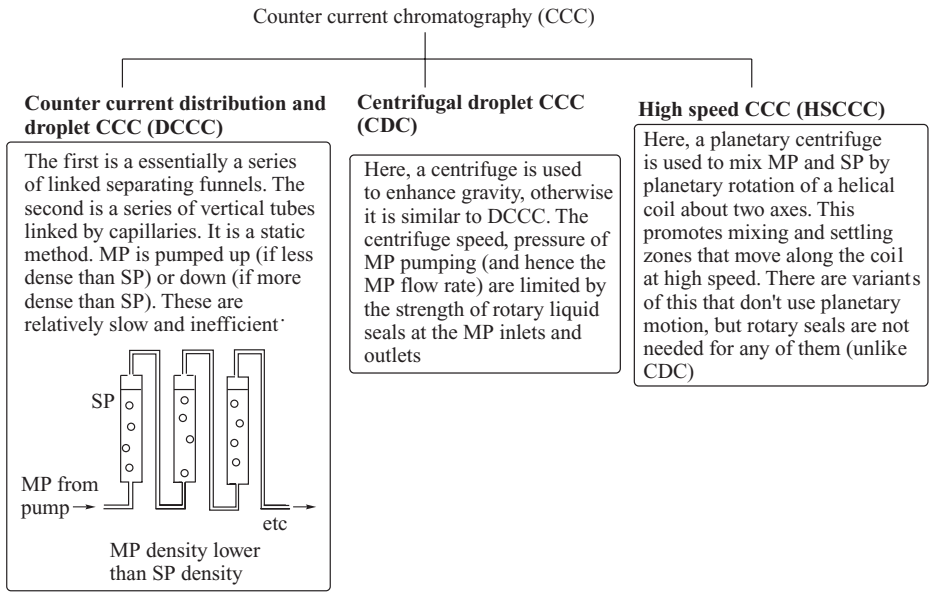


Figure 4.3.18 *Versions of counter current chromatography*

immiscible phases (known as the mobile and stationary phases or head and tail phases, according to how the coil is wound around the holder and the direction of the rotating axis) have an auxiliary solvent that is miscible in both phases to aid partitioning.

Counter current chromatography can be used to both fractionate complex mixtures and to isolate individual components, although the latter is usually performed along with other techniques, such as column chromatography or HPLC. In this way, CCC (usually HSCCC) has helped in the identification of many new constituents of alcoholic beverages (especially wines), mainly by NMR and mass spectrometry.

Application of CCC to alcoholic beverages appears to be in two main areas; fractionation and isolation of flavor precursors (often as glycosides) and fractionation, characterization and isolation of phenolic compounds, especially anthocyanins and their derivatives. Baderschneider and Winterhalter (1997), Roscher and Winterhalter (1993) and Winterhalter *et al.* (1997) used CCC, along with other techniques to fractionate and identify a number of glycosidic components of Riesling grapes or leaves. The aglycones of many of these compounds are flavor compounds or possible flavor compound precursors. In some cases, (e.g. Roscher and Winterhalter, 1993), vine leaves were used as a source of natural products, when it was known that compounds common to vine leaves and grape juice are found at much higher levels in the leaves. In the above study, multilayer coil counter current chromatography (MLCCC) was used to separate leave glucosidic compounds into eight fractions, each of which was tested on its ability to generate 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), an important flavor component of mature Rheinreisling wines, giving rise to 'petroleum' notes. 3,4-dihydroxy-7,8-dihydro-β-ionone-β-D-glucopyranose was indicated as a major TDN precursor. MLCCC is a variant of HSCCC. More recently, Baderschneider and Winterhalter (1997) used similar methods to isolate a new aroma precursor, 2-ethyl-3-methylmaleimide-*N*-β-D-glucopyranose, from Riesling wine. MLCCC was used to isolate the fraction containing this compound, which was subsequently acetylated, purified by HPLC and characterized by spectroscopic methods. Similarly, the glucose ester of (*E*)-2,6-dimethyl-6-hydroxyocta-2,7-dienoic acid was isolated from Riesling wine by CCC and subsequent acetylation and purification by

flash chromatography and HPLC. It is possible that the compound is a precursor of wine lactone, a powerful flavor compound found in many wines (Winterhalter *et al.*, 1997).

However, the majority of applications of CCC to alcoholic beverages is in the analysis of phenolic compounds in red and white wines. Firstly, some less well studied phenolic compounds of Riesling wine (benzoic acid derivatives, cinnamic acid derivatives, flavonoids and lignans) were isolated in various fractions using MLCCC (Baderschneider and Winterhalter, 2001). Many compounds were characterized by NMR, MS and UV spectroscopy, including 12 benzoic acid and cinnamic acid derivatives, two other phenyl propanoids, seven stilbenes, dihydroflavonols, four dihydroflavonol glycoconjugates, six lignans and three neolignans, with many compounds being reported for the first time in Riesling wine. Using similar methods, seven novel stilbenes, including dimeric species, had previously been isolated from Riesling wine (Baderschneider and Winterhalter, 2000).

Secondly, HSCCC, with the aid of semi-preparative HPLC, has been used to isolate novel anthocyanin derivatives (pigments) in red wines, such as pinotin A in the wines of Pinotage, an important South African variety (Schwarz *et al.*, 2003). The new compound, a malvidin-3-*O*-β-D-glucopyranoside, 4-vinylcatechol adduct, was fully characterized by LC-ESI-MS^n and one and two dimensional NMR (Section 4.4.1).

Still in the realm of grape and wine pigments, centrifugal precipitation chromatography (CPC) was adapted from protein fractionation by ammonium sulfate precipitation to fractionate polymeric pigments from red wine and black tea (Degenhardt *et al.*, 2001). In CPC, the separation column is made up of two high density polythene disks equipped with mutually mirror imaged spiral grooves. A dialysis membrane is sandwiched between the disks, which form two identical channels separated by the membrane. The disk and membrane assembly is mounted on a seal-less continuous flow through centrifuge set to 2000 rpm. The column was entirely filled with hexane or MTBE. The sample was introduced through a 1 ml sample loop. Rotation was applied and then the upper channel was eluted with a linear hexane (or MTBE)/ethanol gradient at 0.5 ml/min using an HPLC pump. The lower channel was eluted with ethanol at 0.06 ml/min with a syringe pump. The sample entered the system from the outer terminal of the lower spiral channel, whereas the MTBE/ethanol gradient was eluted through the upper channel in the opposite direction. Elution was monitored by UV absorption. Solvent gradients are generated in the separation channel by the action of circular motion, where polymeric pigments are precipitated in hexane or MTBE rich solvent and experience increasing ethanol concentration. Thus, repetitive precipitation and dissolution of the analytes occurs along the channel. HPLC analysis of early fractions clearly showed the presence of lower molecular weight phenolic compounds, as well as higher molecular weight species, whereas the lower molecular weight species were missing from the late fractions. The main limitation of CPC was its low sample capacity; scaling up from analytical scale to semi-preparative would enable determination of the degree of polymerization and identification of the monomeric units could be determined by NMR, MS and other analyses.

A good example of a scaled up MLCCC procedure is the fractionation of anthocyanin classes from grape pomace and skins (Vidal *et al.*, 2004). Here, a *tert*-butyl methyl ether:*n*-butanol:acetonitrile:water acidified with trifluoroacetic acid (2:2:*x*:5) solvent system was chosen, with step gradient elution. The amount of material loaded on the system varied between 100 mg and 2000 mg, the coil volumes varying between 100 ml and 700 ml accordingly. Monomeric anthocyanins were separated into their glucosides and corresponding acetylated, coumaroylated and caffeoylated derivatives and identified by UV spectrometry, mass spectrometry and RPHPLC retention times. Isolation of comparatively large quantities of pure anthocyanins is useful in that the compounds can be used as references for analytical methods, as subjects for model studies (e.g. for oxidation or polymerization reactions) and for sensory analysis.

Another good example of the application of HSCCC to wine analysis is the rapid isolation of malvidin 3-glucoside (a major pigment in grape skins and new red wine), following sample clean up on an Amberlite XAD-7 column, in quantities and purities that are sufficiently high for use as an analytical standard (Degenhardt *et al.*, 2000). At the time of writing (2009), a 1 mg/ml solution of malvidin 3-glucoside in ethanol costs around $200 per ml.

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4.4

Spectroscopic Methods

Exchange of atomic and molecular energy (i.e. absorption and emission) is quantized – it can only occur in discrete packets or quanta. All types of spectroscopy (except some forms of mass spectrometry) involve the absorption of a quantum of electromagnetic radiation (Figure 4.4.1) by the molecule, thus promoting an energy transition in the molecule from the ground state or a lower energy state to an excited state. The energy of a quantum of radiation, and hence the energy difference between lower and upper atomic or molecular energy levels is given by Equation 4.4.1.

$$E = h\nu = \frac{hc}{\lambda} \tag{4.4.1}$$

where ν is the frequency, λ is the wavelength of radiation, c is the velocity of light and h is Planck's constant.

The type of transition depends on the energy (and hence frequency and wavelength) of the quantum absorbed; relatively high energy ultraviolet and visible radiation (Section 4.4.3) promotes electronic transitions, whereas less energetic infrared radiation (Section 4.4.2) promotes vibrational and rotational transitions (Figure 4.4.1). Even less energetic microwaves and radio waves promote electron spin and nuclear spin transitions (respectively), in the presence of an applied strong magnetic field (Section 4.4.1).

Many spectroscopic techniques (e.g. atomic absorption, infrared and UV-visible spectroscopy) involve measurements of the absorption of electromagnetic radiation by atoms or molecules. This is known as absorption spectroscopy. Emission spectroscopy (e.g. atomic emission and fluorescence spectroscopy) involves the measurement of emitted radiation. For a great many substances, under a reasonable range of conditions (e.g. in solution over a range of concentrations), Beer's law is obeyed, which gives a linear relationship between the extent of absorption or emission and the concentration of the absorbing or emitting species.

Relaxation of excited molecules back to a lower energy state can occur in a number of ways, which include radiationless transitions as well as emission transitions, as shown in Figure 4.4.2. Here, the radiationless relaxation transitions are shown occuring between vibrational levels after absorption of visible radiation by the molecule. Although these transitions are rapid, they are slow compared with emission transitions, which then occur at lower energy (longer wavelength) than the absorption. This phenomenon is known as fluorescence; it can be seen from Figure 4.4.2 that the fluorescence emission spectrum will be more or less a mirror image of the absorption spectrum, but occurring at longer wavelength. Theoretically, all organic molecules are capable of fluorescence, but in practice generally those molecules with extended conjugated

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
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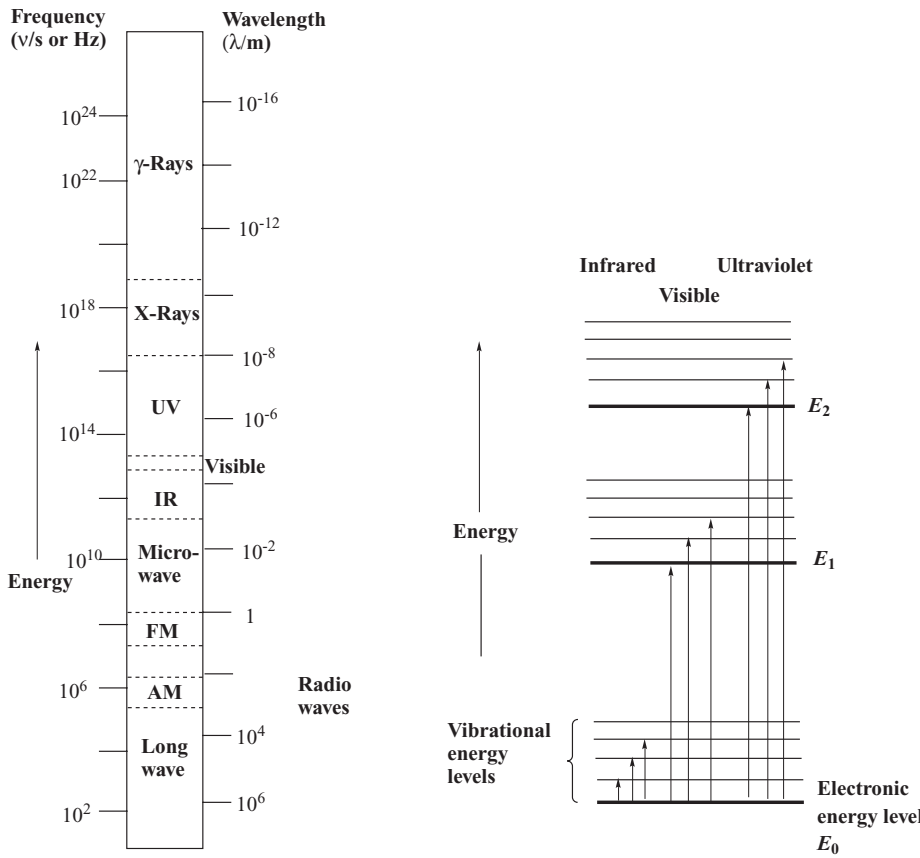


Figure 4.4.1 *The electromagnetic spectrum and energy levels involved in infrared and UV-visible spectroscopy, showing some transitions following absorption of radiation. Rotational levels (not shown) occur between each vibrational level*

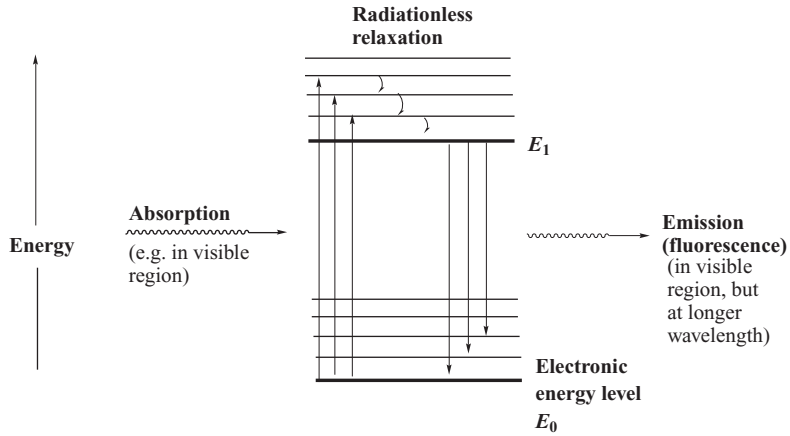


Figure 4.4.2 *Energy changes that occur during fluorescence. See also Figure 4.4.1*

or aromatic groups give the most readily observed fluorescence spectra. The intensities (absorbance) of fluorescence spectra are usually high compared with the corresponding absorption spectra, thus giving an advantage in sensitivity, which can be useful in the analyses of trace components (Section 4.4.3).

Spectroscopic methods are of prime importance in the analysis of alcoholic beverages, as in other areas of science; they enable the identification, quantification and often the determination of the structures of components. These, in turn, enable a better understanding of some of the molecular processes that occur during the production and consumption of alcoholic beverages, such as fermentation, maturation (including the influence of oak), fining, filtration, foam production and interactions between molecules that contribute to organoleptic qualities.

A basic knowledge of spectroscopy at the undergraduate level is assumed for this section, so there is no detailed account of any spectroscopic method given here. Instead there are introductory paragraphs that are designed to give an outline account and set the scene for each technique as applied to the analysis of alcoholic beverages. Within these paragraphs certain features that are considered especially important are dealt with in a bit more detail.

4.4.1 Nuclear Magnetic Resonance

Like electrons, certain atomic nuclei possess spin, and this circulation of charge generates a magnetic moment along the axis of spin. These nuclei are called 'magnetic nuclei' and are characterized by an overall 'spin' (more correctly, spin quantum number, symbol I) that is nonzero in value. By far the most important nuclei in alcoholic beverages are ^{1}H and ^{13}C (both with $I = {}^{1}/_{2}$), although ^{2}H (D) ($I = 1$) is important in certain applications (see below). For detailed discussions on NMR spectroscopy, the reader is referred to Lambert *et al.* (1998), Silverstein *et al.* (2005) and Nelson (2003).

The NMR phenomenon arises from transitions between energy levels, just as in other types of spectroscopy. In NMR, transition occurs when nuclei in a strong magnetic field B_0 are subjected to a radiofrequency field B_1 (frequency ν), normally applied in the x direction, with B_o in the z direction.

Because the energy levels are so close together (even in the strongest magnetic fields available, separation is in millijoules, mJ), thermal motions tend to equalize the energy level populations. For example, for ^{1}H in a field of 7 Tesla at room temperature, the excess fraction in the lower energy level is only 5×10^{-5}. The near equality of population of the two levels is ultimately responsible for the insensitivity of NMR, compared with other spectroscopic techniques. Compounding this are relatively low sensitivities and natural abundances of key nuclei such as ^{13}C (1.1%) – ^{12}C is nonmagnetic – coupled with the low concentrations of many important components of alcoholic beverages. However, advances in electronic engineering and computer technology have largely minimized these problems and NMR spectroscopy takes its rightful place in the armory of analytical methods for the study of alcoholic drinks and other foodstuffs.

Nowadays, it is much more common to have the RF field (B_1) applied as a short pulse, rather than continuously. This is the basis of Fourier transform (FT) NMR, as opposed to continuous wave (CW) NMR spectroscopy. Rather than considering individual nuclei and their magnetic moments, it is more convenient to consider the bulk substance under investigation, where it is assumed that all the nuclei are of one type, with $I = {}^{1}/_{2}$. In the presence of a strong magnetic field, the nuclei distribute themselves between the two energy levels, so that there is a slight excess of nuclei in the lower level, with spins aligned with the applied field. This results in a bulk magnetization M, where M is a vector with a fixed z component and no x or y components. If the population levels are disturbed (by an RF pulse B_1 in the x direction), then M becomes a vector with a fixed z component, but with x and y components that vary sinusoidally, 90° out of phase – that is, executing a circular motion or precession (Figure 4.4.3). In actuality, perturbation causes a more complex double precession (known as nutation) of magnetization vectors and the whole subject benefits by referring to

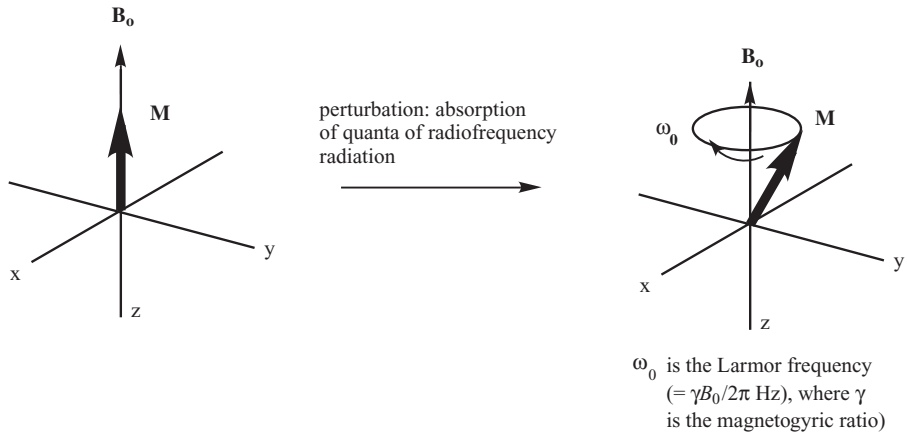


Figure 4.4.3 *Precession of bulk magnetization vector (M) about the z axis upon application of an RF pulse along the x axis*

a rotating frame of reference (x', y' axes), rather than the 'fixed' laboratory frame of reference. If the Larmor frequency and RF pulse frequency are identical, then after the pulse, the magnetization will remain fixed in the $x'y'$ plane of the rotating frame: it will rotate at a frequency of $\gamma B_0/2\pi$ (Hz) with respect to the fixed laboratory xy plane.

The coil that supplies the RF field is wrapped around the (laboratory) x axis (perpendicular the applied field B_0) containing the sample, along with the receiver coil. Hence, the precessing magnetization about the (laboratory z axis) will induce an oscillating current in the receiver coil that can be detected; this is the NMR signal. However, this signal has a limited lifetime (though long compared with pulse duration), since it dies as the nuclei reassume their equilibrium distribution between the two energy levels. This process of signal loss after pulsed excitation (perturbation) is called free induction decay (FID). The signal resulting from just one pulse is likely to be very weak and will be hard to differentiate from the general electronic background ('noise'). Consequently, the pulse, followed by decay time sequence is repeated many times (until the 'signal to noise ratio' is acceptable). The data is accumulated in the computer memory, where noise, being random, gradually diminishes, whereas signals become more intense. Fourier transform can then be carried out on the final FID data to produce the familiar frequency spectrum.

Because a normal organic sample will have nuclei of the same type (e.g. ^{1}H or ^{13}C) in different magnetic environments (corresponding to different locations in different components in the sample), these will have slightly different Larmor frequencies. Hence a pulse of RF field at the nominal precession frequency (say that of ^{1}H or ^{13}C in TMS $(CH_3)_4Si$), will be slightly offset from the actual resonant frequencies of the nuclei in the sample molecules. Consequently, the FID patterns for each environmentally identical set of nuclei will be different, depending on this offset or frequency difference. This gives rise to a complex combined FID pattern for the sample that is unraveled by the application of Fourier transform into a series of peaks at different frequencies, with respect to TMS (chemical shift), corresponding to the various magnetic environments that exist for the nuclei in the sample molecules.

The final position of the magnetization will depend on the time duration of the RF pulse (t_p), which is usually a few microseconds. The flip (or tip) angle (θ) through which the magnetization is flipped from the z axis is given by Equation 4.4.2.

$$\theta = \gamma B_1 t_p \tag{4.4.2}$$

Since in practice, the pulse duration that is needed to produce a particular flip angle varies somewhat from day to day, it is standard practice to refer to this duration by the effective flip angle rather in time units. Thus a pulse width is often described as 90° (in degrees) or $\pi/2$ (in radians), rather than in microseconds.

In practice, the relationship between the pulse width and the flip angle is observed by varying the pulse duration and examining the resultant NMR signal intensity. Since the magnetization is measured in the *xy* plane there will be a sinusoidal variation in the intensity with pulse width, as shown below.

Pulse width	$\pi/2$	π	$3\pi/2$	2π
Intensity	Maximum +	0	Maximum –	0

The most common representation of an NMR experiment, in terms of pulse and acquisition times (etc.), is shown in the Figure 4.4.4, illustrated for one of the simplest kinds of NMR experiments, where the pulse width tips the magnetization on to the y' axis (90°). Repeated acquisition of the FID data and Fourier transform of the accumulated data in the time domain at the end of the experiment gives the familiar 1D NMR spectrum in the frequency domain, showing separate peaks at different chemical shifts for each magnetically different nucleus. Such spectra, with spin–spin coupling normally included for ^{1}H, but removed or subdued for ^{13}C, using broadband decoupling (or nowadays using a form of WALTZ proton decoupling) or *J*-scaling (respectively), are often sufficient for the characterization of smaller, less complex molecules. More complex molecules, with more C–H connectivities, usually need more sophisticated 1D techniques for full characterization, such as Distortionless Enhancement by Polarization Transfer (DEPT), Incredible Natural Abundance Double Quantum Transfer Experiment (INADEQUATE), Insensitive Nuclei Enhanced by Polarization Transfer (INEPT) or Nuclear Overhauser Effect Difference spectroscopy (NOED) – or 2D techniques such as (homonuclear) correlation spectroscopy (COSY), heteronuclear correlation spectroscopy (HETCOR), homonuclear multiple bond coherence spectroscopy (HMBC), heteronuclear multiple quantum coherence spectroscopy (HMQC), nuclear Overhauser effect spectrocopy (NOESY) or total correlation

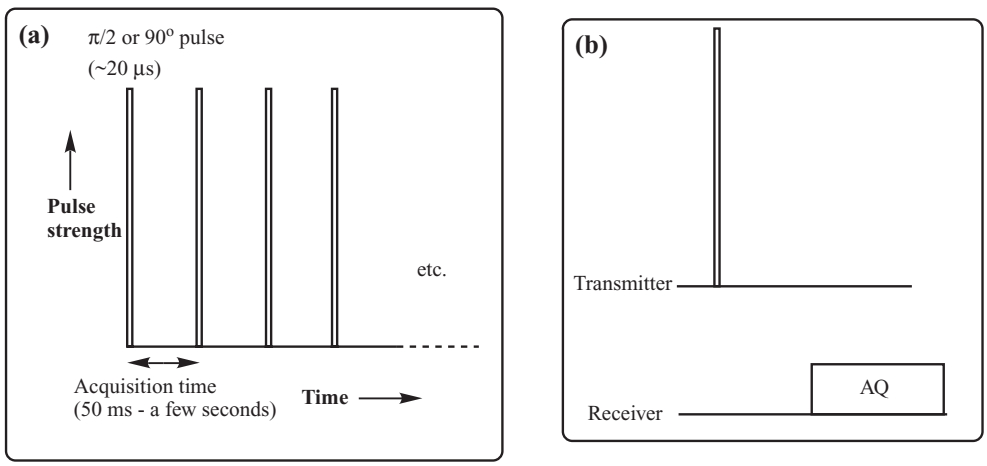


Figure 4.4.4 *Representation of the most basic NMR experiment. Not drawn to scale. (a) shows detail of pulse and acquisition times, (b) is a shorthand presentation. Other shorthand versions exist - e.g. with transmitter and receiver representations on same line and with a FID picture for AQ*

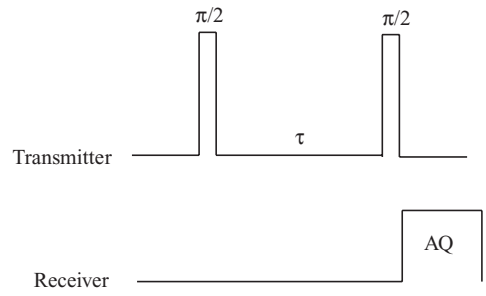


Figure 4.4.5 *Pulse sequence for COSY-90 experiment*

spectroscopy (TOCSY). These techniques, although involving much more complex pulse sequences than that shown in Figure 4.4.4, are now routine.

Multidimensional NMR techniques have been developed to cope with such complex examples: in particular, H–H Correlation Spectroscopy, better known as COSY and H–non-H Correlation Spectroscopy, known as HETCOR. A COSY spectrum is usually a plot of ^{1}H chemical shift on both the *x* and *y* axes, whereas a HETCOR spectrum is usually a plot of ^{1}H chemical shift on one axis against the chemical shift of some other nucleus (usually ^{13}C) on the other axis. The peaks in the whole COSY spectrum (plotted as contour lines derived from a 'stack plot') lie along a diagonal line with cross peaks, representing coupled nuclei. To analyze a COSY spectrum, firstly a diagonal is drawn through the contour lines. The contour lines that are not on the diagonal (called 'cross peaks' or 'off-diagonal peaks') contain the coupling information.

The basic COSY (strictly COSY-90 or COSY-$\pi/2$, since other versions are used) pulse sequence is shown in Figure 4.4.5. Here, τ is an incremental delay. Considering a single line in a complex multiplet, a pulse of $\pi/2$ causes the magnetization vector to precess in the *xy* plane at a frequency ν_1 that depends on the its chemical shift and on each of the couplings with which the multiplet is involved (Figure 4.4.6).

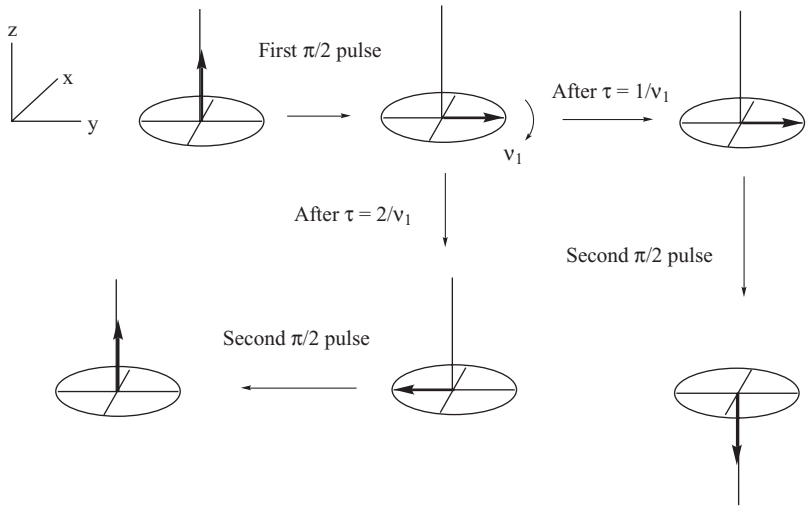


Figure 4.4.6 *Signal intensities and time delay (τ) between COSY-90 pulses*

If the evolution time, $\tau = 1/\nu_1$, then at the end of that time, the vector will be aligned along y and so the second $\pi/2$ pulse will rotate the vector down along the $-z$ axis (see below). This results in inversion of the population distribution for that pair of energy levels and for all the connected transitions in the other resonances to which the proton is coupled (Figure 4.4.6). On the other hand, if $\tau = 1/2\nu_1$, then after that time, the vector will be aligned along $-y$ and the second $\pi/2$ pulse will return the vector to the z axis, resulting in normal population distribution (Figure 4.4.6).

Intermediate values of τ give intermediate results and repeating many experiments with incremented τ results in a set of spectra that have normal chemical shift values and normal coupling, but each peak contains coded information about the frequencies of all other peaks to which it is coupled. The second Fourier transformation then gives a spectrum with ^{1}H chemical shifts in each dimension. The normal spectrum is now found along the diagonal because the intensity of each transition oscillates at ν_1 during t_1 and because its detected frequency in t_2 is also ν_1. The off diagonal 'cross peaks' determine which chemical shifts are connected to which, because the intensity of the ν_1 transition is modulated during t_1 by all the transitions to which it is connected.

HMBC is a particular 2D HETCOR technique used to correlate (determine the connectivity between) ^{1}H and ^{13}C peaks for nuclei separated by (usually) two or three multiple bonds, whereas HMQC indicates direct C–H coupling. HMBC is often used to assign quaternary carbon atoms.

Additionally, the stereochemistry of complex beverage molecules, such as dimeric and oligomeric sugars, can be deduced from NOESY, a technique that makes use of through space interaction between magnetic nuclei (in the same molecule), known as the nuclear Overhauser effect (NOE). Signal enhancement or diminution depends on the magnetogyric ratios of the interacting nuclei and on their proximity; NOE is more pronounced for nuclei that are spatially close.

Signal enhancement due to NOE is an example of cross polarization, a through-space effect in which polarization of spin states of one type of nucleus (say ^{1}H) caused by irradiation (B_1) induces polarization of spin states of another nucleus (say, ^{13}C or ^{1}H). In general, the maximum NOE enhancement is given by Equation 4.4.3.

$$\mathrm{NOE}_{\max} = \frac{1}{2}\left(\frac{\gamma_{\mathrm{irr}}}{\gamma_{\mathrm{obs}}}\right) \tag{4.4.3}$$

Here, γ_{irr} is the magnetogyric ratio of the nucleus being irradiated and γ_{obs} is that for the nucleus under observation. The total (maximum) line intensity is given by $1 + \mathrm{NOE}_{\max}$. Hence, in the case of proton decoupled ^{13}C NMR spectra, the ^{13}C signal can be enhanced up to 200% by the irradiation of protons. This value is a theoretical maximum; most actual ^{13}C lines exhibit less (sometimes much less) than maximum enhancement. Two dimensional NOESY spectra contain cross peaks that represent resonances from nuclear spins that are spatially close. The one dimensional nuclear Overhauser effect difference spectroscopy (NOED) technique is carried out by selecting particular resonances in a molecule for irradiation with an RF pulse, observing the effect on other resonances in the NOE spectrum, and then subtracting the normal NMR spectrum from this. Only those peaks that have significant NOEs with the resonance of interest (the irradiated one) are revealed in the NOED spectrum.

High resolution NMR (mainly ^{1}H and ^{13}C) has been applied extensively to the study of alcoholic beverages and some of the processes involved in their production since the 1970s. The major applications are:

- Identification or structure elucidation of specific components (often with the aid of other spectroscopic techniques, such as mass spectrometry)
- Quantitative determination of specific known components
- Time course studies (e.g. of fermentations, oxidations, etc.)
- Determination of D/H ratios by site specific isotopic fractionation-NMR (SNIF-NMR).

The reader is referred to the reviews of Martin (1988), Rapp *et al.* (1988), Rapp and Markowetz (1990a) and Košir and Kidrič (2002) for general accounts of the application of NMR spectroscopy to alcoholic beverages.

Many of the NMR reports on alcoholic beverage analysis involve the use of statistical or chemometric analysis for interpretation of results (e.g. Petrakis *et al.*, 2005; Conte, 2008; Son *et al.*, 2009).

Identification or structure determination of specific components (often after extraction from the wine sample) includes acetic acid (Weekley *et al.*, 2003) and other acids (Avenoza *et al.*, 2006), amino acids (Košir and Kidrič, 2001; 2002), red wine pigments (Tamura *et al.*, 1994; Bakker and Timberlake, 1997; Fulcrand *et al.*, 1998; Cabrita *et al.*, 2000; Degenhardt *et al.*, 2000; Es-Safi *et al.*, 2000; Atanasova *et al.*, 2002; Schwarz *et al.*, 2003; Mateus *et al.*, 2006), terpenoids (and their glycosides) (Baltenweck-Guyot *et al.*, 1996; 2000; Collado *et al.*, 1996; Bonnländer *et al.*, 1998) phenolic substances (Escribano-Bailón *et al.*, 1996; Guyot *et al.*, 1996a; Yeap Foo *et al.*, 1998; Saucier *et al.*, 1997; Dufour and Bayanove, 1999; Lu and Foo, 1999; Baderschneider and Winterhalter (2000); Remy-Tanneau *et al.*, 2003) and a variety of aroma compounds (many of which are terpenoids or norisoprenoids) (Guth, 1996; Versini *et al.*, 1996; Guth, 1997; Dufour and Bayanove, 1999; Marchand *et al.*, 2002). Also, additives and residues have received some attention from NMR spectroscopists (Rapp *et al.*, 1986; Mojsiewicz-Pienkowska *et al.*, 2003).

NMR spectroscopy (especially ^{13}C NMR) is a powerful tool for the simultaneous qualitative and quantitative determination of beverage components in whole samples. Identification and quantitation by this method are possible for components with known chemical shifts, but structure elucidation of unknown components is not; instead, these generally need to be isolated from the sample as pure compounds and analyzed separately. Typically, the sample is prepared by mixing beverage (1.0 ml) and D_2O (0.2 ml) or D_2O (0.2 ml) with 1,3-propanediol as internal standard for quantitative work. For abundant constituents (>1 g/l), the sample can be analyzed whole, but for minor analytes (<1 g/l), some method of focusing (e.g. by vacuum distillation) is required. For example, a standard 1D ^{13}C NMR spectrum of a wine sample after less than an hour of data accumulation will readily show ethanol, glycerol, carboxylic acids and the major sugars, fructose and glucose (Rapp and Markowetz, 1990b), although the spectra of the latter are complex due to the presence of various anomeric forms. Thus, glucose exhibits 12 signals arising from the α- and β-glucopyranose anomers, whereas fructose gives 30 signals arising from α- and β-fructofuranose, α- and β-fructopyranose and keto anomers. On the other hand, minor sugars such as arabinose, galactose, mannose, rhamnose and trehalose are detected more easily in the wine concentrate. Likewise, amino acids, anthocyanins, esters, minor acids, lactones and polyols and can be identified in wine concentrates (Košir and Kidrič, 2001) or even intact wines (Košir and Kidrič, 2002; Brescia *et al.*, 2003). One and two dimensional NMR (Gradient-selected COSY, TOCSY, HMQC and HSQC) were used to identify 17 amino acids and γ-aminobutyric acid in freeze-dried Slovenian wine samples (Košir and Kidrič, 2001). A WET pulse sequence was used to suppress strong proton signals arising from water (4.80 ppm), ethanol (3.64 ppm), glycerol (3.62 ppm) and other relatively abundant components. Later, Košir and Kidrič (2002) used 1D NMR spectra of amino acids in white wines and chemometric analysis to determine the grape varieties responsible for the wines. The use of signals from seven amino acids allowed good statistical separation of wines according to vine variety, whereas if the signals from butylene glycol, glycerol and succinic acid were included in the chemometric analysis, separation of wines between coastal and continental regions of Slovenia was achieved.

Alcoholic beverages are of such compositional and molecular complexity that even using the most modern high field NMR instruments to investigate the complete beverage, signal overlap, poor signal intensity or lack of coupling information can lead to incomplete or ambiguous assignments. In order to improve this situation, diffusion ordered spectroscopy (DOSY) has been applied to the analysis of differences in constitution of Port wines of varying ages (Nilsson *et al.*, 2004a; 2004b). DOSY is used to separate signals on the basis of their diffusion coefficients, which are related to the hydrodynamic radii (apparent molecular size) of the molecules giving rise to the signals. The DOSY technique offers a means of integrating diffusion encoding into multidimensional pulse sequences. A series of pulsed field gradient stimulated echo NMR spectra is

obtained with increasing gradient pulse strength. These signals then decay at rates that are determined by their diffusion properties. The version of DOSY (2DJ-IDOSY) pioneered by Nilsson *et al.* (2004b), using a simpler, more sensitive pulse sequence, improved the signal to noise ratio by a factor of 2 and reduced experimental time by a factor of 4. Using this technique, Port wines of different ages were found to differ largely by the relative concentrations of amino acid, organic acids, an unidentified (possible) disaccharide and large aromatic species, probably polymeric polyphenols (Nilsson *et al.*, 2004a). In particular, the older wines had lesser amounts of these compounds, probably as a result of significant precipitation during the course of maturation.

Quantitative ^{1}H NMR has also been used in the analysis of organic acids and amino acids in beer (Nord *et al.*, 2004), polyphenols in cider apple juice (Berregi *et al.*, 2003a; 2003b) and methanol and other components in zivania, a traditional Cypriot pomace spirit (Section 3.7.3) (Petrakis *et al.*, 2005). In the last mentioned study, the NMR data in the region 5.5–1.1 ppm, for many versions of zivania and many other European spirits, were used for processing by canonical discriminant analysis (CDA). The method, validated against a comparable GC method, gave a satisfactory degree of prediction and classification between zivania brands and other European (including Greek) spirits.

Contaminants, such as methanol, and illegal additives, such as diethylene glycol (Rapp *et al.*, 1986), are easily detected in a standard ^{13}C NMR spectrum of the whole sample.

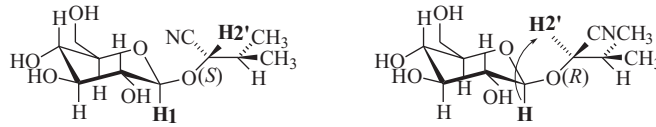
The methods described above, although carried out on whole samples, may require the breaching of a larger sample (e.g. in a bottle, can or cask) and transfer of the small quantity of liquid to an NMR tube, and consequently are invasive and not strictly nondestructive. At the extreme, an NMR probe and spectrometer were developed to study the contents (particularly of ethanol and acetic acid) of intact bottles of wine (Weekley *et al.*, 2003). Careful adjustment of both cryogenic and room temperature magnetic field shims on a 2.01 Tesla 310 mm room temperature superconducting solenoid imaging magnet resulted in an extremely homogeneous magnetic field over the entire bottle of wine giving reasonable resolution (4 Hz line width). Moreover, careful adjustment of the room temperature shims compensated for different bottle shapes, volumes and cork types.

Using a special pulse sequence, ^{1}H signals were found at 4.8 ppm (singlet; water), 3.6 ppm (quartet; $CH_3C\mathbf{H_2}OH$), 2.0 ppm (singlet; $C\mathbf{H_3}CO_2H$) and 1.0 ppm (triplet; $C\mathbf{H_3}CH_2OH$). In this way, using appropriate standards in full bottles, the ethanol and acetic acid contents of wines of different vintages were estimated. Thus, it is possible to determine the quality of an old vintage wine without opening the bottle; the higher the level of acetic acid, the more likely the wine has undergone spoilage, possibly by ingress of oxygen and bacteria through a poor quality, perished or poor fitting cork.

There are numerous examples of the application of NMR spectroscopy to the characterization of new compounds isolated from alcoholic beverages or their precursors, usually by chromatographic methods; only examples of literature where structure determination plays a major role are considered here. Usually, a combination of NMR techniques, involving both ^{1}H and ^{13}C nuclei, is used, such as COSY, DEPT, HETCOR, HMBC, HSQC, NOED, NOESY, SEFT and TOCSY, and often in conjunction with other spectroscopic methods.

Within the realm of carbohydrates, an early example involves the use of NOE combined with molecular mechanics, used to differentiate between heterodendrin and *epi*-heterodendrin, two diastereoisomeric cyanogenic glycosides present in barley (*Hordeum vulgare*) seeds (Lankhorst *et al.*, 1995). *Epi*-heterodendrin in barley is of considerable interest because it is thought to be an important source of the carcinogenic ethyl carbamate in whisky (Section 3.2.2). The NOE between the anomeric proton H1 and the side chain proton H2′ is larger for *epi*-heterodendrin than for heterodendrin, thus indicating a shorter distance between these protons in the former diastereoisomer (Figure 4.4.7). This assertion was supported by molecular mechanics calculations.

Pigments and phenolic compounds in general, especially from colored fruits and their alcoholic beverages, have received considerable attention from NMR spectroscopists. Here, NMR spectroscopy has been



Heterodendrin *Epi*-heterodendrin

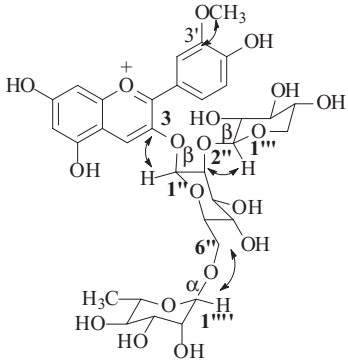
Figure 4.4.7 *Use of NOE to differentiate between heterodendrin diastereoisomers. Lankhorst* et al. *(1995)*

particularly useful in assigning the sugar moieties of anthocyanin and polyphenol glycosides and in determining the structures of oligomers and other condensation products. Novel anthocyanin triglycerides, such as the 3-(6″-α-rhamnosyl)-2″-β-xylosyl-β-glucosides of cyanidin, peonidin and petunidin, were found in the berries of *Vaccinium padifolium*, a member of the blueberry family (Cabrita *et al.*, 2000). The observation of cross peaks from HMBC spectra was used to establish the linkages in the glycoside moieties, as shown in Figure 4.4.8(a) for peonidin 3-(6″-α-rhamnosyl)-2″-β-xylosyl-β-glucoside, although a combination of many techniques, including COSY and TOCSY, were used to assign all the ^{1}H and ^{13}C resonances for these anthocyanins and others in *V. padifolium*. Similarly, Lu and Yeap Foo (1999) identified gallic acid 3-β-glucopyranoside, gallic acid 4-β-glucuronide and 2-hydroxy-5-(2-hydroxyethyl)phenyl-β-glucopyranosides as novel polyphenolic compounds in Chardonnay pomace.

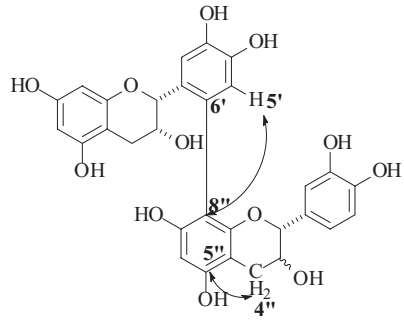
^{13}C and ^{1}H NMR spectroscopies have been used to establish the structures of biphenyl linked flavan-3-ol dimers isolated from Chardonnay pomace (Yeap Foo *et al.*, 1998). In particular, the structures epicatechin [6′→8′]-epicatechin and epicatechin [6′→8′]-catechin (Figure 4.4.8(b)) were established by TOCSY and observation of long range C, H couplings via HMBC (e.g. between H-5′ and C8′; H-4′ and C-5′). Similarly, NMR (along with ESI-MS and molecular mechanics) showed the existence of acetaldehyde mediated flavan-3-ol dimerization in a model wine solution (Saucier *et al.*, 1997). Four isomers were observed for the dimerization of catechin, corresponding to acetaldehyde-bridging at 6-6, 8-8, 6-8 and 8-6 on the A ring of the two catechin monomers, with the 6-8 bridged dimer appearing to be the most abundant isomer, according to HPLC analysis.

NMR spectroscopy has been especially valuable in the determination of the structures of condensation products of anthocyanins, flavan-3-ols and other compounds (including aldehydes, ketones and simple phenols) in grapes (including pomace), wine and model wine solutions. Particularly in the case of ageing red wine, NMR has played a key role in demonstrating that anthocyanins undergo numerous reactions at different rates with wine constituents to produce new pigments, which are more stable than the original anthocyanins. Prior to the discovery of pyranoanthocyanins and similar compounds (including vitisin type derivatives), it was thought that the color changes accompanying red wine aging were due to condensation reactions between original anthocyanins (flavylium ions) and flavan-3-ols (catechins), as well as between flavan-3-ol monomers. Although it is now believed that pyranoanthocyanins are the main pigments in young (say, 1–2 year old) red wines, and therefore the molecules that under condensation during subsequent aging, it is still likely that significant contributions to color changes during the earlier stages of maturation are provided by the reactions mentioned in the previous sentence.

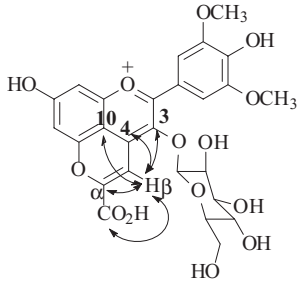
Anthocyanins and catechins, in the presence of acetaldehyde in a model red wine solution were shown to produce dimers and higher oligomers, which were isolated by preparative HPLC or column chromatography and partially characterized by 1D and 2D NMR spectroscopy and FAB-MS (Escribano-Bailón *et al.*, 1996). Although flavan-3-ol dimerizations (and higher oligomerizations) may contribute something to the orange hue of aged red wine, it is more certain that they contribute to the yellow hue of aged white wine (Es-Safi *et al.*, 2000). Simulated aging of a model white wine solution containing (+)-catechin and glyoxylic acid



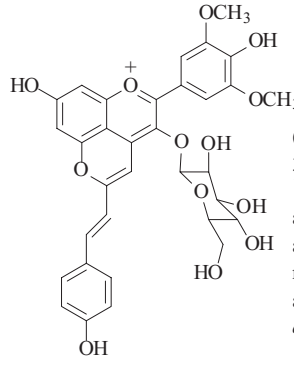
(a) Peonidin 3-(6"-α-rhamnosyl)-2"-β-xylosyl-β-glucoside, showing some HMBC cross-related nuclei. (Cabrita *et al.*, 2000).



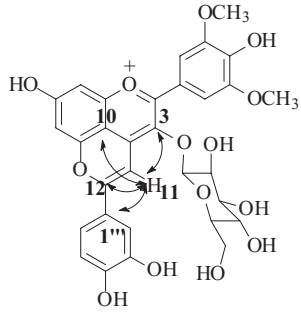
(b) Biphenyl linked flavan-3-ol dimers, showing some HMBC correlations (Yeap Foo *et al.*, 1998).



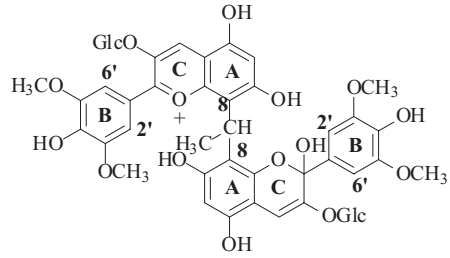
(c) Pyranomalvidin 3-glucoside, showing HMBC correlations for the new pyran ring β proton (Fulcrand *et al.*, 1998).



(d) Vinylpyranomalvidin 3-glucoside-phenol. ^{1}H resonances were assigned using 1-D NMR and *g*COSY; ^{13}C resonances by *g*HMBC and *g*HSQC (Mateus *et al.*, 2006).



(e) Malvidin 3-glucoside 4-vinylcatechol adduct from Pinotage wine, showing selected HMBC correlations. (Schwarz *et al.*, 2003)



(f) Ethyl-linked malvidin 3-glucoside dimer shown in flavylium-hydrated flavylium form. NOESY showed through-space couplings between the central ethyl moeity and H2', H6', OCH_3 of rings B. (Atanasova *et al.*, 2002).

Figure 4.4.8 *Some pigment and polyphenol structures determined largely by NMR. Original notation used*

(a hydrolysis product of tartaric acid) led to the formation of two types of colorless dimers consisting of two flavanol units linked by carboxymethine bridges. After isolation of these dimers and further incubation in alcohol free solution, these were converted to yellow xanthylium salts ($\lambda_{max} \sim 440$ nm and 460 nm) by dehydration, followed by oxidation. NMR spectroscopy showed the colorless dimers to be 6-6, 6-8 (two forms) and 8-8 linked catechins via glyoxylic acid; for example, HMBC for the 6-8 dimers (Figure 4.4.9) indicated correlations between C8a and H2 (for both catechin units), C8a and H4 (for both catechin units),

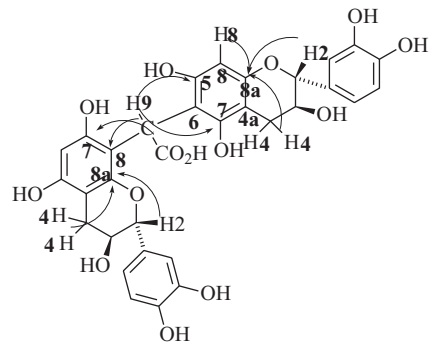


Figure 4.4.9 *HMBC correlations in 6-8 glyoxylic acid-linked dimer of (+)-catechin. Es-Safi* et al. *(2000)*

C8a and H9 (bridge methine proton) (first left hand unit), C8a and H8 (second unit), C5, C7 and H9 (second unit), and C7, C8a and H9 (first unit).

Similarly, Remy-Tanneau *et al.* (2003) demonstrated that an ethanolic (1:1) solution of malvidin 3-glucoside and (–)-epicatechin (or (+)-catechin) kept at 35 °C for 16–19 days formed significant quantities of colorless anthocyanin-flavan-3-ol dimers. The major dimer formed from the anthocyanin and (–)-epicatechin was shown by HMBC to be malvidin 3-glucoside[C2-O-C7,C4-C8]epicatechin (i.e. a dimer with two linkages between the monomers). Figure 4.4.10 depicts this dimer with the major HMBC correlations that imply the linkage positions.

Anthocyanin pyruvates or pyranoanthocyanins (see Figure 4.4.8(c) for one example), whose characteristic colors lie to the blue side of true anthocyanin colors (i.e. $\lambda_{max} < 528$ nm), were shown to be the major pigments in red wines during the first two years of maturation, from reactions of anthocyanins with pyruvate in model solutions and by comparison with real wine components (Bakker and Timberlake, 1997; Fulcrand *et al.*, 1998). Similar reactions were observed between anthocyanins and acetaldehyde, and these derivatives with pyran rings are sometimes known as vitisins. For pyranomalvidin 3-glucoside (Figure 4.4.8(c)), a negative NOE was observed between H8 and H2′ (H6′) and also between Hβ and H2′ (H6′), in agreement with the positions of the hydrogen atom and carboxyl group on the new pyran ring (Fulcrand *et al.*, 1998). Long

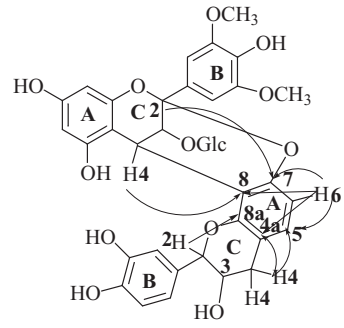


Figure 4.4.10 *Major HMBC correlations for the malvidin 3-glucoside [C2-O-C7, C4-C8] (−)-epicatechin dimer. Remy Tanneau* et al.*, 2003*

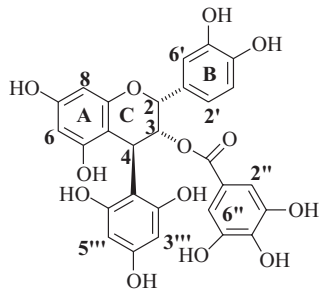
range heteronuclear correlation experiments (HMBC) indicated a variety of correlations consistent with the proposed structure (Figure 4.4.8(c)).

More recently, bluish pigments ($\lambda_{max} > 528$ nm), discovered in Port wines were shown to be vinylpyranoanthocyanin-flavanols (Mateus *et al.*, 2003) and vinylpyranomalvidin 3-glucoside-phenol, the latter supported by comparison with a fully NMR characterized sample from a model solution reaction (Mateus *et al.*, 2006) (Figure 4.4.8(d)). A similar pigment was isolated from anthocyanin rich extracts of Pinotage wine by high speed countercurrent chromatography (HSCCC – see Section 4.3.4) and characterized by LC-ESI-MS and NMR (Schwarz *et al.*, 2003). One dimensional ^{1}H and ^{13}C NMR, as well as DEPT, were used to assign resonances, whereas HMBC and HSQC were used to assign all ^{1}H-^{13}C connectivities in the molecule (Figure 4.4.8(e)).

Anthocyanins in red wine can also undergo acetaldehyde mediated self-condensation (dimerization). A new pigment ($\lambda_{max} = 518$ nm) found in a model wine solution containing malvidin 3-glucoside and acetaldehyde was shown by NOESY, COSY, HMBC and HSQC to be the structure shown in Figure 4.4.8(f) – this is the major form at wine pH (Atanasova *et al.*, 2002). Each of the main forms (diflavylium, flavylium-hydrated flavylium and flavylium-quinoid) was detected by LC-ESI-MS. The occurrence of this pigment in red wine was demonstrated by HPLC.

Proanthocyanidins in grape skins and wine are important contributors to astringency, a certain amount of which is necessary for the correct 'mouthfeel' of red wine and other drinks such as cider and perry. Many of these are high molecular weight polymers and it is known that they have a wide diversity of structures, particularly with regard to linkage type and positions between the monomeric subunits. Partly because of this and partly because of the difficulty in obtaining pure samples of the higher polymers, structure determination is difficult, hence acid catalyzed hydrolysis of proanthocyanidins and analysis of the cleavage products by NMR and other techniques can provide some insight into their monomer composition and linkages. A standard procedure is to hydrolyze the polymer in the presence of benzyl mercaptan or phloroglucinol to trap electrophilic flavonoid species (formed from the cleavage of extension subunits–nonterminal units) and so to reveal cleavage positions and consequently linkage positions in the original polymer. Kennedy and Jones (2001) used HPLC to separate the hydrolysis products of skin and seed proanthocyanidins from Chardonnay grapes. A major phloroglucinol trapped hydrolysis subunit (isolated using preparative HPLC) is shown in Figure 4.4.11, along with the NOE correlations that were used to characterize it (HMBC was also used in the structure elucidation).

Like polyphenols, aroma compounds and their glycosidic precursors (nonvolatile themselves, but important potential sources of aroma) are important components of alcoholic beverages and consequently have received some attention from NMR spectroscopists.



NOE correlations (from NOESY and ROESY) for protons

Proton	NOE correlated protons
2	3, 2', 6'
3	2, 4, 2', 6'
4	3, 2", 6"
2'	2, 3, 2, 6"
5'	6'
6'	2, 3, 2', 5', 2", 6"
2"	4, 2', 6'
6"	4, 2', 6'

Figure 4.4.11 *Acid hydrolysis product of grape proanthocyanidin. Kennedy and Jones (2001)*

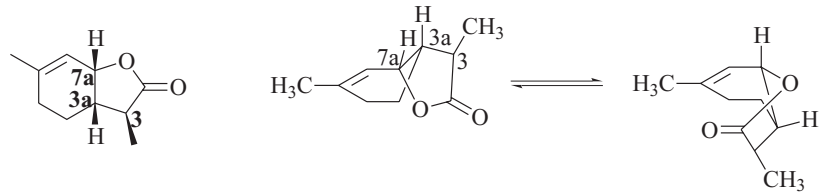


Figure 4.4.12 *Configuration and conformations of wine lactone. Guth (1996)*

Wine lactone (3*S*,3a*S*,7a*R*)-3a,4,5,7a-tetrahydro-3,6-dimethylbenzofuran-2(3*H*)-one (Figure 4.4.12) is powerful odorant (coconut notes) found in white wines, particularly those of 'aromatic' varieties, such as Scheurebe (Riesling type) and Gewürztraminer (Traminer type) (Guth, 1996). Comparison of spectroscopic (NMR, IR, MS and circular dichroism) and chromatographic data (chiral GC) of wine lactone isolated from different white wines and with those of the eight stereoselectively synthesized diastereoisomers showed that wine lactone had the structure given in Figure 4.4.12. In the NMR spectroscopic evaluation, the spin systems of the diastereoisomeric lactones were identified from TOCSY spectra and individual protons were assigned by quantum filtered COSY spectroscopy. In particular, the coupling constant $^{3}J_{7a,3a}$ (6.8 Hz) was consistent with equatorial–axial, rather than equatorial–equatorial or axial–axial configuration of the protons at the ring junction.

Analysis of HPLC and TLC fractions of glycosidic extracts of Alsace Gewürztraminer wines by NMR and MS has revealed a number of isoprenoid and other glycosides and a phenylpropenoid glycerol (Baltenweck-Guyot *et al.*, 1996; 2000). The products were acetylated prior to their analysis by GC-MS and NMR. NOESY and HMBC spectra gave through space and through bond connectivities (respectively) thus enabling the determination of the nature and assemblage of the sugar moieties, as well as confirming the structures of the aglycones, as outlined in Figure 4.4.13 for a glycoside of *cis*-furan linalool oxide. Other glycosides characterized included those of (*E*)-6,9-dihydroxymegastigma-4,7-dienone (a precursor of β-damascenone), benzyl alcohol and phenylethanol.

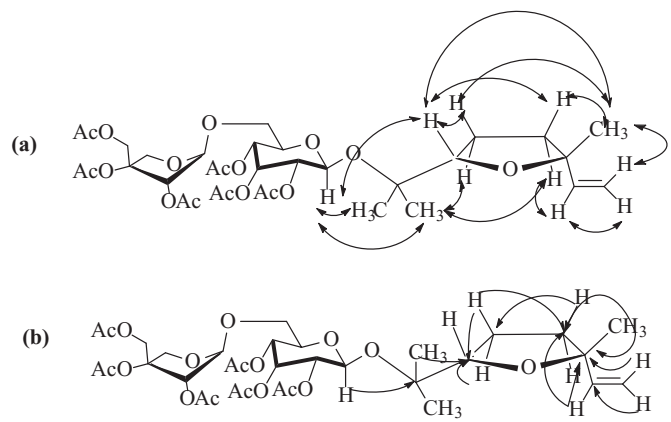
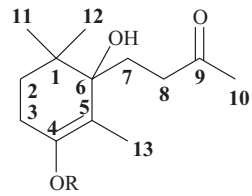


Figure 4.4.13 *Most important NOESY connectivities (a) and HMBC connectivities (b) for the aglycone moeity of peracetylated* β-D-*apiofuranosyl-(1-6)-O-*β-D-*glucopyranoside of cis-furan linalool oxide. Baltenweck-Guyot* et al. *(2000)*



R = glycosyl

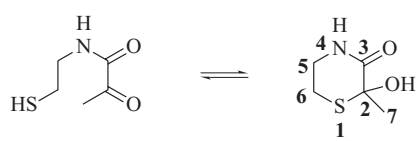
Coupling constants (J_{HH})	Hz
$J_{2,3}$(ax-ax)	12.3
$J_{2,3}$(eq-ax)	4.9
$J_{2,3}$(eq-eq)	2.3
$J_{3a,3b}$ (H2 uncoupled)	13.0
$J_{7a,7b}$ (H8 uncoupled)	17

^{13}C resonances were assigned *via* inverse detection or inverse long range correlations.

Figure 4.4.14 *Selected NMR data for megastigm-4-en-9-one aglycone, a TDN precursor in Riesling grapes. Versini* et al. *(1996)*

1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN), like many other C_{13} norisoprenoids, is a major contributor to the aroma of wine made from 'aromatic' grape varieties, such as Riesling, where it contributes to what is commonly known as kerosene or petroleum odor (especially noticeable in aged wines). Like other odorous norisoprenoids, it is likely that TDN is synthesized during fermentation and aging from norisoprenoid (ultimately carotenoid) precursors. Several such precursors have been suggested for TDN, their common feature being the production of some TDN upon treatment with acid. A megastigm-4-en-9-one glycoside has been suggested as an important precursor in Riesling grape juice (Versini *et al.*, 1996). The structure of the aglycone (Figure 4.4.14) was determined by NMR spectroscopy, especially HMBC (inverse long range ^{1}H–^{13}C correlation), ROESY (the rotating framework version of NOESY) and TOCSY.

The Maillard reaction (Section 2.6.2) is a complex series of reactions that occur between amino acids and carbonyl compounds (including sugars) in foodstuffs, including alcoholic beverages. The reactions can occur to a certain degree at temperature below 37 °C, but are more pronounced at higher temperatures. Many of the products are colored or possess characteristic aromas and hence make particularly important contributions to the organoleptic properties of alcoholic beverages that have had heat treatment, or some of whose ingredients have undergone some kind of heat treatment. Included here are Madeira (Section 2.10.6), certain Sherries (Section 2.10.2), dark beers (Section 2.6.2) and pasteurized drinks, particularly overpasteurized beers. Many important Maillard products are nitrogen, oxygen or sulfur-containing hetetocycles and include 2-acetylthiazole (cooked cereal notes, found in some wines) and 2-acetyl-2-thiazoline (roast meat notes, found in some highly pasteurized beers). Using a wine-like model solution containing cysteine and methylglyoxal, Marchand *et al.* (2002) were able to identify *N*-(2-sulfanylethyl)-2-oxopropanamide (Figure 4.4.15) as a precursor of 2-acetylthiazole from MS, ^{1}H and ^{13}C NMR data. HMBC was used for long range ^{13}C–^{1}H correlations.



HMBC correlations

^{13}C	^{1}H
2	1, 5
3	-
5	1
6	2
7	5

Figure 4.4.15 *HMBC correlations and structure of 2-acetylthiazole intermediate in Maillard reaction between cysteine and methylglyoxal. Marchand* et al. *(2002)*

Although most polyphenols, especially polymeric species, are considered to be too involatile to contribute much directly to wine aroma, they may make an indirect contribution to flavor by influencing the 'free' concentration of certain types of flavor compounds. They also make an important contribute to taste (mouth-feel) by forming complexes with saliva proteins, thus giving the sensation of astringency. Using a dynamic headspace technique on a water-alcohol model solution, Dufour and Bayanove (1999) were able to show that the flavan-3-ols, (+)-catechin and (–)-epicatechin, reduced the volatility of isoamyl acetate, ethyl hexanoate and benzaldehyde more than that of more hydrophobic limonene, whereas a wine tannin fraction decreased the volatility of benzaldehyde somewhat, had no effect on the ester aroma compounds and actually increased the volatility of limonene. Measurement of ^{1}H chemical shift changes on addition of ligand (aroma compound) to a substrate (a flavan-3-ol or tannin fraction) enabled the estimation of dissociation constants in 1:1 binding models.

In recent years, NMR spectroscopy has been used in kinetic or time course studies of a number of aspects relating to alcoholic beverages, including metabolic processes such as fermentation (Avenoza *et al.*, 2006; Son *et al.*, 2009), oxygenation (Conte, 2008) and reactions between (+)-catechin and aldehydes (Nonier *et al.*, 2007). Son *et al.* (2009) were able to use ^{1}H NMR to investigate metabolic changes occurring during alcoholic fermentation of grape juice and to evaluate the impact of three yeast (*Saccharomyces cerevisiae*) strains (RC-212, KIV-1116 and KUBY-501) on the wine metabolite profile. Using pattern recognition chemometric techniques, differences were observed for each fermentation, the differentiation being based upon the concentrations of 2,3-butanediol, citrate, glucose, glycerol, malate, *N*-methylnicotinic acid, polyphenols, proline, pyruvate, succinate and tartrate. TOCSY, HMBC and HSQC were used to acquire the NMR data. Lyophilized wine samples (1 ml) were dissolved in D_2O (0.4 ml), 400 nM oxalate buffer (pH 4.0) (0.14 ml) and 5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (0.06 ml). The latter provided a chemical shift reference. The supernatant after centrifugation was subjected to NMR experiments using a NOESYPREAST pulse sequence to suppress the residual water signal. This study demonstrated the ability of NMR, coupled with multivariate statistical analysis, to differentiate between the same must fermented by different yeast strains and so NMR could be used as both a fermentation monitor and a check on yeast authenticity.

Avenoza *et al.* (2006) used ^{1}H NMR to monitor the change in malic and lactic acid concentrations in fermenting grape must, with and without added *Oenococcus oeni* for malolactic fermentation. Samples were dissolved directly in D_2O, without prior freeze drying, because of the high sugar contents of the fermenting musts compared with finished wine. Hence the NMR spectra were recorded with a standard pulse sequence (zgpr with p19 at 6 dB and flip angle 90°) for presaturation of the water signal at 1875 Hz. The results compared favorably with those using enzymic/colorimetric methods, with NMR having the advantages of being more robust and requiring minimal sample preparation or manipulation.

A preliminary ^{1}H NMR spectroscopic method has been used to observe molecular changes occurring during microoxygenation of red wine (Conte, 2008). Wine from the same vintage was kept in two identical stainless steel tanks, one of which was micro-oxygenated at the rate of 4 ml of oxygen per ml of wine per month. Samples were taken every week for five weeks. After adjustment of pH to 2.0, the wine samples were extracted with diethyl ether and the residues, after evaporation of solvent, were dissolved in $CDCl_3$ and subjected to 1D ^{1}H NMR spectroscopy. The data was analyzed using multivariate statistical analysis, where it was found that signals corresponding to acetaldehyde, diethyl oxalate, acetoin, L-alanine, diethyl malonate, diethyl oxalate, diethyl succinate and ethyl acetate (all 1.8–1.1 ppm) differentiated between micro-oxygenated and control samples.

Nonier *et al.* (2007) used 1D ^{1}H NMR spectroscopy (and other methods) to study the differences in rates of reaction over a period of 54 days at 20 °C (in the dark) between (+)-catechin and a variety of aldehydes that were known to be derived from toasted oak. Thus the model wine solution containing these substrates was meant to simulate the production of pigments in white wine aging in oak casks. NMR experiments in DCl/D_2O clearly showed that the catechin reactive sites were at C6 and C8. Likewise, disappearance

of the aldehyde proton signal of furfuraldehyde and the C6, C8 protons of (+)-catechin during reaction between these two species, indicated that adducts were formed via catechin C6, C8 and aldehyde, possibly as bridged dimers like those formed between catechin and acetaldehyde. (+)-Catechin was found to react with furfuraldehyde, 5-hydroxymethylfurfuraldehyde and 5-methylfurfuraldehyde much more readily than with the phenolic aldehydes, syringaldehyde and vanillin. UV-visible spectroscopy and HPLC showed that the increase in solution absorbance at 440 nm was closely linked to the formation of products formed via reaction between catechin and aldehydes.

Solid state ^{13}C NMR (with cross polarization and magic angle spinning, CP/MAS) has been used to study the natural lacquer-like pigmented deposits that adhere to the inner surface of around 5–10% of bottles of aged red wines (Waters *et al.*, 1994). The deposit often covers the entire inner surface, and although it does not appear to influence the wine quality, is usually quite firm and is easy to pour out the wine from (with due care), it is nonetheless usually regarded as a negative commercial attribute. Waters *et al.* (1994) found the ^{13}C CP/MAS NMR spectra of deposits to include peaks at 174 ppm (C=O of peptides), 155–116 ppm (O-aryl and aryl region of aromatic amino acids such as tyrosine and histidine, as well as polyphenol carbon atoms), 76–56 ppm (sugar carbon atoms) and 40–20 ppm (aliphatic amino acid side chain carbon atoms). These results suggested that the deposit is composed largely of a protein–polyphenol complex, which also contains sugar molecules, probably as glycoside moieties of the polyphenol polymers. Amino acid analysis of the deposits following acid hydrolysis indicated that ~22% of the mass of the deposit could be accounted for by proteinaceous material, the remainder probably being all polyphenols (with glycoside moieties). The major amino acids in the deposit were (in order of importance) glycine, aspartic acid/asparagines, serine and threonine.

Polyphenols are important components of foodstuffs and alcoholic beverages that are generally thought to give health benefits (Chapters 5.5 and 5.8). Apart from possible direct antioxidant properties, it is believed that they act by forming complexes with proteins, thereby influencing cell signaling pathways. Solid state ^{2}H and ^{31}P NMR spectroscopies have been used to study the *in vitro* interaction between 4-deuteroepigallocatechin gallate and phospholipid bilayers (dimyristoylphosphatidylcholine liposomes) (Kajiya *et al.*, 2008), where it was shown that not only is this polyphenol incorporated into the bilayer, but it is able to move freely on the membrane surface. Hence in an *in vivo* situation, the polyphenol would be able to find its specific binding protein, known to exist in membranes. Epigallocatechin gallate is a tea polyphenol, and although it has been shown that the extent and type of *in vitro* interaction between flavan-3-ols and lipid bilayers is dependent on structure, amongst other factors, it is not unreasonable to suppose that similar interactions occur between cider or wine polyphenols and membranes.

Site specific isotopic fractionation-NMR (SNIF-NMR) is a version of deuterium (^{2}H) NMR developed by G.J. Martin and coworkers in the 1980s that has found particular application in the determination of origin of alcoholic beverages (and other foodstuffs) and in the detection of fraud. Natural deuterium is not distributed uniformly throughout the world and natural hydrogen isotopic distributions in compounds (say, water and plant sugars) are the result of a number of biochemical, chemical and physical processes. For example, the ^{2}H content of water ranges from ~90 ppm in Antarctica to ~160 ppm at the equator, because water vapor from warmer climates is richer in H_2O than HDO, the resulting precipitation in cooler regions is also depleted of HDO. Since plants synthesize sugars from water and carbon dioxide, both geographical and meteorological factors influence the D/H ratio in the sugars. Furthermore, given fixed geographical and meteorological factors, the D/H ratio in plant sugars depends on the biosynthetic pathway from water and carbon dioxide utilized by the plant: the Calvin (C3) or Hatch–Slack (C4) cycle (Section 4.4.5). Hence the D/H ratio in plant sugars is a function of the plant biochemistry, geographical and meteorological factors, and so could be used to identify botanical and geographical origin. This of course can be determined by isotope ratio mass spectrometry (IRMS) (Section 4.4.5), but this technique is not site specific and only gives a global value of D/H for a particular compound.

Deuterium NMR is capable of giving site specific values of D/H, but the procedure is rather complex for molecules such as plant sugars, which contain relatively large numbers of magnetically different hydrogen atoms. Fortunately, the microbiological conversion of sugars to ethanol (alcoholic fermentation) and subsequent processes such as distillation do not significantly influence the individual D/H ratios, and it has been shown that measurements on ethanol deuterium sites CH_3CHDOH (methylene) and CH_2DCH_2OH (methyl) can be directly related to those in the original sugars that produced the ethanol (Martin *et al.*, 1983). The deuteroxy (CH_3CH_2OD) signal overlaps with that of HOD and hence is usually ignored.

SNIF ^{2}H NMR (e.g. NMR frequency 61.4 MHz at 9.4 Tesla) experiments on alcoholic drinks are usually carried out with WALTZ proton decoupling, a fluorine locking device and sometimes with an accurately known mixture of C_6D_6 and C_6H_6 as reference in a concentric capillary NMR tube. Alternatively, a standard of known isotope ratio (e.g. tetramethylurea; D/H = 136 ppm) can be used in place of hexadeuterobenzene and hexafluorobenzene can be used as the locking material. Because of the inherent magnetic insensitivity of the ^{2}H nucleus, and resulting poor signal to noise ratio, SNIF-NMR should normally be used on neat distilled beverages; wines and beers need careful distillation in a spinning band column (Martin *et al.*, 1988).

The parameter R is often used to express the relative ^{2}H distribution in the ethyl fragment of ethanol, as given by Equation 4.4.4.

$$R = \frac{3h(\mathrm{II})}{h(\mathrm{I})} \tag{4.4.4}$$

Here h(II) is the height of the methylene ^{2}H signal and h(I) is the height of the methyl signal, with respect to the height of the reference signal. Water involved in the fermentation has a significant effect on the ^{2}H content of the methylene site, whereas the sugar is the main influence on the deuterium content of the methyl site of ethanol. If the deuterium in ethanol were distributed statistically (if there was no isotope fractionation), h(II)/h(I) would be 2/3, giving R a value of 2.0. In practice, R varies between ~2.20 and ~2.80 and can be used to differentiate some, but not all, sources of sugar in alcoholic drinks, because of data overlap. A new parameter C describing relative deuterium concentration is defined by Equation 4.4.5 (where h(std) is the peak height of the standard and f is a correction factor for ethanol concentration).

$$C = \frac{h(\mathrm{I}) + h(\mathrm{II})}{h(\mathrm{std})\, f} \tag{4.4.5}$$

A combination of R and C values can be used to distinguish between ethanol in spirits derived from different sugar sources, as shown in Table 4.4.1.

Alternatively, the D/H ratios can be calculated via Equation 4.4.6 and are expressed as ppm (Zhang *et al.*, 1998).

$$\left(\frac{D}{H}\right)_i = \frac{N_{\mathrm{D},i}}{P_i N_{\mathrm{H}}} \tag{4.4.6}$$

Here, D_i and H_i are the numbers of deuterium and protium atoms at site i, $N_{D,i}$ is the number of monodeuterated isotopomers of type i, N_H is the number of fully protonated molecules and P_i is the number of equivalent hydrogen positions at site i. A curve fitting algorithm based on a complex least squares treatment of the ^{2}H signal was used for the quantitative evaluation of monodeuterated isotopomers.

SNIF-NMR has been successful in the detection of fraud and the authentication of origin (Ogrinc *et al.*, 2003). SNIF-NMR is usually based on the measurement of ^{2}H/^{1}H ratios at the methyl and methylene sites of ethanol, after it has been distilled from the wine, but methylene sites of glycerol after distillation, solvent

Table 4.4.1 *Relative deuterium data for ethanol derived from different sugar sources*

Sample	Sugar source of ethanol	Mean R (with typical standard deviation)	Mean C (with typical standard deviation)
Synthetic alcohol	Fossil fuel	2.262 (0.007)	1.464 (0.003)
Cognac	Grape	2.528 (0.007)	1.173 (0.004)
Applejack	Apple	2.552 (0.007)	1.140 (0.004)
Cachaca and rum	Sugar cane	2.312 (0.006)	1.232 (0.004)
Tequila	Agave	2.235 (0.001)	1.203 (0.001)
Vodka*	Potato	2.697 (0.011)	1.083 (0.004)

*Most vodka is produced from cereals, especially wheat
R and C are defined by Equations 4.4.4 and 4.4.5

extraction and triacetylation can also be used (Zhang *et al.*, 1998). Combined with IRMS, SNIF-NMR can be used to detect chaptalization that used sugar from either C_3 plants (e.g. cane sugar) or C_4 plants (e.g. beet sugar). By combining $\delta^{13}C$ values (from IRMS – see Section 4.4.5) with the normalized hydrogen isotope ratio – $(D/H)_I$ – of wine ethanol, an 'adulteration triangle' can be constructed (Figure 4.4.16).

Similarly, by analyzing the data from SNIF-NMR and IRMS experiments, using a variety of statistical methods, it has been possible to check the authenticity of large numbers of samples from a number of wine growing regions (Košir *et al.*, 2001; Ogrinc *et al.*, 2003).

Electron spin resonance (ESR) is a sister technique to NMR, involving transitions of unpaired electron spins in an applied magnetic field when subjected to microwave (rather than radio wave) radiation. It has been used for some time to predict the flavor stability of beers by observing the production of free radicals (species with an unpaired electron) in the presence of a spin trapping agent, such as *N-tert*-butyl-α-phenylnitrone (Nyborg *et al.*, 1999). High concentrations of free radicals in beer are associated with staling, part of which is caused by oxidation and which is characterized by a deleterious change in flavor profile (Section 2.6.12). Beer contains a number of compounds (e.g. melanoidins, phenols and proteins) that are able to remove highly

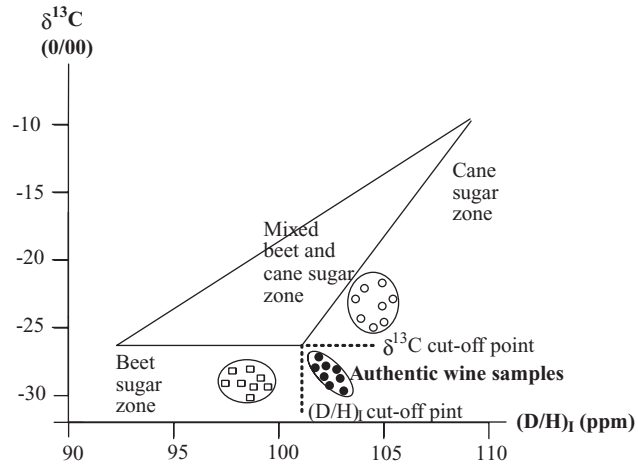


Figure 4.4.16 *Adulteration triangle in a plot of $\delta^{13}C$ versus $(D/H)_I$ for wine ethanol. □ wine samples made with added beet sugar; ○ wine samples made with added cane sugar. Ogrinc* et al. *(2003)*

reactive oxygen and carbon radicals by forming relatively stable free radicals (Section 5.8.7), thus acting as radical scavengers or antioxidants. Spin trapping agents, such as nitrones and *N*-oxides, when added to beer react with free radicals that are in excess of the radical scavenging ability of the beer, to give a stable nitrodioxide or nitroxide radical whose increase in signal strength can be monitored in the ESR spectrometer. This occurs after a certain time lag period; the shorter this period, the more likely (and the quicker) the beer will undergo staling and the lower the flavor stability. If this happens, the brewery must find and put right the cause of increased radical formation; perhaps by adjusting the process to give lower levels of dissolved oxygen and lower levels of metal cations (especially Fe and Mn).

4.4.2 Infrared Spectroscopy

The infrared region of the electromagnetic spectrum is divided for convenience into three subregions according to arbitrary (and therefore approximate) wavelength ranges. These are near, mid and far infrared regions – named by their relationship to the visible region. At the low energy end, the far infrared region ($\sim$400–10 cm^{-1} or 1000–30 μm) lies adjacent to the microwave region and is useful for rotational spectroscopy. The mid infrared region ($\sim$4000–400 cm^{-1} or 30–2.5 μm) is used to study the fundamental vibrations and associated rotational-vibrational structure; this is the region most widely used to study organic functional groups, such as those in the component molecules of alcoholic beverages. The high energy near infrared region ($\sim$14000–4000 cm^{-1}, 2.5–0.8 μm or 2500–800 nm) lies next to the visible region and can be used to study overtone or harmonic vibrations. For detailed discussions on IR spectroscopy, the reader is referred to Lambert *et al.* (1998) and Silverstein *et al.* (2005).

The bonds in molecules are constantly vibrating, that is to say, stretching or bending (deforming), so that when a bond in a molecule absorbs a quantum of infrared radiation (whose frequency corresponds to the vibrational frequency of the bond) that bond vibrates with greater amplitude. This causes a vibrational transition (from lower to higher vibrational energy level), but only occurs if the vibrational motion is accompanied by a change in the permanent dipole moment of the bond (as in nonsymmetric bonds) because the changing electric dipole needs to interact with the sinusoidally changing electromagnetic field of the incident infrared photon in order for a vibrational transition to occur. The energy of the vibrational transition of a bond in a molecule (and hence the wavelength of the IR radiation absorbed) depends upon the following:

- Atomic identity (C–H, O–H, etc.)
- The types of bonds between (single, double, triple) between atoms
- Intramolecular interactions (the molecular neighborhood of the vibrating bond)
- Intermolecular interactions (state of matter of the sample).

This is the basis of infrared spectroscopy. Nowadays, the big majority of IR analyses are carried out using Fourier transform (FT) instruments rather than dispersive instruments (the IR equivalent of continuous wave NMR instruments).

The FT instrument is rather more complex in that it uses a Michaelson interferometer rather than a monochromator and it also requires a computer to convert the interference patterns, by Fourier transform, into conventional frequency (or wavelength) spectra. The interferometer consists of a beam splitter and two mirrors, one fixed and the other moveable (Figure 4.4.17(i)). The beam splitter transmits half the radiation and reflects the other half when incident radiation strikes it at a 45° angle (Figure 4.4.17(ii)). The transmitted and reflected beams from the beam splitter strike the two mirrors that are oriented perpendicular to each beam, and are reflected back to the beam splitter. When only one mirror is in place (Figure 4.4.17(iii)), the beam reflected from the mirror returns to the beam splitter, which sends half the radiation to the detector and

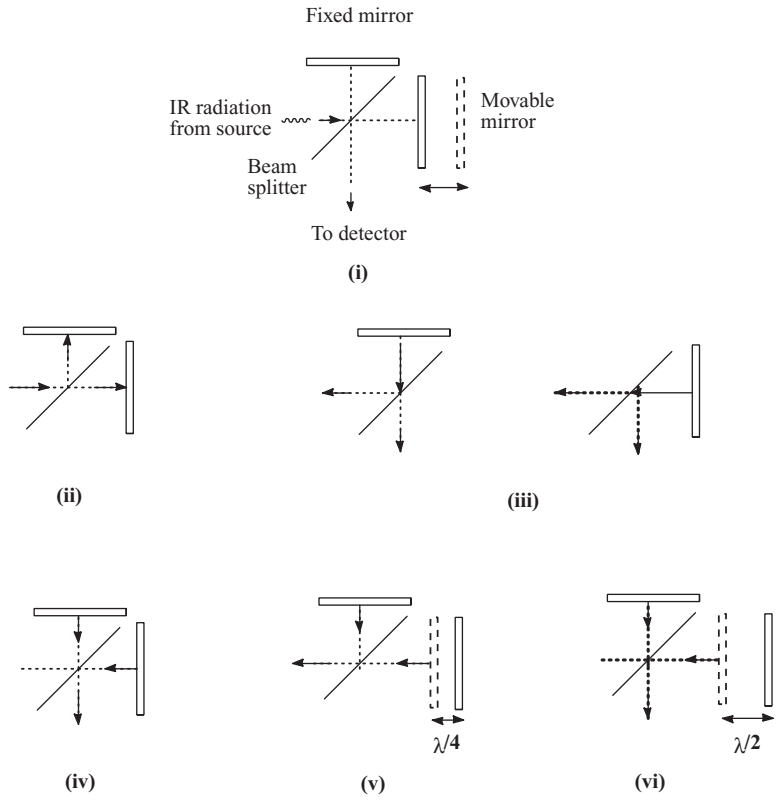


Figure 4.4.17 *FTIR spectroscopy: the interferometer section of an FTIR spectrometer*

the other half back to the source. If both mirrors are in place (see Figure 4.4.17(iv)–(vi)), interference occurs at the beam splitter where there is overlap of the radiation from the two mirrors. When the two mirrors are equidistant from the beam splitter (the 'retardation' is zero), for all wavelengths, constructive interference occurs for the beam passing to the detector, whereas destructive interference occurs to the beam going back to the detector (Figure 4.4.17(iv)). When the retardation is λ/2 (Figure 4.4.17(v)), when the moving mirror has moved λ/4 away from the equidistant position, destructive interference occurs to the beam going to the detector. Conversely, when the retardation is λ (corresponding to the position of the moving mirror at λ/2 – see Figure 4.4.17(vi)), constructive interference occurs to the beam passing to the detector. The detector thus senses a cosine variation of intensity that depends on the retardation and a plot of such a response versus retardation is known as an interferogram.

In practice, the interferogram of the polychromatic source can be considered as the sum of all the cosine waves from each wavelength and they are each detected simultaneously. This interferogram, saved in the computer memory, is converted to a conventional spectrum by Fourier transform. Because several 'scans' can be performed, the signal to noise ratio is superior to that of a dispersive instrument.

Several sampling techniques are available for FTIR spectroscopic analysis, depending on the physical state of the sample. Liquid samples can be sandwiched between two plates of a highly pure salt (commonly sodium chloride, potassium bromide or calcium fluoride). The plates are transparent to the infrared light and hence will contribute nothing to the spectrum, but most salt plates are highly soluble in water, so the sample and

washing reagents must not contain water. Solid samples can be finely ground with pure anhydrous KBr, or made into a slurry (or mull) with a liquid hydrocarbon such as nujol, or alternatively dissolved in a suitable solvent and run in a solution cell made of NaCl, KBr or other material. In the latter two cases (and others) the spectrum of the mulling agent or solvent is subtracted by computer from the spectrum of the sample.

Both solid and liquid samples can be measured in close contact to a crystal of high refractive index, such as diamond, germanium or zinc selenide. This technique is called attenuated total reflectance (ATR) and uses a property of total internal reflection called the evanescent wave. A beam of infrared light passes through the ATR crystal so that it is reflected at least once off the internal surface in contact with the sample. This reflection forms the evanescent wave which extends into the sample by a few micrometers. The beam is then collected by a detector as it exits the crystal.

Standard bench top FTIR instruments have been used or adapted successfully for use in alcoholic beverage analysis, although a dedicated spectrometer made by Foss Electrics (Denmark) meant for routine screening of samples is used in many analytical laboratories. The WineScan FT 120 instrument is usually equipped with an autosampler that pumps the sample through a cuvette of optical pathlength 37 μm. Zeroing and cleaning are built into the automation program, where it is possible to achieve an analysis time of 30 s per sample. However, in the above instrument, the number of scans generated for each sample, the selection of spectral regions (5012–926 cm^{-1}) and the data processing are fixed by the manufacturer.

The application of FTIR spectroscopy to alcoholic beverage analysis is extensive, especially during the past decade, with the advent of increasingly sophisticated spectrometric, data treatment and chemometric software. Because of its speed and simplicity, FTIR has been used for quality control or authentication of grape must (Swanepoel *et al.*, 2007), wine (Palma and Barroso, 2002; Cocciardi *et al.*, 2005; Urbano Cuadrado *et al.,* 2005; Tarantilis *et al.*, 2008; Garde-Cerdán *et al.*, 2010), distilled spirits (Palma and Barroso, 2002; Cocciardi *et al.*, 2005; Lachenmeier *et al.*, 2005; Lachenmeier, 2007), brewer's grits (Pojić *et al.*, 2009), wort (Titze *et al.*, 2009), beer (Llario *et al.*, 2006; Lachenmeier, 2007), Chinese rice wine (Yu *et al.*, 2009) and peat (for the production of Scotch whisky) (Chapman *et al.*, 2001; Harrison *et al.*, 2006). Similarly, FTIR has been successful in the quantitative determination of specific wine components (Gorinstein *et al.*, 1992; Vonach *et al.*, 1998; Moreira and Santos, 2004; Nieuwoudt *et al.*, 2004).

Other specific components, such as anthocyanins and polyphenols in wine (Fernández and Agosin, 2007; Soriano *et al.*, 2007; Jensen *et al.*, 2008) and carbohydrates in beer and wine (Coimbra *et al.*, 2002; 2005; Boulet *et al.*, 2007; Niu *et al.*, 2008) have received attention from FTIR spectroscopy. Both the near infrared and mid infrared regions are the widely used for the analysis of alcoholic drinks, unlike other areas of food science or agricultural science, where NIR spectrocopy tends to dominate.

The NIR region of the electromagnetic spectrum, situated between the visible and mid infrared regions, covers the wavelength range between *ca*. 780 nm and 2526 nm. Recently, NIR reflectance spectrometers with capability extending into the visible region have become available for research and industrial use, where they have proved to be of considerable value (McCaig, 2002).

Quality control methods that use FTIR spectroscopy generally require the construction of a database of optimized calibration models. Statistical analyses are used to correlate these data with IR data for the same set and to compute regression models and errors of prediction for the chosen parameters (e.g. total acidity, total sugars, percentage ethanol by volume, etc.). Validation is then carried out on a different set of samples. Differentiation/determination of origin methods generally follow a similar route, often using principal component analysis and other statistical analyses of a selected region of the IR data for each compound. It is in these areas that FTIR has proved to be an excellent screening method; it is fast, easy (with no or little sample preparation) and capable of indicating the possibility of fraud or inadequate quality. However, it is an indirect method in that it uses a calibration based on previously determined results and hence it generally lacks the confidence for the presentation of evidence of the fraudulent activities of alcoholic drinks producers. In these cases, reference or official methods (which are invariably lengthier and more demanding)

should be used to confirm those from FTIR analysis. Given the considerable advantages mentioned above, it is likely that FTIR spectroscopy will become more widely used for routine quality control screening in the alcoholic beverage and other food industries.

There appears to be only one recent report on the application of FTIR spectroscopy to the quality control of grape must (Swanepoel *et al.*, 2007). Here, total soluble solids (a measure of the sugar content, expressed as °Brix), pH and titratable acidity (expressed as g/l tartaric acid) were measured using an exploratory data analysis of the IR spectra of 1170 South African must samples (647 for °Brix, 252 for pH and 271 for titratable acidity). After optimization, the standard error of prediction was 0.46 °Brix, 0.05 pH and 0.49 g/l tartaric acid, thus indicating that FTIR is a useful method for the assessment of major grape must parameters, as well as for general quality control. Although these three parameters can be easily and rapidly measured by other methods, three separate experiments are required, whereas FTIR spectroscopy is able to measure all three rapidly in a single experiment.

Palma and Barroso (2002) were able to use FTIR data for the differentiation and classification of Sherries and Jerez brandies according to maturation age, as well as for the differentiation and classification of brandies from France, South Africa and Spain. It was possible to differentiate fino Sherries from all six 'scales' or levels of aging of a single aging batch of criaderas and solera (Section 2.10.2). Likewise Jerez brandies of three ages could be differentiated and these could be distinguished from French and South African brandies. The best differentiation of Sherries was obtained by utilizing the part of the IR spectrum that differed most between samples, the region ~930–3000 cm^{-1}. The major statistical techniques that were applied to the data were principal components analysis (PCA), linear discriminant analysis (LDA) and partial least squares regression (PLSR) analysis. The first principal component was related to the aging process, whereas the second principal component recorded another reason for the differences between sample spectra.

Near IR as well as mid IR spectroscopy was used to determine a variety of parameters (including % ABV, total acidity, total sulfur dioxide and lactic acid content) for 180 wines from different regions of Spain and from different vine varieties (Urbano Cuadrado *et al.*, 2005). The NIR spectra were obtained at 400–2500 nm using folded transmission through a ring cup with 0.1 mm path length. Reflection was provided by a gold surface placed at the bottom of the cup. The mid IR spectra were obtained at 3000–800 cm^{-1}, using attenuated total reflectance (ATR with a ZnSe crystal). PCA was used to correlate wine parameters for the samples (measured by a variety of methods) with the optimized NIR and/or MIR spectral data. After assessment of outliers, PLSR was used to generate correlation equations with a determination coefficient (R^2) and then validation was performed to generate standard error prediction. In general, NIR spectroscopy provided better results because of more favorable signal to noise ratios. However, use of both NIR and MIR data improved the determination of glycerol and total sulfur dioxide.

In the same year as above, Cocciardi *et al.* reported the application of a new FTIR method for the analysis of wine and distilled spirits. The method used a bench top FTIR instrument fitted with a single bounce attenuated total reflectance (SB-ATR) sample handling accessory (as opposed to a CaF_2 transmission cell or traditional multireflection ATR). The mid IR region was used for analysis (within 3000–850 cm^{-1}; the actual range used depended on the component or property to be analyzed) The new method applied to the determination of ethanol in distilled spirits (11 samples) gave results that were comparable with those obtained from transmittance FTIR (with a flow through cell) and superior to those obtained with FT-NIR. A PLS calibration of a 72 sample database for the prediction of 11 different components and wine parameters by SB-ATR/FTMIR spectroscopy was developed and validated using an independent set of 77 samples. Good coefficients of correlation (R^2) between reference and predicted values for the validation set were obtained for most parameters, except citric acid, total sulfur dioxide and volatile acids contents. It is possible that combined use of NIR and MIR data may have improved the correlation with total sulfur dioxide content, as for Urbano Cuadrado *et al.* (2005).

The highest quality tequila ('100% agave') must be bottled in Mexico, but the 'mixed' version (which can be made by distilling maguey juice with up to 49% (w:v) of added sugar) can be exported in bulk and bottled in foreign countries (Section 3.5.5). Although Tequila is protected under the North American Free Trade Agreement (NAFTA), adulteration and fraud (e.g. via addition of other types of alcohol, such as grain spirits, or by mixing different types of tequila) are commonplace. Principal component analysis was applied to ion chromatographic data (Cl^-, NO_3^-, SO_4^{2-}, $CH_3CO_2^-$ and oxalate $(COO^-)_2$ content) and FTMIR data from the region 4996–930 cm^{-1} (omitting the spectral regions of water absorption: 3696–2971 cm^{-1} and 1887–1447 cm^{-1}) (Lachenmeier *et al.*, 2005). Differentiation between Mexican bottlings (100% agave Tequila) and foreign bottlings (mixed Tequila) was observed, but no discrimination between categories of Tequila (añejo, blanco, reposado etc.) was possible from this data. Interestingly, the Mexican bottlings had high acetate contents and invariably possessed oxalate, whereas the foreign bottlings had lower acetate levels, higher chloride and sulfate levels and no oxalate. On the other hand, 100% Tequilas possessed significantly higher concentrations of methanol and isobutanol than mixed Tequilas.

Nieuwoudt *et al.* (2006) used FTIR to evaluate the major fermentation products of a variety of pure *Saccharomyces cerevisiae* cultures. Calibration equations for the quantification of ethanol, glucose, glycerol, reducing sugar and volatile acidity contents of South African Chenin Blanc fermenting musts and synthetic musts were derived from FTIR spectroscopy by the use of PCA and other statistical analyses of the data. A high degree of prediction (that is, with low standard error of prediction) were obtained for all the chosen parameters/components and the accuracies compared favorably with those associated with the respective reference methods used for the quantification of these parameters/components. Thus, FTIR spectroscopy was shown to provide a rapid and reliable screen for the fermentative properties of wine yeast strains.

Earlier, the same group had used PCA applied to FTIR for the design of calibration sets in order to predict glycerol levels in wine and to detect and classify outlier samples (Nieuwoudt *et al.*, 2004). Emphasis here was placed on the use of PCA to identify the major sources of variation in the FTIR spectra of wine samples and on the early detection and classification of poorly predicted and outlier samples. Judicious choice of calibration sets was considered necessary in order to design calibration sets that are robust and, at the same time, accurate.

The last mentioned aspects (above) were also investigated by Moreira and Santos (2004). Here poor accuracy of prediction (e.g. for total acid content, volatile acid content and total sugar content of dry wines) was attributed to spectroscopic interferences. Strongly absorbing bands due to abundant components, such as ethanol, organic acids and others produced major interferences in the analysis of low concentration components such as volatile acids and sugars in dry wines. The probable reason for poor prediction lies in the choice of samples for the calibration set; for example, sweet wines (with high sugar content) and dry wines (with low sugar content) were in the same set. It was suggested that a possible way of minimizing this problem would be to perform specific calibrations using different sample sets for different wine types (e.g. red, white, dry and sweet wines) thereby minimizing variation between samples and decreasing the extent of spectroscopic interference.

Although NIR spectroscopy has been used for some time for the determination of protein and water content, as well as the amino acid composition of barley and malt, more recent work has involved the application of information gained from wavelength studies of isolated barley hordein in order to improve prediction and reduce error in the estimation of total protein in barley and malt (Fox *et al.*, 2002). Strong correlations for grain protein (as determined by the Dumas method – see Section 4.6.3) and NIR wavelengths were found in the NIR region at six wavelengths between 1116 and 2300 nm (inclusive), indicating the usefulness of choosing a standard closely related to the parameter of interest in improving calibration robustness and hence the predictive ability of the method. Using a calibration set of 44 and a validation set of 19, multiple linear regression and partial least squares regression analysis applied to the results gave standard errors of prediction between 0.15% and 0.27% for barley and malt protein content. Clear differences could be seen in the NIR

spectra of barley and malted barley hordein, the largest difference occurring in the 1920–1940 nm region probably resulting from hydrolysis of hordein during malting and loss of moisture during kilning.

More recent work has suggested IR spectroscopy can be used for routine process control regarding the fat content of brewer's grits, an important consideration for brewers who use a high adjunct grain bill (Pojić *et al.*, 2009). Extensive statistical analysis showed the NIR method compared favorably with a standard chemical method, which cannot be readily applied to process control because of its high demand on time.

Similarly, FTIR spectroscopy has recently been evaluated for its ability to determine important quality parameters of wort (Titze *et al.*, 2009). All parameters relating to density could be determined with high accuracy, after accurate calibration using 256 'Congress' (EBC) wort samples. Additionally, total nitrogen content could be determined with good accuracy, but the method was of limited use in the determination of protein related parameters, because (for example) soluble nitrogen content was found to be dependent upon the harvest year during calibration of the that parameter. Other parameters, such as viscosity and color, could not be determined by this method. However, repeatability (over 130 days) was good and it was judged that FTIR spectroscopy has much promise as a routine wort and beer analyzer, saving both time and money.

FTIR spectroscopy has also been used for the rapid screening of beer samples (Duarte *et al.,* 2004; Llario *et al.,* 2006; Lachenmeier, 2007 and references therein), which must be degased prior to analysis. Llario *et al*. (2006) used ATR-FTIR spectroscopy to assess the important quality parameters of % ABV (by ethanol estimation), original extract and real extract (by carbohydrate estimation) (Section 2.6.2), using optimized spectral ranges for each component/parameter in order to minimize both prediction and calibration errors. Choice of ATR sampling minimized the influence on the overall spectra by the absorptions due to water molecules. The most important spectral region for all the parameters was 1200–1000 cm^{-1}, corresponding to C–O stretches. The calibration and validation sets were mostly pale bottom-fermented beers, whence the prediction errors were 1.5% for ethanol, 2.8% for real extract and 1.9% for original extract. Duarte *et al.* (2004), on the other hand, used FTIR only for classification of beers, using calibration and validation sets that included a wider variation of beers: bottom-fermented (Section 2.6.6), top-fermented (Section 2.6.5) and alcohol free beers (Chapter 2.13).

Lachenmeier (2007) used transmittance FTIR combined with multivariate data analysis as a rapid screen for quality control and assessment of authenticity of a range of spirits (including German fruit spirits) and beers (including bottom- and top-fermented beers). A partial least squares method was used to correlate the FTIR spectral data (which used the region 926–5012 cm^{-1}, but excluded the regions containing water absorptions) with results from reference methods. Validation using an independent set of samples showed strong correlations with reference values and high accuracies were obtained for the spirit parameters density, ethanol, ethyl acetate, isobutanol, methanol, 2- and 3-methyl-1-butanol and 1-propanol. Similar high accuracies were achieved for the beer parameters, density, ethanol content, lactic acid content and original gravity. Other beer parameters, pH, bitterness unit and color (EBC), showed lesser correlation and accuracy, but their determinations were still useful in screening analyses. Once again it was concluded that FTIR is useful for quality control of alcoholic beverages because quantitative assessment of important components or parameters as well as chemometric classification are simultaneously achievable.

In recent years, NIR spectroscopy (extended into the visible region) has been combined with fiber optics for the noninvasive quality control assessment of alcoholic drinks in intact bottles. For example, Nordon *et al.* (2005) have determined alcohol content in 200 ml flat clear glass bottles, Cozzolino *et al.* (2007) have assessed alcohol content, total SO_2, free SO_2 and pH of commercial wine in intact bottle, and Yu *et al.* (2007) have used this system to discriminate amongst rice wine samples in square brown glass bottles according to age. The specific spectrometer systems set up by these authors are suitable for on site wine quality determination, but the feasibility of visible-near infrared (Vis-NIR) spectroscopy and statistical analysis using least squares support vector machines (LS-SVM) for on line quality control determination of Chinese rice wine composition has been investigated by Yu *et al.* (2009).

Yu *et al.* (2009) designed a circle light fiber spectrometer system to determine transreflectance spectra of rice wine samples in unlabeled, sealed brown glass bottles on a conveyer belt. Statistical equations were established between reference data (% ABV, total titratable acidity and pH) and Vis-NIR spectra by LS-SVM. The statistical analysis method compared favorably with more widely used methods such as PCA, PCR and PLSR, with correlation coefficients for validation (r_{val}) of 0.915, 0.888 and 0.872, and lower root mean square error of validation (RMSEP) of 0.168 (% v:v), 0.146 (g/l) and 0.033 for the above-mentioned parameters. Based on the results, it was concluded that the Vis-NIR spectrometer system was suitable for on line wine quality determination, and LS-SVM was a reliable multivariate method for NIR analysis.

Using the methodologies described in the previous paragraphs, FTIR spectroscopy can be used to discriminate between wine samples of different grape varieties and also between red wines that have been subjected to different aging régimes. Tarantilis *et al.* (2008) have used similar techniques to classify Greek red wines according to grape variety using C-18 SPE extracts (Section 4.2.4) containing anthocyanins and polyphenols. AT/FTIR was used in the region 1800–900 cm^{-1} and spectra of extracts from wines of the varieties Agiogitiko, Merlot and Xinomavro were used in a library or data bank as 'fingerprints' for comparison with spectra of SPE extracts of unknown wines. The method was able to discriminate Greek wines made from these three varieties of *V. vinifera*.

NIR spectroscopy has been used to discriminate between Spanish red wines according to the extent of aging in oak casks: crianza $<$ reserva $<$ gran reserve; and according to type of oak cask used: American, French or combination (Garde-Cerdán *et al.*, 2010). NIR spectra of 510 samples and the concentrations of eight oak volatile compounds and ethylphenols (determined by SBSE-GC-MS; see Sections 4.2.4 and 4.3.2) were used as a database. Samples were grouped according to oak type used and also according to storage time, and partial least squares regression (PLSR) gave good calibration statistics ($R^2 > 0.86$) and allowed both discrimination of fresh samples and prediction of desirable oak volatile content and undesirable ethylphenol content.

All of the above examples involved measuring the IR spectra of alcoholic beverages, (or in one case, grape must), where the emphasis was on quality control, confirmation of origin (differentiation between samples according to type or origin) or prediction of component concentration. In the next examples, more emphasis was placed on quantitative determination of particular components of beverages, rather than on classification or discrimination. In an earlier report (Gorinstein *et al.*, 1992), FTIR (KBr discs; 4000–400 cm^{-1}) was used on lyophilized Israeli white wine samples (ethanol was removed at reduced pressure and the residue was freeze dried) to determine a number of components, including phenolic compounds. Polar phase HPLC, gel filtration and electrophoretic techniques (for protein assay) were also used in this study.

Real time HPLC-FTIR was applied for the first time to the analysis of alcohols, carbohydrates and organic acids in six Austrian wines (Vonach *et al.*, 1998). An anion exchange resin was used as the stationary phase, with 5 mM aqueous sulfuric acid as the mobile phase. A transmission flow cell of 25 μm pathlength and CaF_2 windows protected by a layer of low density poly(ethylene) from exposure to acidic solutions. The presence of a low wave pass filter allowed measurements in the spectral region 1600–900 cm^{-1}, where C–O stretches of alcohols and carbohydrates ($\sim$1050 cm^{-1}) and of carboxylic acids (1260 cm^{-1}) occur. The C=O stretching region of carboxylic acids ($\sim$1740–1700 cm^{-1}) was not used because of interference from water absorption at 1640 cm^{-1}. The results compared well with those from official methods, usually enzymic methods (a distillation method for ethanol and a gravimetric procedure for tartaric acid).

More recently, FTNIR (transmission, in the spectral range 800–2500 nm) has been used to identify and quantify the major four oligosaccharides (isomaltose, isomaltotriose, maltose and panose) in Chinese rice wine (Section 2.7.1) (Niu *et al.*, 2008). The reference method was HPLC in ion exchange mode, using pulsed amperometric detection, and the database was derived from the analysis of 40 rice wines from vintages spanning 1996–2005. PLSR with cross validation was used to build the calibration model. Although the calibration accuracy was satisfactory for the four sugars, the validation accuracy was not, indicating that

further studies are needed, possibly using a wider database to improve the specificity, prediction, accuracy and robustness of the models.

FTIR spectroscopy has been particularly focused on the identification and quantification of phenolic compounds in wine and in the analysis of carbohydrates in a number of alcoholic beverages. Fernandez and Agosin (2007) developed an FT/MIR spectrometric chemometric technique for the quantitative analysis of red wine tannins, via C-18 SPE extraction and elution with methanol. The IR sampling mode was ATR, whereby the extract (50 μl) was added to the ZnSe crystal and the methanol was evaporated in vacuum before running the spectrum. Calibration models were constructed using protein precipitation and phloroglucinolysis as analytical reference standards. After spectra preprocessing, different partial least squares models were tested, where PLS regression with full range second derivative spectra gave the most accurate determination of tannin concentration (root mean standard error of calibration = 6.7%, root mean standard error of prediction = 10.3%, $R = 0.958$). Moreover, the mean degree of polymerization of proanthocyanidins in the extracts could be estimated accurately by a similar method. It is expected that these models for tannins, along with other MIR calibrations for other important wine phenolic compounds, still to be developed, will in future be used as routine analyses in wineries.

The presence of higher concentration/strongly absorbing species (such as ethanol and organic acids) tends to complicate the direct analysis of phenolic compounds in wine by FTIR spectroscopy. Nevertheless, by discarding noninformative, 'noisy' spectral regions, Jensen *et al.* (2008) were able to limit the mid infrared spectral widow to 1485–1425 cm^{-1} and 1060–995 cm^{-1} for optimum quantitative analysis of both grape based polyphenols (largely proanthocyanidins) and hydrolyzable tannins derived from maturation in oak casks. The most important spectral regions for the analysis of wine tannins were deduced by the application of four different variable selection methods, including an iterative backward elimination of changeable size intervals partial least squares program, to standard 5012–926 cm^{-1} spectra. To help with the quantification of tannins in wine, the FTIR spectra of 20% aqueous ethanol solutions of (+)-catechin, grape tannin and oak tannin were recorded, along with a red wine spiked with different levels of grape tannin. The calibration model (using 128 red wines of wide variation) was based on the results of a tannin assay according to a slightly modified method of Harbertson *et al.* (2003) (Section 4.4.3). The four variable selection methods resulted in spectral regions that were used to develop calibration models and gave improved prediction of tannin content: RMSEP = 69–79 mg catechin equivalents (CE)/l; $r = 0.93$–0.94 compared with that derived from a calibration model developed using all variables: RMSEP = 115 CE/l; $r = 0.87$).

Turning the attention away from tannins, an FTIR spectroscopic method has been developed for the analysis of anthocyanins in young red wines (Soriano *et al.*, 2007). A reference HPLC method (using analytical standards of the most common grape/young wine anthocyanins) and a UV method were used to construct the calibration set from 350 young Spanish red wines from various regions and from a variety of vine varieties. The validation set was composed of 40 different young red wines covering the range of anthocyanin concentrations found in the calibration set. Partial least squares regression was used to perform the calibrations, where the prediction error (SEC) was 0.15–23.79 mg/l. Validation equations developed to correlate reference and FTIR results revealed a systematic error in the determination of specific anthocyanins (e.g. cyanidin 3-glucoside and petunidin 3-glucoside acetate), which had to be eliminated by application of a correction factor. Nevertheless, the method was considered suitable for measuring anthocyanin levels in young red wines.

Carbohydrates, especially polysaccharides, in alcoholic beverages have been determined by FTIR methods similar to those described above. Polysaccharides were extracted from wine by rather complex procedures involving firstly reduced pressure distillation (for removal of ethanol), dialysis (to remove tartaric acid and other small polar molecules) and then concentration, freezing and freeze drying to give a powder consisting of a mixture of polysaccharides (the 'polymeric material') (Coimbra *et al.*, 2002; 2005). Polysaccharides were then precipitated by the addition of different amounts of absolute ethanol to the polymeric material and, after

centrifugation, both residue and supernatant were kept for analysis, the residues being dissolved in water, rotary evaporated, frozen and freeze dried. The monosaccharide composition was determined by Saemon hydrolysis, conversion of the sugars to volatile alditol acetates, followed by GC analysis. Uronic acids were determined by colorimetry (Coimbra *et al.*, 1996). It was this data that was used to compose the calibration set by chemometric correlation with FTIR spectral data from the same samples. Boulet *et al.* (2007), on the other hand, used 15 polysaccharides with characterized structures previously purified from a red wine.

Single-reflectance ATR using a ZnSe crystal (Coimbra *et al.*, 2002; 2005) or a Ge crystal (Boulet *et al.*, 2007) appears to be the IR sampling mode of choice, with scans in the spectral region 4000–400 cm^{-1} (Coimbra *et al.*, 2002; 2005) or 1850–950 cm^{-1} (Boulet *et al.*, 2007). In the former reports, the 1200–800 cm^{-1} spectral region was used for analysis, whilst in the latter report, each spectrum was subjected to linear standardization at absorbance values from 0 to 1 for the wavenumbers 1800 and 1040 cm^{-1}, respectively. As in other cases, a variety of chemometric methods were used to analyze the data: PCA, canonical correlation analysis (CCA) and a PLS regression model (Coimbra *et al.*, 2002), PLS with orthogonal signal correction (OSC) (Coimbra *et al.*, 2005) and PLS (Boulet *et al.*, 2007). In all the cases outlined above it was possible to characterize wine polysaccharides, although quantification of the main families will need to overcome strong colinearity between the polysaccharide reference spectra and intrafamily spectral variability (intensified by the presence of esterified uronic acid units in the polymer chains) in order to achieve improved accuracy and robustness of the models (Boulet *et al.*, 2007). Similarly, it was necessary to use OSC-PLS to improve the predictive ability of the model for quantification of mannose from mannoproteins (Coimbra *et al.*, 2005).

Beta-glucans, oligomers or polymers of D-glucose, are produced by the action of hydrolytic enzymes on barley starchy endosperm cell wall cellulose during malting and subsequent processes involved in brewing. Oligomers can be released into the wort, and if not efficiently degraded, will be detrimental to general brewing performance (Section 2.6.2). NIR spectroscopy has been used to determine the β-glucan content of milled or homogenized samples of barley or malt, as well as in individual barley or malt grains, in an attempt to develop a rapid method for assessing endosperm modification of malt (de Sà and Palmer, 2006, and references therein). Three hundred and nineteen kernels were analyzed in reflectance mode in the spectral region 900–1700 nm and the same grains were analyzed for β-glucan content by an enzymatic/colorimetric reference method (Section 4.4.3). The standard of error of cross validation between the NIR method and the reference method was satisfactory, suggesting that NIR can be applied to β-glucan determination in whole grains, but is less reliable if the β-glucan content is very low.

Wine polysaccharides are derived from two sources: grape berry cell walls and yeast cell walls. In the former, arabinans and arabinogalactan proteins constitute the largest families, followed by rhamnogalactouronan II, whereas in the latter, mannoproteins and β-glucans are the most abundant. Winemaking procedures such as must clarification with added pectolytic enzymes, skin contact (maceration), fining and filtration can influence the polysaccharide content of the finished wine. Using the methods described above, Coimbra *et al.* (2002) were able to differentiate between extracts of white wines made by different methods (e.g. with and without clarification and/or maceration).

Peat is a very important factor in the production of Scotch whisky and a few other whiskies, since the inclusion of malt dried over peat fires or dried by hot air infused with peat smoke adds valuable aroma and flavor dimensions to the product (Section 3.2.3). In an earlier study, an attempt was made to use FTMIR spectra of surface peat layers from different areas within a specific location to distinguish between vegetative types of peat, to relate chemical and biological properties of peat and to predict chemical and microbiological properties of peat (Chapman *et al.*, 2001). Canonical variation analysis showed that the FTIR spectra of peat from three major vegetation zones were significantly different, indicating nonidentical chemical composition of peat at the three sites. PLS analysis gave a good correlation between the spectra and physicochemical properties such as pH, ash and total nitrogen content. In particular, it was found that variations in the spectral regions associated with the vibration of secondary amide groups accounted for much of the variation in total

nitrogen content. However, PLS models gave poor prediction of chemical and microbiological properties of moorland and forest peat.

It has long been suspected, but not known for certain, that peats originating from different areas of Scotland have different chemical constitutions and hence influence the organoleptic character of the whisky. FTIR spectroscopy has been used as a rapid screening method to assess the discrimination of a large number of peat samples from six different Scottish locations: four from basin or valley bogs (two from Islay, one from Aberdeenshire and one from Speyside) and two from blanket bogs (one from Islay and one from Orkney) (Harrison *et al.*, 2006). Peat samples were previously differentiated using Curie-point pyrolysis mass spectrometry, a destructive method that has serious reproducibility problems. In this study, FTMIR spectra of peats from both blanket and basin/valley bogs were analyzed by a supervized principal component analysis program (one that uses a priori knowledge about class structure within the modelling process), in particular, discriminant function analysis (DFA). Initially, without using a priori knowledge, the results did not cluster clearly, indicating that variability within geographic location was more significant that that between locations. However, encoding the data with the knowledge of the six locations of the peat samples led to a clearer differentiation, although the two basin peats from Islay could not be distinguished. These results suggested that the different chemical composition of the peat samples from different locations may contribute different chemical characteristics to peated malt and hence to peated Scotch whisky. However, it remains to be seen whether such methods can discriminate between the pyrolysis products of the same samples of peat; only then will a relationship between peat type and whisky aroma/flavor be demonstrated.

Like IR spectroscopy, Raman spectroscopy has great potential for multicomponent and other chemometric analyses used to distinguish samples and to determine authenticity of origin, as it readily provides structural fingerprinting data. Additionally, Raman spectroscopy is a rapid, nondestructive technique that requires little sample preparation. However, to date (2009) it has never had the popularity of IR spectroscopy for the analysis of alcoholic drinks. Frausto-Reyes *et al.* (2005) used Raman spectroscopy to distinguish silver Tequilas from aged Tequilas (nine samples and four references) with principal component analysis of the data. Fluorescence background of the Raman spectra (Raman spectra of organic compounds usually have a fluorescence background) was also used for classification. A Raman system with back scattering geometry was used, with linearly polarized radiation of 514.5 nm for excitation. A holographic filter was used to eliminate Rayleigh emission light and an interference filter was used to reject the plasma frequencies of the ecitation laser.

4.4.3 Colorimetry, UV-Visible Spectroscopy and Fluorometry

UV-visible spectroscopy involves the absorption of ultraviolet and/or visible light by molecules, which promotes electronic transitions in the molecules. In practice, radiation in the approximate range 190–800 nm (from the 'near UV' to almost the near infrared) is most useful; that is to say this range is widely applicable and gives most information. Spectroscopy involving the 'far UV' region of the electromagnetic spectrum, although useful, requires rather more specialized equipment. For general accounts on UV-visible spectroscopy, see Lambert *et al.* (1998) and Silverstein *et al.* (2005).

The wavelength of ultraviolet-visible light absorbed by a molecule depends on the electronic energy levels within the molecule, which in turn depend upon certain structural features in the molecule. The general term for a structural feature within a molecule that absorbs radiation is chromophore. In UV-visible spectroscopy, the chromophores are multiple bonds (e.g. C=C, C=O) (giving $\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$ and other transitions), heteroatoms (e.g. O, S, I) (giving $n \rightarrow \sigma^*$ and other transitions) and transition metals (often as complexes with organic analytes). Single bonds (e.g. C–C, C–O) are also chromophores, but the transitions ($\sigma \rightarrow \sigma^*$, $n \rightarrow \sigma^*$) are so high in energy that absorption occurs well into the UV region (vacuum UV region), making them

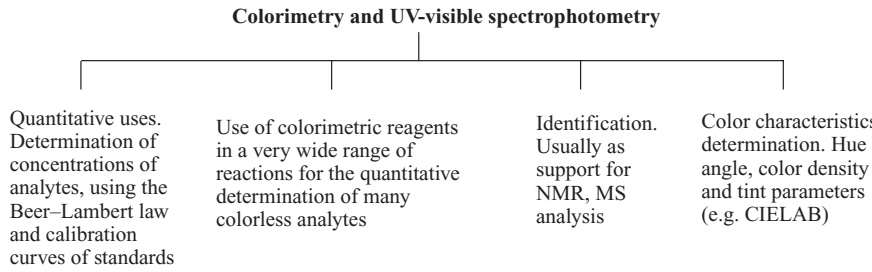


Figure 4.4.18 *Major applications of UV-visible spectrophotometry to the analysis of alcoholic beverages*

inaccessible by standard spectrophotometers (190–800 nm). The most amenable chromophores are those with extended conjugation (e.g. carotenoids) or aromatic character (e.g. porphyrins or phenolic compounds) or containing certain transition metal species; here the wavelength of absorption is either in the near UV region (say 210–350 nm) or visible region (360–800 nm). The study of colored analytes (those that absorb between 360 and 800 nm) is often known as colorimetry.

Of all the many spectroscopic techniques that are applied to the analysis of alcoholic beverages and their raw materials, colorimetry/UV-visible spectrophotometry (the last term often abbreviated to spectroscopy) is probably the most widely employed, even discounting its use as a major HPLC detection technique. Indeed, here the latter use will not be discussed, having been covered in Section 4.3.3.

The major ways in which UV-visible spectroscopy is used in the analysis of alcoholic drinks and their raw materials is summarized in Figure 4.4.18. To give a detailed account of the involvement of UV-visible spectroscopy in every aspect of alcoholic drinks analysis is beyond the scope of this book. Instead, focus will be on specific methods for the determination of specific analytes, with the emphasis being on colorimetric (or fluorometric) reactions (those that involve change of color, or introduction of a chromophore or fluorophore, such as that introduced by a chromogenic or fluorogenic reagent). Major uses of UV-visible spectroscopy and fluorometry (not including its use as a mode of detection in HPLC) in alcoholic beverage analysis include color assessment and quantitative analysis of specific compounds or families of compounds. The latter applications are numerous, so only a selected number of the more important ones will be discussed here in any detail.

Measurement of Color

Color can be measured most simply by use of a set of color standards, originally colored glass discs (comparator discs) developed by Lovibond for use with his early comparative colorimeter (the Tintometer – see Section 1.2.5) in the determination of the color of beer wort, beer and caramel or syrup adjuncts. Although updated versions of the Lovibond colorimeter are still used in breweries and wineries, and the American Society of Brewing Chemists has based its color scale for wort and beer on the Lovibond scale (°Lovibond/ASBC), more sophisticated ways for determining color are in use today. These methods involve the measurement of color intensity (absorbance) of the beverage at one or more wavelengths, using a spectrophotometer.

For the simple assessment of color, the European Brewery Convention (EBC) suggested 430 nm as being most appropriate for the pale Pilsner style beers that dominate continental Europe, whereas the Institute of Brewing (now the Institute of Brewing and Distilling) in the UK chose 530 nm because maximum variation in ale color occurs at this wavelength.

The SRM requires the measurement of the absorbance (A_{430}) of the sample in a 1 cm cuvette at 430 nm, in which case the SRM value is defined by Equation 4.4.7, where D is the dilution factor.

$$\text{SRM} = 12.7\, DA_{430} \tag{4.4.7}$$

SRM is related to the older Lovibond scale and the European EBC scale (which also uses A_{430} and a 1 cm pathlength) via Equations 4.4.8 and 4.4.9, respectively.

$$\text{SRM} = 1.3546\ \text{Lovibond} - 0.76 \tag{4.4.8}$$

$$\text{SRM} = 0.508\,\text{EBC} \tag{4.4.9}$$

Thus, pale lager beers, pale ales and pale wheat beers, brown beers, porters and stouts tend to have SRM values of *ca.* 2–6, 6–10, 12–24, 24–30 and 35+, respectively, with the corresponding EBC values being approximately double these.

These simple color assessments work well if there is no haze ($A_{700} < 0.039A_{430}$) and if the beer has 'average spectral characteristics,' which applies to the big majority of beers; the major exceptions are those, such as cherry or raspberry ales, that have been necessarily made with colored adjuncts.

A more recent approach has been to devise an augmented SRM that has no restriction on the spectral characteristics of beers and the color can be determined under any viewing conditions, unlike the CIE L*a*b* method (see below) (de Lange, 2008). Based on the absorbance spectra of 99 beers, the absorbance at any wavelength of any beer with average spectral characteristics can be computed from Equation 4.4.10.

$$A(\lambda) = \frac{\text{SRM}}{12.7}\left\{0.018747\exp\left(\frac{\lambda - 430}{13.374}\right) + 0.98226\left(\frac{\lambda - 430}{80.514}\right)\right\} \tag{4.4.10}$$

The transmission spectrum of any beer (without spectral restrictions) is given by Equation 4.4.11, where ζ_i are essentially eigenvectors of the covariance matrix of the normalized transmission spectra of the beers that were used to construct Equation 4.4.10. $A(\lambda)$ has the form shown in Equation 4.4.10.

$$T(\lambda) = \log^{-1}[A(\lambda) - c_1\zeta_1 + c_2\zeta_2 + \cdots] \tag{4.4.11}$$

The coefficients c_1 and c_2 are augmentation coefficients to account for the deviation of a particular normalized spectrum from the average normalized spectrum. For a beer with close to average spectral characteristics they are small, but for nonaverage beers, such as a cherry ale, they are much more significant and have to be included. Thus a simple SRM determination (via Equation 4.4.10) of cherry ale would give a value of around 15 (dark amber), with no indication of its red color, whereas application of Equation 4.4.11, with three coefficients (1.8, 0.8 and –0.1) yields a much accurate color assessment.

Red wine color can be described by measurements at 420, 520 and 620 nm, representing yellow, red and blue contributions to the overall color. Young red wines have strong absorbances at 620 nm, arising from the quinoid forms of anthocyanins (Figure 4.4.19) and their derivatives, whereas aged red wines have a greater absorbance at 420 nm, because of the presence of condensed polyphenols. Indeed the hue of a red wine can be described by the ratio of the absorbances at 420 and 520 nm (A_{420}/A_{520}) (Ribéreau-Gayon *et al.*, 2000a); it is ~0.5–0.7 for young wines and increases with aging, reaching ~1.3 as an upper limit. Also, a function known as brilliance (Equation 4.4.12) can be used to describe red wine color. It relates to the shape of the spectrum: young red wines have narrow maxima at 520 nm (bright red), whereas older samples have broader,

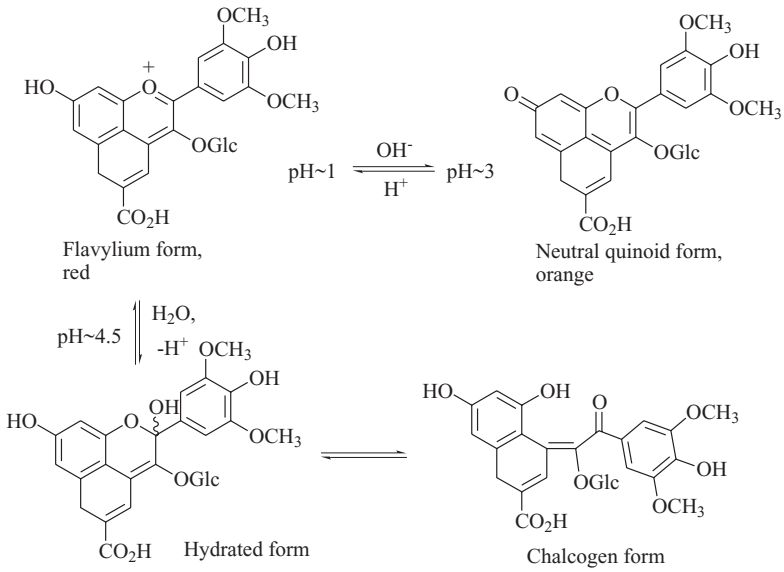


Figure 4.4.19 *Some molecular forms of anthocyanins in young red wines. Illustrated for vitisin A, an abundant anthocyanin derivative of young red wine. Asenstorfer and Jones (2007)*

less well defined maxima at 520 nm (brick red), giving brilliance values between 40 and 60 and below 40, respectively.

$$\text{Brilliance (\%)} = \left[1 - \left(\frac{A_{420} + A_{620}}{2A_{520}}\right)\right] \times 100 \tag{4.4.12}$$

More recently, tristimulus values (Hughes and Baxter, 2001) have been developed to describe the color of beverages in terms of the transmittance at three wavelengths that correspond to the maximum response of human retinal cone cells for red (600 nm), green (550 nm) and blue (450 nm) light perception. This system of measurement was devised by the Commission Internationale de l'Eclairage (CIE). The transmission spectrum of a beverage in a 10 mm cuvette relative to a water reference in a spectrophotometer with a standard illuminant light source (usually the D_{65} standard) is used to derive the three CIE color matching functions (tristimulus values). These are notated X (red), Y (green) and Z (blue). However, these values are usually transformed into three uncorrelated color parameters L^*, a^* and b^*, which give a representation of the color in three dimensions, known as the CIE L*a*b* space (Figure 4.4.20). In this representation, hue is a continuum of colors (between yellow and blue) around the equator of the sphere. Young red wines should have a red-blue hue, Pilsner beers a yellow hue and pale ales a yellow-red hue. Value, which is located along the North–South axis, measures the brightness of color. Many young red wines should have value measurements in the southern hemisphere. Finally, chroma, the distance from the North–South axis to the circumference, describes the depth or strength of color. A young wine from Chateau Latour should have a chroma measurement close to the circumference in the southern hemisphere, in the blue-red hue region. Lambrusco or young Valpollicella, on the other hand, will have a chroma characteristic closer to the North–South axis, indicating less depth of color.

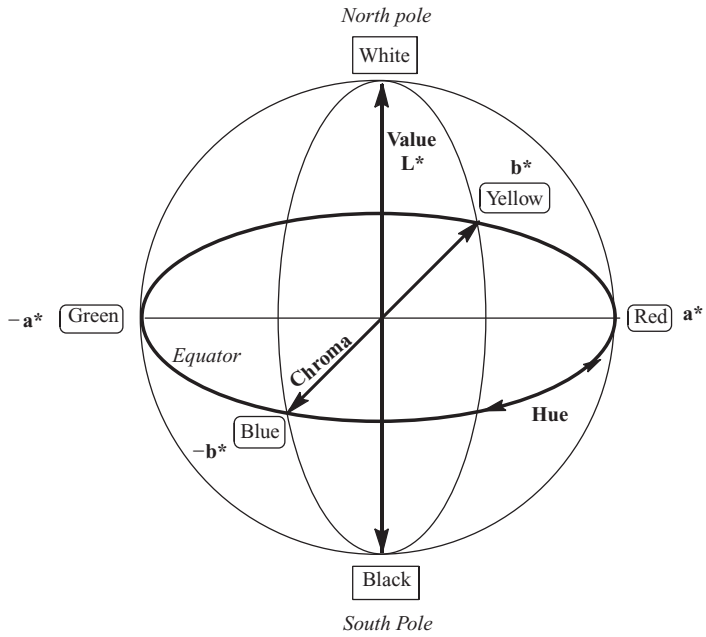


Figure 4.4.20 *The CIE L*a*b* color space. Hue is a continuum of colors around the equator. Value describes brightness and chroma relates to color strength :* $c = (a^* + b^*)^{1/2}$. *The hue angle is defined as* $h = tan^{-1}(b^*/a^*)$. *It describes the color nuance*

Color characteristics have been used to investigate new grape and wine anthocyanin pigments. One of the earliest studies involved the characterization of color stable anthocyanin derivatives in maturing red wines (Bakker and Timberlake, 1997; Romero and Bakker, 2000a; 2000b). Vitisins A and B, condensation products between malvidin 3-glucoside and pyruvic acid and acetaldehyde (respectively), were found to give UV-visible spectra that showed hypsochromic shifts (i.e. to lower wavelength) of ∼19 nm and ∼38 nm (respectively, depending on the solvent) from malvidin 3-glucoside, the major anthocyanin of grape juice. With increasing pH, the tristimulus L^* value increased for malvidin 3-glucoside, indicating loss of color intensity, whereas vitisins A and B largely retained their color between pH 2 and pH 5. Similarly, the hue angles for the two vitisins were constant between pH 2 and pH 4. Both these aspects demonstrated the superior color characteristics and color stability of the derivatives over the simple anthocyanin.

Similarly, tristimulus parameters have been used to study the aging of red wines (e.g. Port wines) (Romero and Bakker, 2000b). Generally, as red wines age their L^* values increased (indicating loss of color) and this was accompanied by an increase in hue angle from purple-red to brown-red. These observations are consistent with the slow formation of condensation oligomers from anthocyanins and flavan-3-ols. However, for the first stage of aging (∼6 months in 600 l oak casks), L^* values and hue angle decreased as anthocyanins reacted with fermentation carbonyl compounds (especially acetaldehyde and pyruvic acid) to produce vitisins. In the aging period beyond six months the vitisins gradually form oligomeric condensation products.

Determination of pK_a Values

Anthocyanins and their derivatives are important wine pigments. They can exist in solution as a number of species, depending primarily on the pH of the medium and the concentration of pigments. The earliest reliable

estimates of the ionization and hydration constants of native anthocyanins such as malvidin-3-glucoside were made by Brouillard and Delaport (1977 – see also earlier papers) using pH and temperature jump experiments and UV-visible spectroscopy. The complex equilibria existing in dilute solutions of anthocyanins and their derivatives over a range of pH have been studied recently by UV-visible spectrophotometry, ^{13}C NMR spectroscopy and paper electrophoresis (Asenstorfer *et al.*, 2003; 2006; 2007). Brønsted ionization constants for malvidin-3-glucoside, vitisin A and 8,8-linked malvidin-3-glucose-ethyl-catechin/epicatechin were determined by fitting absorbance data for each analytical wavelength to Equation 4.4.13 (Asenstorfer *et al.*, 2006).

$$A_{\mathrm{p}} = \frac{A_0 + A_1 10^{\mathrm{pH}-\mathrm{pK}(1)} + A_2 10^{2\mathrm{pH}-\mathrm{pK}(1)-\mathrm{pK}(2)+\cdots}}{1 + A_1 10^{\mathrm{pH}-\mathrm{pK}(1)} + A_2 10^{2\mathrm{pH}-\mathrm{pK}(1)-\mathrm{pK}(2)+\cdots}} \tag{4.4.13}$$

Here, A_p is the predicted absorbance at a particular wavelength estimated at a particular pH value. It results from the absorbance of the equilibrium mixture of the acid, A_0, and its ionization products, A_1, A_2, etc. and is determined by the sum of the respective absorbances and partial molar fractions of the acid and its ionization products.

The pK_a values were found to depend on structure: for example the first pK_a value, $pK_a(1)$, was 1.76 for the malvidin-3-glucoside, but 0.95 for vitisin A and 2.33–2.44 for the various dimer stereoisomers. These pK_a values were found to agree well with those determined by paper electrophoresis (Asenstorfer *et al.*, 2006) and the assignment of equilibrium species (flavylium, hydrated, quinoid base, etc.) was confirmed by ^{13}C NMR spectroscopy for vitisin A (Asenstorfer and Jones, 2007); at wine pH (~3.2–3.6) the most important structures are the flavylium ion the neutral quinoid forms, with the latter predominating (λ_{max} ~498 nm) (Figure 4.4.19).

In *dilute* solution at wine pH, the major forms of anthocyanins and their derivatives (such as vitisin A) tend to be colorless hydrated or orange quinoidal forms (Asenstorfer *et al.*, 2003; 2006; Asenstorfer and Jones, 2007). The obvious bright red color of young red wines is attributed to the much higher concentrations of anthocyanins and derivatives, resulting in extensive association, via π-stacking or dipole–dipole interaction, (or copigmentation) between a number of species, including the quinoidal forms of malvidin-3-glucoside and vitsin A.

Colorimetric and Fluorometric Reactions and Titrations

There are many colorimetric and fluorometric reactions that are utilized to determine the concentration of a specific component of an alcoholic beverage (e.g. acetic acid, D-glucose, (–)-malic acid, tartaric acid, (+)-lactic acid and (–)-lactic acid) or families of components (e.g. amino acids, phenolic compounds or reducing sugars). Additionally, there are colorimetric methods for the determination of most metals found in alcoholic beverages or their raw materials and precursors.

Many of the methods for individual organic components depend on an enzymic reagent carrying out the reaction that enables UV-visible absorption measurement. These can often be bought as kits (e.g. Boehringer Mannheim (now Roche), Fluka, Megazyme or Sigma), which contain the enzymic reagent, buffer solutions and other reagents required for the analysis, along with detailed instructions and standard solutions of the analyte to be determined. They are usually straightforward to operate and of good accuracy and precision, but they do suffer from limited shelf life, particularly of the enzyme reagent(s). As with any other analytical method, the test solutions will probably need to be diluted so that measurements fall within the calibration range of the kit.

Total Phenolic Content (TPC)

TPC of musts and beverages is usually measured by the Folin–Ciocalteu method, mostly the version developed by Singleton and Rossi (1965). A mixture of phosphomolybdic ($H_3PMo_{12}O_{40}$) and phosphotungstic acids ($H_3PW_{12}O_{40}$) in alkaline solution is known to oxidize most phenols to give a blue coloration which can be measured at 760 nm. The oxidizing agent is a polymeric species containing tetrahedral phosphate units linked to octahedral molybdate and tungstate units, where the metals in oxidation state +6 and are readily interchangeable. Upon oxidation of phenols (as the anions or phenoxides, in alkaline solution) some of the metal +6 oxidation states are reduced to +5 states and it is this combination of mixed oxidation state complexes that is thought to give rise to the coloration.

In one version of this method (Ribéreau-Gayon *et al.*, 2000a), red wine (1 ml), diluted five or tenfold with distilled water, is mixed with reagent (5 ml), 20% aqueous sodium carbonate and made up to 100 ml with distilled water. After 30 min, the absorbance of this solution in a 1 cm cell is measured at 760 nm and TPC is calculated according to Equation 4.4.14. Values lie between 10 and 100 for red wines.

$$\mathrm{TPC_{F-C}} = 20A_{760}(\mathrm{DF}) \tag{4.4.14}$$

where DF is the dilution factor.

In another version (Amerine and Ough, 1980b), red juice or wine samples are diluted × 5, whereas pale samples are used undiluted. To the sample (1.00 ml) in a 100 ml volumetric flask, is added distilled water (60 ml) and Folin–Ciocalteu reagent (5.00 ml). After 30 s, aqueous sodium carbonate (15 ml) is added and the solution is brought to volume with distilled water. After a 2 h stand at ∼20 °C, the absorbance is measured at 765 nm against a blank made up as above, but without the sample.

The total phenolic content of each sample (taking into account any dilution factor), is determined using the standard plot for gallic acid standards (e.g. 50–500 mg/l) and is expressed in terms of mg/l of gallic acid (gallic acid equivalents).

A much simpler, speedier method for measurement of TPC was suggested by Ribéreau-Gayon *et al.* (2000a). It does not involve the use of a colorimetric reaction, but is a simple measurement of absorbance at 280 nm (Equation 4.4.15, where DF is the dilution factor).

$$\mathrm{TPC_{280}} = A(280\,\mathrm{nm})(\mathrm{DF}) \tag{4.4.15}$$

$\mathrm{TPC_{280}}$ values lie between 6 and 120. The method relies on the fact that the benzene rings of the majority of phenols have an ultraviolet absorption at 280 nm. It is the simplest method for the determination of phenols, but is subject to error if the phenol does not absorb at 280 nm, as in the case of cinnamic acids and some of their biological derivatives, such as chalcones. Similarly, if there are nonphenolic aromatic compounds present that do absorb at 280 nm, then this will lead to an overestimate of phenolic content. In practice, for red wines, both these interferences are known to be very small.

Procyanidin Content

Procyanidins are polymeric flavanols, which yield anthocyanidins ($\lambda_{max} \sim 520$ nm) upon heating with hydrochloric acid under oxidative conditions (Section 5.8.6). They are important components of many alcoholic beverages, particularly red wines. The method of Glories *et al.* (1988) requires the preparation of two samples (red wine diluted × 50; white wine undiluted or diluted × 2). One sample (4.00 ml) is mixed with water (2.00 ml) and pure 12 M HCl (6.00 ml) and heated at 100 °C in a water bath for 30 min, after

which 95% ethanol (1.00 ml) is added to solubilize the red pigments that are formed and the absorbance at 520 nm is measured (A_1) (1 cm optical path length). The other sample is treated as above, but is not heated (absorbance A_2). By comparison with an oligomeric procyanidin solution derived from grape seeds, the procyanidin concentration of the sample is obtained by use of Equations 4.4.16 and 4.4.17.

$$\text{For young wines, } \mathrm{PC}(g/\mathrm{l}) = 16.16A_1 - 24.24A_2 - 1.71\mathrm{AC} \tag{4.4.16}$$

$$\text{For older wines, } \mathrm{PC}(g/\mathrm{l}) = 16.16\mathrm{A}_1 - 32.32\mathrm{A}_2 - 3.86\mathrm{AC} \tag{4.4.17}$$

where AC is the free anthocyanin content (in g/l), as determined by the method described below, for example.

Tannin Content

Estimation of tannin content (procyanidin and similar flavonoid polymers plus hydrolyzable tannins) can be carried out using a modification (Jensen *et al.*, 2008) of the method of Harbertson *et al.* (2003). This method involves the precipitation of tannins with bovine serum albumin (BSA). The precipitate is redissolved and the tannin concentration is measured by a color reaction with ferric chloride. Typically, the wine sample is diluted in a model wine solution containing 12% ethanol (v:v) and tartaric acid (5 g/l), whose pH has been adjusted to 3.3 with aqueous NaOH. The test solution and BSA are mixed and allowed to stand for 30 min before centrifugation at 14000g. The supernatant is poured off the tannin–protein pellet, which is redissolved in SDS/TEA buffer solution (1.5 ml) and a background absorbance measurement on the spectrophotometer is performed on this – A(BG). This solution (1 ml) is then reacted with $FeCl_3$ solution (11.4 mM $FeCl_3$ in aqueous 11.4 mM HCl) (125 μl) and the absorbance (A) is measured after 10 min. Dilutions of sample in the model wine solution are adjusted to give a tannin response (calculated as 1.125A – A(BG)) between 0.3 and 0.75. The tannin content is reported in mg of catechin equivalents/l from a linear plot of the color reaction between catechin and $FeCl_3$ (absorbance = 0.006258 × [catechin (mg/l]; $R = 0.9997$).

Anthocyanin Content

Estimation of anthocyanin content of musts and wines is important in many wineries. There are two widely used colorimetric methods, the pH shift method and the sulfur dioxide bleach method, of which the latter is generally thought to be more reliable (Ribéreau-Gayon *et al.*, 2000a). Purple colored beverages, like red wines, black raspberry juice and mulberry juice, contain anthocyanins as both free molecules and condensed or polymerized species. All of these species absorb at *ca.* 520 nm. This method relies upon the fact that bisulfite reacts with free anthocyanins, but not with the condensed species, to form products that do not absorb at 520 nm. So, by measuring the difference in absorption at 520 nm (i.e. before and after the addition of bisulfite), it is possible to estimate the free anthocyanin content of beverages (Amerine and Ough, 1980a). This method is insensitive to the presence of SO_2 in the beverage (a commonly used preservative in commercial juices and wines) and so is thought to be more reliable than the pH shift method (Ribéreau-Gayon *et al.*, 2000a).

The method of Amerine and Ough (1980a) is described in Figure 4.4.21. Firstly, all samples (but not standards) are accurately diluted × 5 and then 1 ml of this diluted solution is used in the procedure described in this figure. After 30 min, 3 or 4 ml of A and B are transferred separately to quartz cuvettes and the difference in absorbance at 520 nm, against distilled water blank is determined. The standard solutions of cyanidin hydrochloride of concentrations say, 100 mg/l, 50 mg/l, 25 mg/l and 10 mg/l, are used for calibration, without prior dilution. A standard curve is prepared and the anthocyanin content of each of the samples, using the standard curve. The results are quoted as mg/l of cyanidin, taking into account the × 5 dilution.

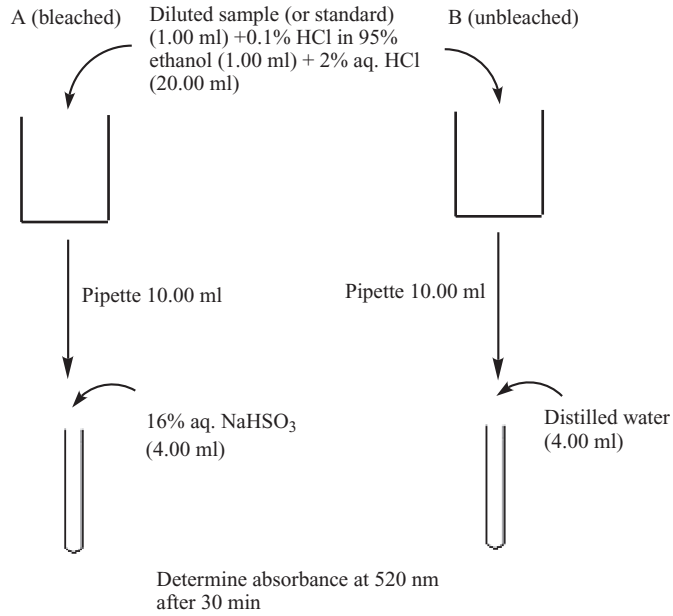


Figure 4.4.21 *Summary of bisulfite bleach method for estimation of anthocyanin content of musts and bevarages*

Individual Acids

Various colorimetric methods are available for the analysis of the important individual aliphatic acidic components of alcoholic beverages and musts, including acetic acid, citric acid, fumaric acid, α-ketoglutaric acid, L-malic acid, D- and L-lactic acids, pyruvic acid and succinic acid (Amerine and Ough 1980b). Similar methods also exist for the determination of sugar acids, such as gluconic acid. These all depend on a reaction of the analyte with a reagent to produce a product that absorbs either in the visible or near ultraviolet region of the electromagnetic spectrum.

Enzymic reagents are particularly favored for these assays and enzymic/colorimetric/UV methods (often using kits from companies such as Boehringer Mannheim, Fluka, Sigma or Megazyme) are widely used in analytical laboratories of the food and beverage industries. Figure 4.4.22 illustrates the principles of several of these enzymic methods. They usually involve the use of dehydrogenase enzymes in the presence of NAD^+ or $NADP^+$ coenzymes in buffered solution, whence the reaction is monitored at ∼340 nm by the formation of NADH or NADPH. An additional enzyme reaction is often used in the assay procedure in order to maximize NADH production, exemplified by the use of glutamate-oxaloacetate transferase in the L-malate assay (Figure 4.4.22) (Möllering, 1985). In the case of fumarate determination, fumaric acid in the sample is first hydrated to L-malic acid by fumarase and then the malate produced here is assayed by the malic acid method (Figure 4.4.22) (Boehringer Mannheim GmbH, 1989).

Samples should be clear and nearly neutral (pH ∼7); turbid samples need filtration before analysis and pH may need to be adjusted by the addition of a small quantity of mineral acid or alkali. Accurate sample dilution is usually necessary prior to analysis, unless the analyte in the beverage is present at low concentration (e.g. <0.2 g/l for malic acid). Lightly colored samples can be analyzed without further sample preparation, but highly colored samples, such as red wine or elderberry must (10 ml) should be stirred with PVP powder (0.1 g) for 1 min and filtered, the clear slightly colored solution being used for the assay. Bound acid concentrations

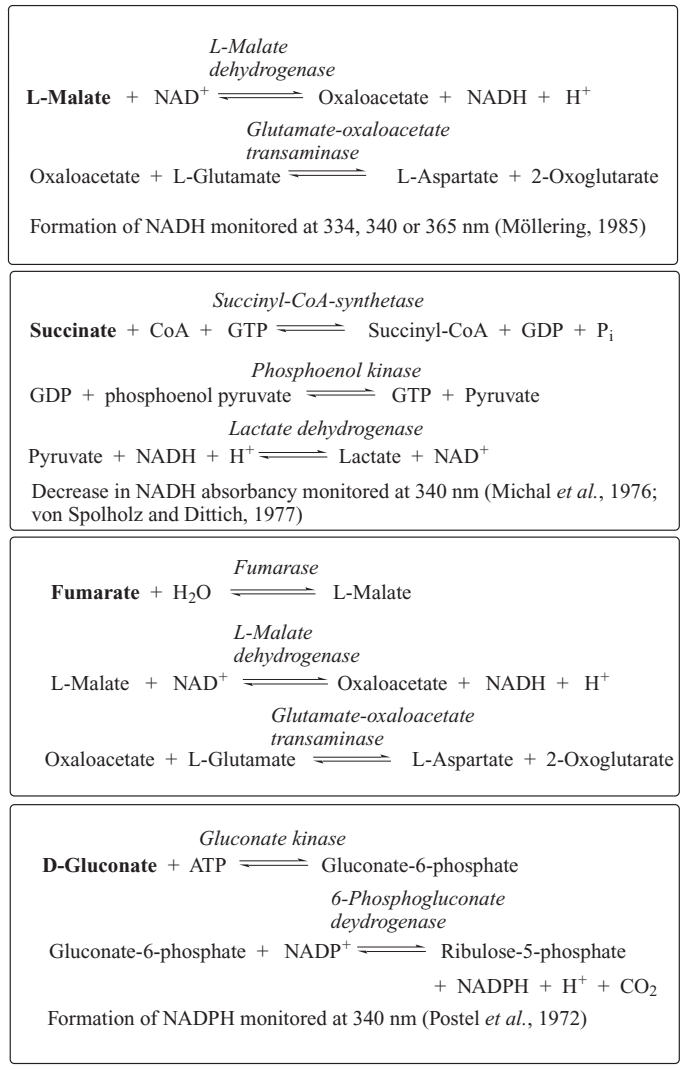


Figure 4.4.22 *Principles of some enzymic methods for the determination of individual acids*

(e.g. as esters) in beverage samples can be determined from the results of free acid and total acid assays. The latter are carried out on samples that have undergone alkaline hydrolysis, followed by neutralization. For example, in the case of malate determination, the sample (20.00 ml) and 2 M sodium hydroxide (6 ml) are heated under reflux (with stirring) for 30 min. After cooling, the solution is neutralized with 1 M sulfuric acid and made up to 50.00 ml with distilled water. This solution (already diluted × 2.5) is then used for the assay.

Sometimes, as in the succinate assay, use of the more direct method is unsatisfactory because of the instability of the dehydrogenase enzyme (here, succinate dehydrogenase), coupled with difficulty of its isolation in pure form (Michal *et al.*, 1976). In cases such as this, a series of coupled enzymic reactions were used and the reaction was monitored by following the fall in absorbance at 340 nm due to NADH (Figure 4.4.22).

Certain individual aliphatic acids are still sometimes determined by nonenzymic colorimetric methods, although in many laboratories chromatographic methods (especially HPLC – see Section 4.3.3) and CZE methods (Section 4.6.1) have largely replaced these. Tartaric acid can be determined by the Rebelein method, which uses the wine red coloration formed between tartrate and ammonium vanadate (Amerine and Ough 1980b and references therein). The standard method involves the following procedure. The sample (3.00 ml of must or 10.00 ml of wine) is mixed with 0.1 M silver nitrate solution in 30% aqueous acetic acid (15 ml) and (for colored samples) decolorizing charcoal (0.5 g). This solution is swirled for 15 s whilst ammonium vanadate (NH_4VO_4) solution (15 ml) is added. (The ammonium vanadate solution is made by mixing NH_4VO_4 (10 g), 1M NaOH (150 ml) and 27% aqueous sodium acetate (200 ml) and making up to 1000.00 ml with distilled water). The solution is filtered, the first 5 ml is discarded and the filtrate (∼10 ml) is allowed to stand for 30 min. Its absorbance is determined in a 1 cm cell at 530 nm against a blank made up as above, but using distilled water in place of the sample. The absorbances of samples are compared with a standard curve prepared using standard tartaric acid solutions carried through the same procedure. The decolorizing charcoal is used to adsorb anthocyanins in colored samples since these interfere with the method (they absorb around 520 nm). This procedure has been improved for colored samples by running further blanks rather than by using decolorizing charcoal (Amerine and Ough, 1980b).

Citric acid may be assayed using the diazo method of Rebelein (Amerine and Ough 1980b). The diazo solution (made from a glacial acetic acid-water solution of sulfanilic acid and aqueous sodium nitrite) needs to be prepared at 0 °C and must be used immediately. This feature, plus its numerous steps (i.e. its labor intensive nature), including centrifugation and the use of many reagents, make the diazo method compare unfavorably with HPLC or CZE methods, despite its good reliability.

Colorimetric methods for the determination of lactic acid involve the oxidation of both enantiomers (hence only total lactic acid can be assayed) to acetaldehyde by cerium (IV) sulfate. The acetaldehyde is then reacted with either sodium nitroprusside/piperidine or *p*-hydroxydiphenyl to give colored derivatives that have absorption maxima at 570 nm and 560 nm, respectively (Amerine and Ough, 1980b). Once again, although these methods generally give reliable results, their nonrobust and labor intensive nature make them unattractive compared with HPLC, CZE or enzymic methods, all of which are able to determine D- and L-lactic acid separately.

Sugars

Although reliable chromatographic methods (especially HPLC and CZE – see Sections 4.3.3 and 4.6.1, respectively) are available for the determination of sugars (including reducing sugars) in alcoholic beverages, musts and wort, colorimetric assays are still sometimes used. Total reducing sugar content of beverages can be determined by titrimetric techniques, such as the Lane–Eynon method (Section 4.6.3).

D-glucose can also be assayed individually using the Sigma D-glucose kit, as for the determination of D-glucose released by the hydrolysis of aroma precursor glucosides (Arévelo Villena *et al.*, 2006). The aforementioned kit involves the use of glucose oxidase to specifically oxidize D-glucose to D-gluconic acid, which after incubation of the test solution with the enzyme and adjustment of pH, the gluconic acid is reacted with *o*-dianisidine to give a red coloration (540 nm), whose absorbance is measured and related to concentration via a calibration curve.

There are enzyme kits available for the colorimetric determination of other sugars and carbohydrates (see catalogues of Boehringer-Mannheim (now Roche), Fluka, Megazyme and Sigma, for example). The Megazyme mixed linkage β-glucan kit can be used to assay the β-glucan content of malt flour, wort or beer. Here, the enzyme lichenase (specific, *endo*-(1-3)(1-4)-β-D-glucan 4-glucanohydrolase) is used to hydrolyze 3→4 links of β-glucan polymers to produce oligomers, which are then hydrolyzed by the enzyme β-glucosidase to give D-glucose. The last named is then oxidized by glucose oxidase and peroxidase to gluconic

acid, which in the presence of 4-aminoantipyrine gives a red coloration at 510 nm. As usual, absorbance measurements are made with respect to a blank carried through the same procedure and the method is regularly calibrated, in this case using either D-glucose standards or control flours of known β-glucan contents. This method forms the basis of a number of recommended or standard methods of various professional bodies, such as the Association of Official Analytical Chemists (AOAC) and the European Brewery Convention (EBC). A method similar to that described above was used to compare glucanase activities of two malting barley varieties and the β-glucan contents of the corresponding malts (de Sà and Palmer, 2004).

A nonenzymic fluorometric method for β-glucan determination uses the reagent Calcofluor in aqueous sodium chloride/glycine buffer at pH 9.5 to produce a fluorescent derivative of β-glucan after 30 min incubation, with excitation at 360 nm, followed by fluorescence at 420 nm (Kanauchi and Bamforth, 2002). Brewers have a special interest here, since extraneous β-glucans in beer wort can lead to processing problems (Section 2.6.2), but at the same time they are a useful source of dietary fiber, giving certain health benefits (Sections 5.7.4 and 5.11.2).

Ammonia, Amino Acids and Proteins

Ammonia is an important constituent of many alcoholic beverages, and particularly of raw materials, such as fruit juices and beer wort. Apart from column chromatography/titrimetric (Section 4.6.3), ion selective electrode methods (Section 4.5.1) and methods mentioned in the next paragraphs, ammonia may be determined using Nessler's reagent, a solution of 0.09 M potassium tetraiodomercurate (II) and 2.5 M potassium hydroxide. Small quantities of ammonia produce a yellow coloration due the formation of the soluble complex $HgO.Hg(NH_2)I$. Color formation can be monitored at 425 nm and its absorbance compared with those of standard ammonia solutions under identical experimental conditions.

Ammonia can also be determined colorimetrically by the phenate test, in which it reacts with sodium phenate (phenoxide) in the presence of hypochlorous acid (HClO) to give the blue soluble indophenol. The reaction is usually monitored at 630 nm. Both the above methods have been used to assay ammonia produced in Kjeldahl digestions for the determination of total nitrogen or protein content of foodstuffs (Section 4.6.3).

Yet another method for the determination of ammonia and urea in foodstuffs makes use of the Megazyme® kit, where ammonia (as NH_4^+) is combined with 2-oxoglutarate in the presence of glutamate dehydrogenase and NADPH to produce L-glutamate and $NADP^+$. NADPH consumption is measured by decrease in absorbance at 340 nm and is related to ammonia content. If urease is then added, urea is hydrolyzed to ammonia, which reacts as described above, so measurement of a further decrease in absorbance at 340 nm can be related to the urea content. All absorbance measurements are made with respect to a blank (with an extra water component in place of the sample) taken through the same procedure.

Total usable nitrogen is a measure of the yeast usable nitrogen content (ammonia, α-amino acids and small peptides) content of beverages, musts and worts. Free amino nitrogen (FAN) is the amino acid nitrogen available to yeast during fermentation. It is often determined by colorimetric methods involving ninhydrin, such as that of the European Brewery Convention, although in reality these methods actually measure ammonia and small peptide content as well as amino acid content. The EBC method involves making the ninhydrin color reagent from ninhydrin (0.5 g), anhydrous Na_2HPO_4 (4 g), KH_2PO_4 (6 g) and fructose (0.3 g) in 100 ml of aqueous solution. For FAN assay, beer (1.00 ml) is diluted to 50.0 ml with distilled water and the diluted sample (2.00 ml) is pipetted into a test tube. After addition of ninhydrin color reagent (1.00 ml), the test tube is heated in boiling water for 16 min, then cooled in cold water before the addition of a diluting reagent (KIO_3 (2 g) in water (600 ml), with ethanol (400 ml) added). The absorbance of this solution is measured at 575 nm against a blank, containing water (2.00 ml) in place of the sample. A glycine standard solution (107.2 mg glycine/100 ml of aqueous solutuion) is used for calibration, following the method described above.

Yeast assimilable nitrogen (YAN), a measure of the ammonia and amino acid content of wine must, is the sum of two separate determinations; ammonia and free amino acids. The former can be estimated by use of the 2-oxoglutarate method (Bergmeyer and Beutler, 1983), whilst the latter can be determined by formol titration (Shively and Henick-Kling, 2001) or by the *o*-phthalaldehyde/*N*-acetyl-L-cysteine spectrophotometric method (Dukes and Butzke, 1998; Shively and Henick-Kling, 2001). Kits are also available for the assay of ammonia and primary amino nitrogen (e.g. see www.megazyme.com).

Reduced volume versions of the ninhydrin method of FAN determination have been applied recently to beer and wine (Abernathy *et al.*, 2009), using ninhydrin solutions that were buffered either by sodium acetate (pH 5.5) for beer or grape juice, or phosphate buffer (pH 6.8, as for the EBC method, above) for beer. These procedures used just 30 μl of sample (or 20 μl of glycine standard) and 200 μl of the pH 5.5 ninhydrin solution or 1 ml of the pH 6.8 ninhydrin solution, thus being economical as well as rapid, accurate and applicable to large numbers of samples.

Data from these methods, along with total nitrogen content data (section 4.6.3) are useful in assessing the fermentability of wine must and beer wort, as well as checking the protein content of malted cereals.

Individual amino acids can be determined by a wide variety of HPLC (Section 4.3.3) and GC (Section 4.3.2) methods, but a routine automated method uses ion exchange column chromatography and an amino acid analyzer, with ninhydrin as a chromogenic reagent (Ribereau-Gayon *et al.*, 2000b) and a similar method uses picric acid solution (Kluba *et al.*, 1978).

There are well established individual colorimetric methods for the determination of arginine and proline in wines and other alcoholic drinks, via color formation with 8-hydroxyquinoline/alkaline sodium hypobromite and ninhydrin (respectively) (Amerine and Ough, 1980c), but these have largely given way to chromatographic methods. More recently, the individual amino acid (lysine and proline) content and biogenic amine (histamine and tyrosine) content of beers have been determined by fluorometric methods on beer extracts, using mostly *o*-phthalaldehyde (with 2-mercaptoethanol and Brij 35 for proline assay) for fluorophore formation (Gorinstein *et al.*, 1999). Absorption of the amino acid/amine *o*-phthalaldehyde complexes were at 340 nm and emission was measured at 430–450 nm. Tyramine was reacted with α-nitroso-β-naphthol and nitric acid containing 2% $NaNO_2$ to form the fluorescent analyte that absorbed at 465 nm and emitted at 540 nm.

Total soluble protein concentration, say of barley extract, malt extract or wort, can be determined using proprietary protein assay kits, such as the BCA kit of Pierce (Rockford, USA), which measures the absorbance of the sample at 562 nm, using bovine serum albumin (2 mg/ml) as the standard. The kit works on the principle that Cu(II) in alkaline medium can be chelated and reduced to Cu(I) by cysteine, lysine, tryptophan and tyrosine residues in proteins, in the presence of bicinchoninic acid (BCA), forming a purple coloration, the intensity of which is proportional to the protein concentration.

Other colorimetric methods for total protein determination include the biuret method, which uses the purple coloration (monitored at 540 nm) caused by interaction of the biuret reagent (often a 20% copper sulfate solution) specifically with peptide bonds, and the rather more sensitive Lowry method, which uses the biuret reagent combined with the Folin–Ciocalteu reagent. Proteins react with these reagents to give a blue solution that can be monitored at ~500 nm for samples with high protein content or ~750 nm for those with lower protein content.

More recently, a microwell plate ninhydrin assay has been applied to the determination of the total protein content of beer by hydrolyzing the protein to amino acids beforehand by heating the sample (200 μl) with 12 M HCl (200 μl) at 95 °C for 18 h (Abernathy *et al.*, 2009). Hydrolyzed bovine serum albumin was used as the calibration standard. The results were 30% lower than those of the Kjeldahl method (Section 4.6.3), which measures nitrogen from all sources. Also, by comparison of the results for undialyzed hydrolyzed beer with those for dialyzed hydrolyzed beer (cut off 3.5 kDa), it was found that only 25% of the total protein in beer is actually derived from peptides of molecular weight greater than 3500 Da.

There are specific colorimetric methods for the assessment of diastase (total amylolytic) activity and individual amylolytic enzyme (e.g. α-amylase, β-amylase and limit dextrinase) activities in malt samples – see also titrimetric methods (Section 4.6.3). Variants of the 3,5-dinitrosalicylic acid (DNSA) method can be used to assess all the aforementioned enzyme activities (see for example Osman, 2002). The general method depends on measurement of the absorbance at 540 nm of the DNSA derivative of reducing sugars released by the action of the enzymes (extracted from, say, malt) on a soluble starch solution. Details of the method are given in Figure 4.4.23. For α-amylase assay, β-amylase is deactivated by heating in the presence of 20 mM calcium chloride solution prior to analysis, whereas for β-amylase determination, α-amylase is deactivated by the presence of 20 mM EDTA in the equilibrated starch solution. A sample (600 μg) of maltose is used as the standard for the colorimetric determination. Standards and controls are treated similarly, except that the enzyme extract is added to the controls after quenching the reaction with 0.1 M NaOH solution.

Limit dextrinase activity can be estimated by the above method, using pullulan (not soluble starch) as the polysaccharide source of reducing sugars and after the deactivation of β-amylase by heating. It can also be estimated, perhaps rather more conveniently, by the limit-dextriZyme (Megazyme, Australia) method (Osman, 2002; McCleary, 1992).

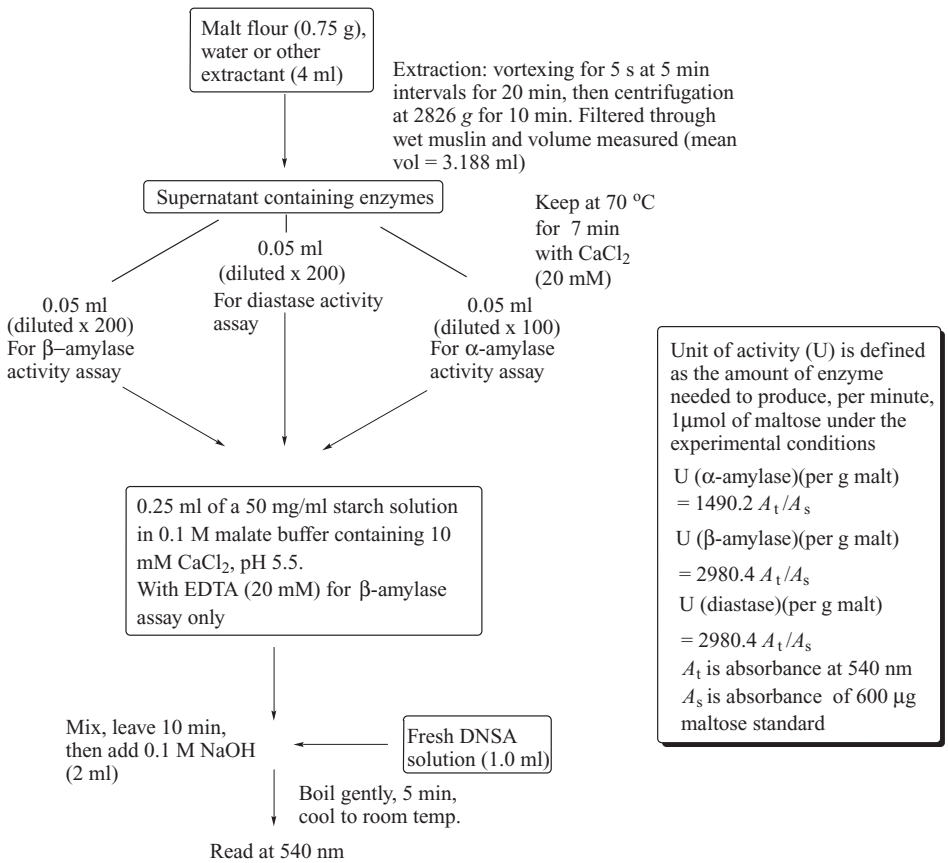


Figure 4.4.23 *Summary of DNSA colorimetric method for the determination of activity of malt amylolytic enzymes. Osman (2002)*

β-Glucanases are enzymes present in malted cereals that hydrolyze the cell wall β-glucans, forming soluble oligomers and β-D-glucose. β-Glucanase activity is important for the efficient release of starch and proteins and removal of oligomers that cause processing problems and can compromise haze stability (Sections 2.6.3 and 2.6.4). β-Glucanase activity can be measured by the Congo Red method, as described by Shengli *et al.* (2009). Here, a β-glucan (0.8 ml of a 30 μg/ml solution) was incubated at 40 °C for 10 min, followed by the addition of beer or wort sample (0.2 ml). After 30 min, the solution was heated to 100 °C for 15 min and then cooled to room temperature. Congo Red (0.2 ml of a 100 g/ml solution) was added and the mixture was diluted with buffer to 2 ml and the absorbance of the supernatant measured at 540 nm. β-Glucanase activity is defined as the amount of β-glucanase needed to hydrolyze 1 μg of β-glucan per min in 1 ml of culture fluid.

Metal Ions

Colorimetric methods are available for the determination of many metals – including those in different oxidation states, such as Fe(II) and Fe(III) – and nonmetals, such as P (as phosphate). Many of these methods have been or are still used as standard or reference (official) methods for alcoholic beverage analysis in certain countries, although for routine analysis, say for quality control purposes, they have been largely replaced by atomic spectrometric (Section 4.4.4) and electrochemical methods (Chapter 4.5). Since there is at least one colorimetric method available for most metal and nonmetallic species found in alcoholic drinks, only an outline of the more important methods that are still used as reference methods or for comparison purposes will be considered here.

Total Iron

Iron (III) reacts with excess of thiocyanates to give a series of soluble and intensively blood-red colored complex compounds. If carried out in the presence of persulfate, this method permits the determination of the total iron [Fe(II) + Fe(III)], since $K_2S_2O_8$ quantitatively oxidizes all the iron to the Fe(III) state.

In a typical experimental procedure for the direct determination of total iron content in alcoholic beverages, the sample, suitably diluted (5.00 ml), is placed in a screw capped bottle and the following reagents are added in sequence: 10% (v:v) H_2SO_4 (1 ml) aqueous 50% (w:v) KSCN (1 ml) and aqueous 1% (w:v) $K_2S_2O_8$. Finally, ethyl acetate (5.00 ml) is added and the bottle is vigorously shaken for 1 min. The biphasic system was allowed to stand for 5 min, to separate the phases into two transparent layers. Portions of 3 ml of the upper red organic layer are transferred into a quartz cuvette of 1 cm pathlength, after filtration using a Whatman No. 42 ashless paper. The absorbance was measured at 495 nm, against a reagent blank. The same procedure is carried out on standard Fe solutions (100–600 μg/l) to obtain a calibration curve.

Ferrous Iron

The nitro-PAPS reagent (Makino *et al.*, 1988), [2-(5-nitro-2-pyridylazo)-5-[*N*-*n*-propyl-*N*-(3-sulfopropyl)-amino]-phenol, disodium salt, dihydrate] has been introduced as a highly sensitive colorimetric reagent specifically for Fe(II), by forming the 1:2 water soluble complex $[Fe(C_{17}H_{19}N_5O_6S)_2]Na_4$ in the 3-8 pH range ($\lambda_{max} = 582$ nm).

In a typical experiment, the sample (1.00 ml) is transferred to a test tube with a pipette, and the following reagents are added in sequence: distilled water (5 ml), aqueous 1.0 M NaN_3 (1 ml), aqueous 1.0 M ascorbic acid (1 ml) and aqueous 1×10^{-4} M nitro-PAPS solution (2 ml). After 10 min, the absorbance of the colored complex is measured at 582 nm, against a reagent blank. Standard Fe(II) solutions are treated in the same way to obtain a calibration curve. The above assay should be carried out in conjunction with a total Fe assay.

Calcium

Numerous methods exist for the determination of this important component (as Ca^{2+}) of alcoholic beverages and their precursors, including EDTA titration (Section 4.6.3) and various complexometric and colorimetric methods, although nowadays the methods of choice tend to be either atomic spectroscopic (Section 4.4.4) or electrochemical (Section 4.5.1) methods.

A simple colorimetric method for the assay of calcium in drinks involves a pH 11 buffer (orthoboric acid (3.6 g) in water: ethanolamine (9:1 v:v)), a color reagent (8-hydroxyquinoline (1 g) + conc HCl (5 ml) + *o*-cresophthalein (40 mg) (in that order), made up to 100 ml with water) and EDTA (disodium salt; 50 g in 1 l of water) (Amerine and Ough, 1980d). To suitable Ca standard solutions (e.g. containing 0–3.0 mg/l) (1.00 ml) is added one drop of water, pH 11 buffer solution (5.00 ml) and color reagent (0.50 ml). After mixing well and allowing to stand for 10 min in a water bath at 25 °C, the absorbance is measured against a blank made as above, but using water (1.00 ml) in place of the standard. The above procedure is carried out on a pale sample (e.g. white wine), diluted 1:20. For a colored sample (e.g red wine, again diluted 1:20 v:v) a double procedure is performed, one exactly as above and the second as above, but adding one drop of the EDTA solution in place of the water. The absorbance value of the EDTA sample is subtracted from that of the water sample. The 8-hydroxyquinoline prevents interference by Mg, whereas the buffer prevents interference from other metal ions.

Copper

There are several colorimetric methods available for the estimation of copper, but those involving (red) complex formation with 2,2′-diquinoline (in the presence of hydroxylamine/sodium acetate to reduce Cu^{2+} to Cu^{+}) or complex formation with diethyldiothiocarbamate are probably the most satisfactory (Amerine and Ough, 1980d). Again, Cu in alcoholic beverages is more often determined by atomic spectroscopy (Section 4.4.4).

Phosphate

Total phosphate can be determined by ashing the sample, evaporating a solution of the ash in 1:5 hydrochloric acid to dryness, redissolving in 1:5 hydrochloric acid and treating aliquots of this solution with ammonium molybdate/aminonaphthosulfonic acid, measuring the absorbance at 600 nm after 30 min (Amerine and Ough, 1980d).

Colorimetric and fluorometric titrations are only occasionally used in the study of alcoholic beverages. Tanaka *et al.* (2002) conducted fluorometric titrations to determine the amount of charge (polyelectrolyte concentration) in whiskies. The amount of charge was attributed to polyphenols derived mostly from oak contact during maturation. The titrant was polydiallyldimethylammonium chloride (poly-DADMAC) and the fluorescence indicator was acriflavine hydrochloride.

Hop Bittering Agent Determination

There are several methods available for the determination of the α-acid and β-acid content of hops. α-Acids are the precursors of *iso*-α-acids, the major bittering compounds of beer (Section 2.6.3). An American Society of Brewing Chemists (ASBC) method involves extracting freshly ground hops with toluene and measuring the absorbance of the resulting filtered extract at 275, 325 and 355 nm (ASBC, 1999). Using the ASBC method with specified standards, the α-acid and β-acid contents of hop samples are calculated using Equations 4.4.18

and 4.4.19, where d is the dilution factor.

$$\alpha\text{-acid content } (\%) = d(-51.56A_{355} + 73.79A_{325} - 19.07A_{275}) \quad (4.4.18)$$

$$\beta\text{-acid content } (\%) = d(55.57A_{355} - 47.59A_{325} + 5.10A_{275}) \quad (4.4.19)$$

Since many hop constituents are prone to deterioration in the presence of oxygen (Sections 2.6.3, 2.6.7 and 2.6.12), it is useful to have a method (other than a sensory method) that can give some indication of the extent of deterioration of the hop sample. One such method (Bamforth, 2002) is based on the fact that the toluene extract of aged hops shows an increased absorbance at 275 nm relative to that at 325 nm. Hence, the hop storage index (HSI), defined as A_{275}/A_{325}, is a measure of deterioration.

Likewise, in Europe, the standard EBC spectrophotometric method for the determination of bitterness (expressed as IBU) in beer (EBC method 9.8) is performed by spectrophotometric determination of an acidified *iso*-octane extract of beer at 275 nm. The absorbance at 275 nm is the sum of the absorbances of all extracted substances, including bittering agents (*iso*-α-acids, unisomerized acids, etc.), as well as polyphenols and others that do not contribute to bitterness. Also a significant, but acceptable, variation in reproducibility results from the extraction process and related manipulations (Benard, 2000).

More recently, two spectrofluorometric methods using chemometrics have been applied to the determination of bitterness in both light and dark beers (Christensen *et al.*, 2005). Partial least squares (PLS) regression models, with full cross validation, were applied for the comparison of autofluorescence and europium induced delayed fluorescence spectra with IBU data generated by application of the standard EBC method to 21 beers. Bitterness uncertainty (reproducibility) was similar to that of the standard method, but the fluorescence methods have advantages in speed, robustness and not requiring solvents.

Dissolved Oxygen

Although a certain amount of contact between oxygen and red wine is thought to be beneficial (even to the extent of applying micro-oxygenation regimes to maturing red wine), giving reduction of astringency and enhancement of color, the quality of white wines is generally compromised by exposure to air because of unfavorable color and flavor changes. Likewise, dissolved oxygen as low as 1 mg/l in beer is bad news, since this quantity can lead to a number of off flavors and can significantly reduce the shelf life of the beer.

There are several colorimetric/spectrophotometric methods for the assay of dissolved O_2 in alcoholic beverages, although most of them were originally developed for testing water samples. These come in convenient kit form (e.g. Chemetrics, Hach, Thermo Scientific) and at least one has a color comparator (Chemetrics), for measurement, in place of using a colorimeter or spectrophotometer. The majority of these are based on the reaction between O_2 and the reduced ('leuco') form of indigo carmine dye, resulting in a color change from yellow to indigo, which can be monitored at 608 nm. Other methods rely on the pink coloration formed on reaction of dissolved O_2 with the reduced form of the dye rhodazine D. Neither method is subject to interference from dissolved gases such as CO_2 or from sulfites, and so can be applied to wine and beer. However, they are subject to interference from pigments and so are generally limited to dissolved oxygen assay of white wine and pale beers. These methods are also destructive of the sample and require careful sampling technique. See also Section 4.5.3 for a description of electrochemical (polarographic) probes for dissolved oxygen, which are widely used in the beer and wine industries.

There has been much interest recently in monitoring oxygen ingress through closures (corks, plastic stoppers, screw caps, crown caps, etc.) into bottled wine (Goode, 2009), as well as in changes in oxygen levels that occur in wine during various processing procedures.

Lopes *et al.* (2005) monitored the diffusion of oxygen into solutions of reduced indigo carmine contained in 375 ml bottles closed with various kinds of stoppers, from natural and technical cork of various grades and types to synthetic closures. The control bottles were sealed with a glass stopper via flame welding. Oxygen ingress was followed over a one year period of horizontal storage of the bottles, thus mimicking storage conditions of real bottles of wine. The oxygen concentrations were determined directly (without sampling) on the bottles using L^*, a^*, b^* measurements (CIELAB76) by scanning the bottles with a spectrocolorimeter equipped with a transmittance accessory. L^*, a^*, b^* values were collected using illuminant D65 and a 10° observer. Hue angle (H^*) was calculated from arctan (b^*/a^*). Oxygen diffusion was shown to be much greater in the first month for all stoppers (except the control, where there was no diffusion), but the overall rate of diffusion was dependent on the stopper material, first grade natural cork being the most resistant to long term oxygen ingress.

More recently, the German company PreSens Precision Sensing has produced a nondestructive probe that includes a photodiode to detect the blue light emitted by an oxygen sensitive sensor when red light is incident upon it. It can be incorporated into a reuseable dot that can be placed inside a bottle to monitor oxygen ingress through the stopper (Goode, 2009). Similar dissolved oxygen sensors (e.g. those of Electro-Chemical Devices Inc. and Mettler-Toledo AG) rely on the quenching of fluorescence emission, for example from a fluorescent layer sandwiched between a light emitter and a light detector (a kind of mini fluorometer). The greater the dissolved O_2 content of the sample the less intense is the fluorescence, and the more rapidly is quenched.

Antioxidant Ability Assays

There are several methods for assessing the *in vitro* antioxidant potential of foodstuffs, including juices, worts and alcoholic beverages. Most of these methods determine the ability of a whole beverage or fractions of a beverage, or even an individual component to quench a free radical species. This ability is usually measured with respect to quenching of the same radical by a reference antioxidant, often tocopherol (vitamin E) or Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). A selection of methods is outlined in the next paragraphs, followed by a commentary regarding their relative advantages/disadvantages, accuracy and precision and extent of intercorrelation.

The ferric reducing ability of plasma (FRAP) assay was developed in 1996 by Benzie and Strain (1996) in order to assess the antioxidant power of human plasma. It has since been applied to assess the antioxidant activity of a range of pure substances and mixtures, such as red wines (Lee *et al.*, 2004). The method compares the ferric ion reducing ability of the antioxidant under investigation with the ferric ion reducing ability of some standard. The original calibration standard was Fe (II), but lately Trolox (a water soluble synthetic vitamin E analog) has been used for this purpose (Lee *et al.*, 2004).

The antioxidant (A) being tested requires a redox potential that is less positive than that of the Fe(III)/Fe(II) half reaction, so that the overall reaction (Figure 4.4.24(a)) has a positive redox potential. The greater the antioxidant power, the more the above reaction lies to the right. The substance *s*-tripyridinyltriazine (2,4,6-tris(2-pyridyl)-1,3,5-triazine or TPTZ) forms complexes with both Fe(III) and Fe(II), but the Fe(II) complex has an intense blue color. Hence, it is possible to deduce the Fe(III) reducing ability of any substance by carrying out the reaction in the presence of TPTZ and by measuring the intensity of the blue coloration (i.e. the absorbance at 593 nm).

1,1-Diphenylpycrylhydrazyl ($DPPH^\bullet$) is relatively stable free radical that exhibits a purple color ($\lambda_{max} \sim 515$ nm) because of the extensive delocalization of the unpaired electron. It can react with hydrogen/electron donors (antioxidants) (Figure 4.4.24(b)). The further the reaction lies to the right, the greater is the radical scavenging ability (which can be related to antioxidant ability) of the antioxidant. Hence, measurement of loss of purple color can be used to estimate radical scavenging ability. One of the problems of this method is, because $DPPH^\bullet$ is a radical that is kinetically (steric hindrance) stabilized (as well as thermodynamically

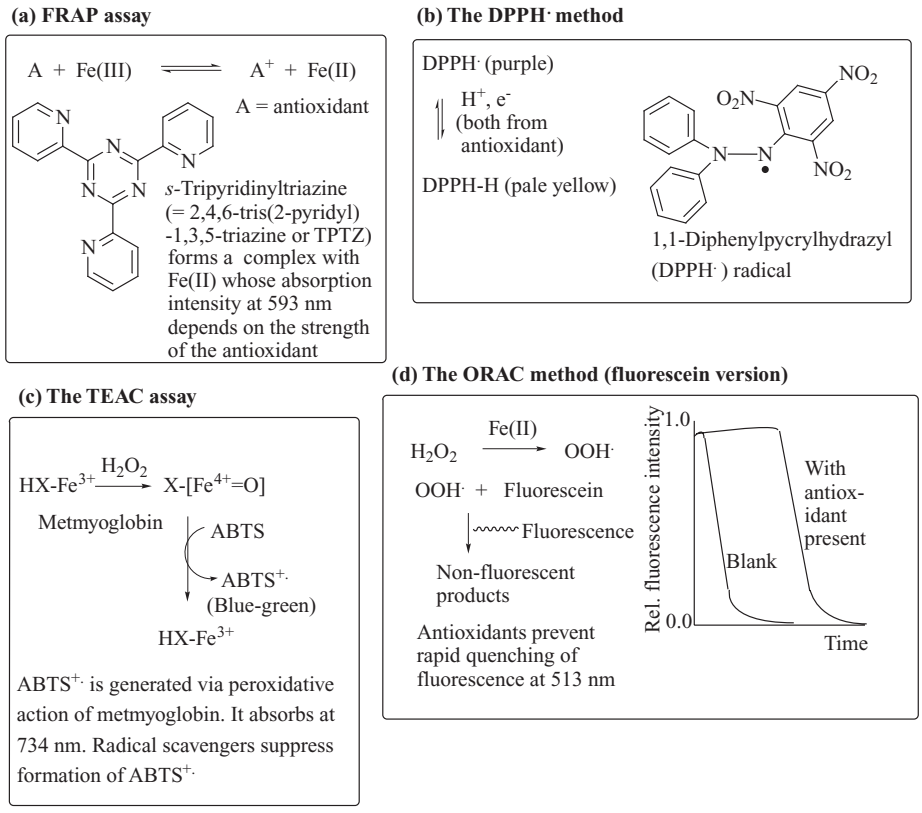


Figure 4.4.24 *Colorimetric methods of antioxidant power determination*

stabilized), it reacts with the wide range of scavenger phenols (and other types) at different rates. In the most basic method, the time interval of 90 min is chosen so that the reaction at room temperature is likely to be complete for all the radical scavenging substances that act as antioxidants in foodstuffs. However, a development of this method that involves measurement of reaction time T_{EC50}, as well as the amount of antioxidant needed to decrease by 50% the initial DPPH concentration (EC_{50}) has been described in the literature (Sánchez-Moreno *et al.*, 1998). The parameter antiradical efficiency ($AE = \{EC_{50}\ T_{EC50}\}^{-1}$) was suggested as being more discriminatory and generally a better measure of antioxidant power than EC_{50}.

The Trolox® equivalent antioxidant capacity (TEAC) assay was developed by Miller *et al.* (1993), and Miller and Rice-Evans (1996). It is based upon the generation of the $ABTS^{+\bullet}$ radical cation when 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is incubated in the presence of a peroxidase enzyme (metmyoglobin) and hydrogen peroxide or in the presence of hydroxyl, peroxyl alkoxyl or inorganic radicals. From the time ABTS, metmyoglobin, buffer and hydrogen peroxide are mixed, the absorbance of the blue-green $ABTS^{+\bullet}$ species is measured at 734 nm with respect to time (Figure 4.4.24(c)). If antioxidants are present before the hydrogen peroxide is added, these scavenge the ABTS radical cations, thus inducing inhibition of absorbance at 734 nm (suppression of color). The unit of measurement is the TEAC, namely the concentration in mmol/l of Trolox with the equivalent antioxidant capacity to a 1.0 mmol/l solution being investigated. The main problem with the TEAC assay is that it does not take the inhibition time into account, thus ignoring a component of antioxidant power (Cao *et al.*, 1995).

The oxygen radical absorbance capacity (ORAC) method was developed originally in 1993 (Cao *et al.*, 1993) from previous work of Glazer and modified to an automated method in 1995 (Cao *et al.*, 1995). It was further modified in 2001 (Ou *et al.*, 2001). In this method, 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) is used to generate peroxy radicals ($OOH^{\bullet}$), which quench fluorescence in β-phycoerythrin (Cao *et al.*, 1993) or a fluorescein salt (Ou *et al.*, 2001). The loss of fluorescence indicates the extent of reaction of the fluorescent probe with the generated peroxy radicals, hence when this reaction is performed in the presence of an antioxidant, the fluorescence will be quenched over a longer time period than without the antioxidant, depending on the potency and concentration of the antioxidant.

After mixing of reagents, fluorescence measurements are made at 37 °C at intervals of time for blank and sample until zero fluorescence. The results are quoted as the ORAC value, namely the net protection area under the fluorescence quenching curve when an antioxidant is present (Figure 4.4.24(d)). It is calculated (in Trolox equivalents) by dividing the area under the sample curve by the area under the Trolox (standard) curve, both being corrected by subtraction of the area under the blank curve. Many other methods for the assessment of antioxidant capacity have been developed over the years: some of these are summarized and compared by McAnalley *et al.* (2003).

Because each of these methods uses a different free radical mechanism (the FRAP method and TEAC assays use single electron transfers) and because of a variety of data analysis methods associated with these assays, it is often difficult to correlate results for the same foodstuff using different methods. At present (2009) the ORAC method seems to be the most favored, possibly because it uses the area under the fluorescence (quenching) versus time curve and hence combines two measurements (inhibition time and degree of inhibition). It is also the only method that directly assays the chain breaking antioxidant activity of substances and mixtures. For these reasons, the ORAC method is thought by many to give the best estimate of antioxidant ability. However, no single method can give a universal antioxidant value of a complex sample, and when choosing a method, many other factors are taken into consideration, such as relative cost (particularly with regard to reagents and instrumentation), time required for analysis and general methodology. Hence, there is plenty of work in this area being performed using the DPPH or FRAP assays, in particular, because of the relative simplicity and speed regarding these two methods.

Intercorrelation between these various methods is often either nonexistent or poor, making to difficult to compare results; indeed scatter plots of TEAC and ORAC antioxidant values for the same substrates show no linear correlation, and only a rather weak (but significant) correlation is observed in the FRAP versus ORAC scatter plot (McAnalley *et al.*, 2003).

There are many reports in the literature on the total antioxidant capacity of alcoholic beverages and musts. There are also reports on antioxidant potential of various fractions and of individual components, along with investigation of correlation of antioxidant capacity with phenolic or other content. The big majority of these reports have focused on phenolic components and the general observation is that highly colored musts and drinks exhibit higher antioxidant ability and have higher contents of phenolic compounds.

Total antioxidant potentials of wines were evaluated with the ABTS (TEAC) method, where a positive correlation between TEAC value and phenolic content (especially gallic acid, (+)-catechin and (–)-epicatechin) was found, as determined by capillary zone electrophoresis (Minussi *et al.*, 2003).

Que *et al.* (2006) have determined the total antioxidant activity, reducing capacity and free radical scavenging capacity of Chinese rice wines (Section 2.7.1) by the thiocyanate method of Mitsuda *et al.* (1966), the ferricyanide method of Oyaizu (1986), and the DPPH method respectively. There were good correlations between these parameters and total phenolic content and also between the concentrations of the ten most important polyphenols in rice wine. Nuomi had the highest and Foshou the lowest antioxidant activity of the wines tested; Guyuelongshan, Hongqu, Shousheng, Foshou and Nuomi.

The thiocyanate method is an older method that measures the prevention by antioxidants of peroxide induced linoleic acid oxidation. Rice wine (120 μl) in 0.04 M potassium phosphate buffer (pH 7.0) (2.5 ml)

was added to linoleic acid emulsion in 0.04 M potassium phosphate buffer (pH 7.0) (2.5ml). The control (5.0 ml) was linoleic acid (2.5 ml) emulsion and 0.04 M potassium phosphate buffer (pH 7.0) (2.5ml). Linoleic acid emulsion (50.0) ml) was prepared with Tween-20 (350 mg) and of linoleic acid (310 μl) and 0.04 M potassium phosphate buffer (pH 7.0) was added up to the volume. The mixed solution (5.0 ml) was incubated at 37 °C in the dark. The peroxide level was determined by reading the absorbance at 500 nm after reaction with 20 mM $FeCl_2$ (in 3.5% HCl) and 30% thiocyanate every 12 h. Inhibition of lipid peroxidation in percent was expressed as antioxidant activity.

The DPPH radical is frequently used for assessment of antioxidant activity, by virtue of its radical scavenging ability, as mentioned previously. The DPPH method, however, is a general method and is not selective with regard to the identities of the radical oxidants. Effectiveness of alcoholic beverages at scavenging *particular* oxidative radicals can be assessed by more specialist procedures, such as the xanthine/xanthine oxidase method for superoxide anion scavenging ability (Lu and Foo, 2001; Que *et al.*, 2006). Here a 0.5 ml of aqueous solution containing rice wine (60–240 μl) was added to a 1.0 ml mixture of 0.4 mM xanthine and 0.24 mM nitroblue tetrazolium chloride (NBT) in 0.1 M phosphate buffer (pH 8.0). A 1.0 ml solution of xanthine oxidase (0.049 units/ml), diluted in 0.1 M phosphate buffer (pH 8.0) was added and the resulting mixture was incubated in a water bath for 40 min at 37 °C. After this time, the reaction was quenched by adding 2.0 ml of 69 mM sodium dodecylsulfate and the absorbance of NBT was measured at 530 nm and compared with that of vitamin C and control (with no antioxidant present) taken through the same procedure.

The effectiveness of wine fractions in trapping peroxyl radicals has been measured using an azo initiator to generate a constant flow of peroxyl radicals (Ghiselli *et al.*, 1998) that cause loss of fluorescence upon reaction with (*R*)-phycoerythrin (R-PE). A 15 nM R-PE solution in 75 mM phosphate buffer (pH 7.0) was incubated at 37 °C with 4 mM 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) in the presence or absence of the wine fractions (80 μl of a 1:200 dilution, pH 7.0). The delay of loss of R-PE fluorescence (t_s) induced by the samples was continuously monitored in a spectrofluorometer. The delay induced by a known amount of trolox (t_T), the water soluble analog of vitamin E, was used as a standard to quantify the results according to the equation, sample antioxidant activity $= t_s - [\text{trolox}]/t_T$.

The ability of wine and wine fractions to scavenge the hydroxyl radical ($OH^{\bullet}$) can be measured by the deoxyribose method (Halliwell *et al.*, 1987) (e.g. Ghiselli *et al.*, 1998). Sample (100 μl of 1:10 dilution at pH 7.4) was added to 10 mM phosphate buffer (690 μl) at pH 7.4 containing 2-deoxyribose (2.5 mM). Iron ammonium sulfate (1.0 mM, 100 μl) premixed with 1.04 mM EDTA was added. The samples were kept in a water bath at 37 °C and the reaction was started by adding 1.0 mM ascorbic acid (100 μl) and 0.1 M H_2O_2 (10 μl). Samples were maintained at 37 °C for 10 min, and then cold 2.8% trichloroacetic acid (1.0 ml) was added followed by 1% thiobarbituric acid (0.5 ml). The samples were boiled gently for 8 min, then after cooling the absorbance was measured at 532 nm.

Because of increasing interest in the contribution of food phenolic components toward good health, much attention during the present decade has been placed on the analysis of total and individual phenols in highly colored fruits, and in the determination of antioxidant capacities of these fruits. Thus, Ehlenfeldt and Prior (2001) studied correlations between oxygen radical absorbance capacity (ORAC), total phenolic content (TPC) and total anthocyanin content (TAC) of the fruit and leaves of 87 highbush blueberry (*Vaccinium corymbusum* L.) varieties, some of which were hybrids of other varieties. ORAC leaf values did not correlate with ORAC fruit values, but significant correlations were found between fruit ORAC, TPC and TAC.

Antioxidant capacities of cultivated rowanberry and other fruit grown (bilberry and lingonberry) in Finland were determined by both the FRAP and DPPH methods (Hukkanen *et al.*, 2006). Antioxidant capacities and TPC of rowanberries were found to high (with the major phenols being chlorogenic acid and neochlorogenic acid), and on the basis their phenolic profiles, principal component analysis was able to separate the varieties of different origin into clusters. Rowanberries are used to for homemade wines and liqueurs, as well as commercial flavored vodkas (Section 3.4.3).

Much further south, measurement of antioxidant capacities, TPC, TAC and ascorbic acid content of blackberries, Cornelian cherries, gooseberries, raspberries and redcurrants showed all (except white gooseberry) to have high TPCs and all had high antioxidant activity, as measured by the FRAP method (Pantelidis *et al.*, 2007). TPC and TAC were highly correlated with antioxidant capacity. Although blackberries had the highest antioxidant capacity for the inhibition of free radicals, Cornelian cherry (a red skinned native wild cherry of Northern Greece) aroused interest because of its high ascorbic acid, anthocyanin and phenolic content. Also, sweet cherries grown in Slovenia had high TPCs and antioxidant capacities, as determined by the ascorbic acid equivalent antioxidant capacity (AEAC) method (Usenik *et al.*, 2008). This time, however, correlation between antioxidant capacity and TPC was found to be cultivar (variety) dependent, although, as in previous studies, darker skinned fruits had higher TACs, TPCs and antioxidant potentials.

Bilberry (*Vaccinium myrtillus*) and blueberry (*Vaccinium* spp.) are small dark skinned fruits that have attracted considerable attention because of their high phenolic contents. They are frequently used to make wines (Chapter 2.11) and liqueurs (Section 3.9.2). Faria *et al.* (2005) examined the ability of bilberry extracts to limit peroxidation (induced by 2,2′-azobis(2-methylpropanimidamide) dihydrochloride) of soybean liposomes, by measuring oxygen consumption and the formation of conjugated dienes (see Section 5.8.4). All the extracts provided protection from peroxidation, but this increased with increased polyphenol content and complexity of the anthocyanin derived pigments. Interestingly the antioxidant power of the extracts, determined using the DPPH and FRAP methods, correlated well with the results from the liposome membrane peroxidation protection assays.

Interest in antioxidant power is not entirely confined to phenolic compounds. Herbs, spices and spice essential oils are used in the preparation of a number of flavored alcoholic beverages, such as apéritifs, flavored spirits (Sections 3.4.2 and 3.4.3), liqueurs (Section 3.9.3) and vermouth (Section 2.12.2). The antioxidant capacities, determined by the DPPH and FRAP methods, of free terpenoid aglycones (mainly chavicol, eugenol, linalool and α-terpineol) from basil, the fresh herb itself and its essential oil were compared (Politeo *et al.*, 2007). The DPPH results suggested that together the free aglycones had good antioxidant properties – comparable with those of the essential oil and the synthetic antioxidant butylated hydroxytoluene (BHT). On the other hand, the FRAP results suggested that the compounds are less effective antioxidants than the essential oil or BHT.

Star anise (*Illicium verum*) and black caraway (*Carum nigrum*), and their powders and essential oils have been examined by the DPPH (and other) methods for their antioxidant activity (Padmashree *et al.*, 2007). Both powders and ethanol/water extracts, as well as their volatile oils, showed strong antioxigenic activity, with star anise volatile oil having higher activity than that of black caraway. Star anise is used to flavor various distilled spirits (Sections 3.4.3 and 3.5.6), some liqueurs (Section 3.9.3) and some vermouths (Section 2.12.2).

Essential oils of basil, cinnamon, clove, nutmeg, oregano and thyme (all important ingredients of some liqueurs and vermouths) were found to have good radical scavenging abilities in the DPPH assay at room temperature, with clove and cinnamon being the most effective (Tomaino *et al.*, 2005). Heating to 180 °C for a short time did not appear to adversely influence the essential oils' antioxidant capacity (except in the case of nutmeg oil: its antioxidant activity increased).

4.4.4 Atomic Absorption and Emission Spectroscopy

Atomic spectroscopy consists of a number of techniques for the determination of elements (mostly metals) in a sample. In principle, both absorption and emission modes are possible, but in practice the former is more widely used in the analysis of alcoholic beverages, so it will be discussed first. See Broekaert (2002) for general reading on flame and plasma atomic spectroscopy.

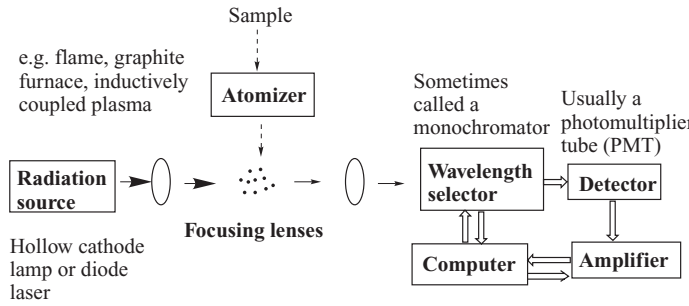


Figure 4.4.25 *Simplified block diagram of an atomic absorption spectrometer*

The basic parts of an atomic absorption spectrometer are shown in Figure 4.4.25. In atomic absorption spectroscopy (AAS), the elements in the sample to be analyzed need to be converted to gaseous atoms; this is done by atomization of the sample in an atomizer. The atoms of a particular element are then subjected to light from a radiation source of a wavelength that corresponds to the energy needed to promote an electronic transition in the atoms of that element. The atoms absorb this energy and are converted to excited electron states for a short time. The spectrometer measures the extent of absorption (by comparison with a blank sample) and relates it to the concentration of the element in the sample via the application of the Beer–Lambert law, once the data for reference solutions of the element have been acquired and the sample has been diluted so that the absorption falls within the range of absorption of the standards. The wavelength of incident radiation is specific to a particular transition in a specific element; radiation of a different wavelength must be used for another element.

The most common source of radiation in atomic absorption spectroscopy is the hollow cathode lamp, a cylindrical structure filled with neon or argon gas at low pressure and containing a cylindrical metal cathode and an anode rod. A high voltage placed across the electrodes causes some gas atoms to be ionized at the anode. Some inert gas cations collide with the cathode internal surface at high speed, causing the ejection of metal species in excited states from the surface. These emit light with the wavelength that is characteristic of that particular metal. It is possible to buy multimetal hollow cathode lamps, which can be tuned in to a particular metal by the operation of a switch on the lamp. Alternatively, diode lasers can be used as a source of radiation.

The mode of atomization generally gives its name to the type of atomic absorption spectroscopy; the major types are flame atomic absorption spectroscopy (FAAS), graphic furnace or electrothermal atomic absorption spectroscopy (GFAAS or ETAAS) and inductively coupled atomic absorption spectroscopy (ICPAAS). The latter atomization technique is more usually associated with atomic emission spectroscopy (ICPAES) or mass spectrometry (ICPMS) (Section 4.4.5). The atomization process produces atoms by a rather complex sequence of events that are summarized in Figure 4.4.26, for flame atomization.

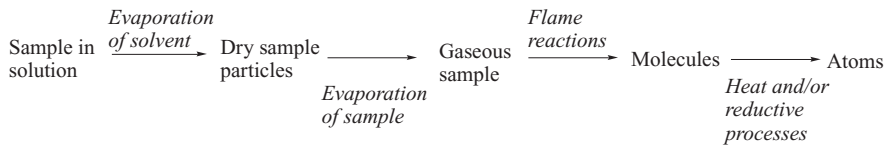


Figure 4.4.26 *Summary of processes leading to the production of atoms in a flame*

The majority of atomic absorption analyses of alcoholic beverages are performed using FAAS. Here, a flame ($\sim 10 \times 2$ cm) is produced from the combustion of a mixture of acetylene and air, giving a maximum flame temperature of ~2000 °C. For samples that need higher atomization temperatures (e.g. samples containing Al, Si and/or P), a nitrous oxide (N_2O)/acetylene flame will give ~3000 °C. The sample is introduced into the flame as a mist from a nebulizer and, provided there is continuous uptake of the solution by the nebulizer, the rate of atom production in the flame will become constant and a steady absorbance can be measured. Background emissions are minimized by a number of instrumental features, including deuterium lamp, Smith–Hieftje and Zeeman corrections. The first named is the most common, which automatically compensates for broadband absorption interferences. FAAS can usually achieve the determination of metals in the μg (10^{-6} g) to ng (10^{-9} g) range.

Graphite furnace atomic absorption spectrometry (GFAAS) – also called electrothermal atomic absorption spectrometry (ETAAS) – involves a flameless atomization method, using a graphite furnace that can achieve high temperatures very rapidly. It is more sensitive than FAAS, being able to determine metals in quantities as low as 1 pg (10^{-12} g). The sample (usually10 or 20 μl) is injected, either manually or via an autosampler into the cold graphite furnace and, by means of an automatic temperature programmer, the solvent and other volatile components are evaporated, usually over a period of a few seconds. The sample is atomized by heating the furnace to ~ 2000 °C over about 5 s. During this time the absorbance signal from the chamber is recorded and displayed on the screen as a function of time. After this, the furnace is heated for 5 s to around 2600 °C, in order to remove residues and prepare for the next sample. During this process, apart from when recording absorbance, the furnace is purged with argon.

Atomic emission spectroscopy (AES) uses the intensity of light emitted by excited atoms in an arc, flame, plasma or spark, while relaxing back to their lower energy states. The earliest and simplest form of AES is flame photometry, which is still used to determine alkali and alkaline earth metals (Na, K, Mg, Ca, etc.) in solution. Atomic emission detectors (AED) can be linked with gas chromatography (Section 4.3.2) and high performance liquid chromatography (Section 4.3.3), as discussed later.

The final atomization method considered here is that of the inductively coupled plasma (ICP), giving rise to ICPAES (or ICPOES) and ICPMS (Section 4.4.5). The plasma is produced in a torch that consists of three concentric tubes, usually made of quartz. The end of this torch is placed inside an induction coil supplied with a radio frequency electric current (~27 MHz). While argon is made to flow between the two outermost tubes of the torch, an electrical spark is applied briefly to introduce free electrons into the gas stream. These electrons interact with the rapidly oscillating magnetic field of the induction coil and are accelerated in alternating directions. These high energy electrons collide with argon atoms, and sometimes a collision causes ionization of argon atoms. These newly released electrons are in turn accelerated by the alternating magnetic field. The process continues until an equilibrium is reached between the release of new electrons in collisions and the recombination of electrons with argon ions. This produces a 'fireball' that consists mostly of argon atoms with a relatively small number of free electrons and argon ions at temperature between 6000 °C and 10 000 °C.

The rapid flow of gas between the two outermost tubes protects the walls from the destructive effect of the plasma, Likewise, a second (slower) flow of argon between the central tube and the intermediate tube keeps the plasma away from the end of the central tube. A third flow (again slower) of gas through the central tube forms a channel that is cooler than the surrounding plasma, but still much hotter than a chemical flame. Samples to be analyzed are introduced into this central channel, usually as a mist of liquid formed by passing the liquid sample into a nebulizer.

ICPEAS is even more sensitive than GFAAS and is similarly capable of determining trace metals and some nonmetals (such as S and Se), present in quantities well below 1 pg, in alcoholic beverages.

Metal species are present at low concentrations in all alcoholic beverages, and although they themselves make negligible direct contributions to flavor, the presence or absence of several species can have significant

effects on flavor and overall quality. For example, the presence of Cu^{2+}, Fe^{2+} and Mn^{2+} at certain levels promote premature staling of beer (Section 2.6.12) and the presence of extraneous Fe species causes a serious fault in wine known as 'casse' (Section 2.9.2). At concentrations higher than 10 mg/l, Fe(III) forms a blue colloidal suspension with tannins and phosphates, affecting both the color and flavor of wine. On the other hand, the presence of certain salts (e.g. $CaSO_4$, $CaCO_3$) in brewing water is known to be beneficial to the quality of certain kinds of beers (Section 2.6.2). Likewise, the presence of certain levels of Cu^{2+} in the wash used to make distilled spirits actually improves the organoleptic qualities by removing volatile sulfur compounds (see for example Section 3.5.3), but acts as a catalyst for the production of ethyl carbamate (Section 3.2.2) and can be present in high concentrations in distillery wastes, thus constituting an environmental problem (Sections 3.2.5 and 5.10.3). Quite apart from these factors, most so called heavy metal ions are harmful to health if present in alcoholic beverages above certain maximum levels (Section 5.10.3), defined by legislation, and hence their analysis is of prime importance. Also of importance are the speciation of metals (e.g. Cr^{3+} or Cr(VI)) present in a beverages, since this has a direct bearing on toxicity, and similarly, the bioavailability of a species (whether it is 'free' or bound to, say protein or polyphenolic compounds) also affects its toxicity (Section 5.10.3).

Generally, less alcoholic samples such as beer, cider and wine need to be treated in one or more of the following ways before analysis, especially if FAAS is used or if the analyte of interest is present at low levels:

- Preconcentration/separation (e.g. by solvent extraction, sorbent or other techniques, the analyte of interest (e.g. Cu) being first converted to a chelate complex, for example with diethylammonium-*N*,*N*-diethyldithiocarbamate)
- Digestion in strongly oxidizing reagents such as concentrated $HClO_4$, HNO_3 or H_2O_2
- Volatile hydride generation, usually using $NaBH_4$ (e.g. for the conversion of Pb species to PbH_4)
- Addition of matrix modifiers, such as $Mg(NO_3)_2$ or $Pd(NO_3)_2$.

These procedures are intended to lower the effect of the complex matrix (for beer, cider and wine this would be water, ethanol and a host of organic components of many different types) on the volatility and atomization of the analyte, thus increasing sensitivity and general quality of analytical method. Distilled spirits, such as rum, tequila or whisky, on the other hand, have much less complex matrices, and their analysis by AAS usually requires no more than dilution with a dilute nitric acid solution and/or the addition of modifiers.

There are many reports on the determination of toxic heavy metals (especially As, Cd, Cu and Pb) in alcoholic beverages by atomic spectroscopy (usually absorption) (Jaganathan *et al.*, 1997; Mena *et al.*, 1997; Azenho and Vasconcelos, 2000; Freschi *et al.*, 2001; Adam *et al.*, 2002; Cvetković *et al.*, 2002; Karadjova *et al.*, 2002; Paleologos *et al.*, 2002; Bakircioglu *et al.*, 2003; Moutsatsou *et al.*, 2003; Kim, 2004; Nascentes *et al.*, 2005; Nóbrega *et al.*, 2005; Husáková *et al.*, 2007; Jurado *et al.*, 2007; Neves *et al.*, 2007; Pohl and Prusisz, 2007; Ajtony *et al.*, 2008; Bramanti *et al.*, 2008; Dessuy *et al.*, 2008; Schiavo *et al.*, 2008; Elçi *et al.*, 2009; Monasterio and Wuilloud, 2009; Pohl, 2009). Similarly, investigation of the Fe content of drinks by atomic absorption spectroscopy has led to a number of reports (Costa and Araújo, 2001; Karajdova *et al.*, 2002; Riganakos and Veltsistas, 2003; Ferreira *et al.*, 2008; Pohl and Prusisz, 2009; dos Santos *et al.*, 2009). There are also several useful reviews on the determination of metals in wine (e.g. Aceto *et al.*, 2002) and other drinks.

Apart from the above, there are reports on the determination of selenium in wine by AAS (Jaganathan and Dugar, 1998), sulfur in grape must (Gump *et al.*, 1996), and on AES (including GC-AES) analysis of organosulfur compounds in wine and spirits (Swan, 2000; Campillo *et al.*, 2009), several wine components simultaneously (Paredes *et al.*, 2006) and haloanisoles in wine and cork (Campillo *et al.*, 2004; 2008).

Some investigators have studied metal profiles resulting from atomic spectroscopic investigation with chemometric techniques in order to differentiate alcoholic drinks and to establish authenticity (Peña *et al.*, 1999; Bellido-Milla *et al.*, 2000; Álvarez *et al.*, 2007; Moreno *et al.*, 2007).

Lead and cadmium in alcoholic beverages are common targets for atomic absorption spectroscopists, since unlike some other heavy metals like copper and iron, there should be no sources in any part of the production process and their presence indicates contamination due to pollution.

A graphite furnace atomic absorption spectrometry method with Zeeman background correction, using ammonium dihydrogen phosphate, magnesium nitrate, and palladium nitrate as matrix modifiers, for the direct determination of cadmium in wines was investigated by Jaganathan *et al.*, 1997. Aqueous nitric acid (10%) containing $Pd(NO_3)_2$ at 100 mg of Pd/l was the most satisfactory matrix modifier. The methodological detection limit was 0.03 μg/l and sensitivity, calculated as the characteristic mass, was 0.4 pg. The use of this matrix modifier also results in excellent reproducibility (10% RSD) at such low levels. Wines (16 worldwide samples) were found have 0.045–1.01 μg/l of Cd.

A method has been developed for the direct simultaneous determination of Cd and Pb in wine by electrothermal atomic absorption spectrometry (ETAAS), using a transversely heated graphite tube atomizer (THGA) with longitudinal Zeeman effect background correction (Freschi *et al.*, 2001). The method was satisfactory for red or white wine diluted 1:1 with 0.028 M HNO_3, but when diluted 1:1 with water containing $Pd(NO_3)_2$ (5 μg) and $Mg(NO_3)_2$ (3 μg) as the modifiers and then performing a two step pyrolysis (10 s at 400 °C and 10 s at 600 °C), the formation of carbonaceous residues inside the atomizer was avoided. The characteristic mass was approximately 0.6 pg for Cd and 33 pg for Pb. The limits of detection (LOD) based on integrated absorbance (0.03 μg/l for Cd, 0.8 μg /1 for Pb) were well within the requirements of the Brazilian Food Regulations, which established the maximum permissible level for Cd at 200 μg /1 and for Pb at 500 μg /1. Brazilian wines (six samples) were found to have <0.03–20 μg/l of Cd and 10.0–55 μg/l of Pb.

A method for the analysis of lead in water and wine using FAAS with a preconcentration step has been reported (Bakircogliu *et al*., 2003). Sample digests in 10% nitric acid were pumped through a column containing an immobilized crown ether with a cavity size selective for Pb^{2+}. The column was then rinsed with 2% HNO_3 and the lead was eluted with 0.1 M aqueous ammonium oxalate and delivered to the spectrometer at 4.0 ml/min by a flow injection valve. The procedure was optimized with respect to sample acidity and volume, loading and elution flow rates, eluent composition and volume, where it was shown that only small improvements in LoD (∼ 3 μg/l for 50 ml loaded at 4.0 ml/min) were obtained for volumes of digested wine greater than 50 ml and there were no advantages of loading at low flow rates. Hence using a loading flow rate of about 20 ml/min, 50ml would be loaded in approximately 150 s, corresponding to a sample throughput of 24/h. The method was evaluated through spike recovery for both water and wine. The lead content of three Port wine samples ranged from not detected to 190 μg/1.

Some years later, Monasterio and Wuilloud (2009) reported an FAAS method for cadmium assay in wine using a supported chelating agent to preconcentrate the analyte prior to spectroscopic analysis. The on line preconcentration system was a microcolumn packed with sheepwool on to which 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol was immobilized. The system was optimized, whence a preconcentration factor of 39 was obtained with 20 ml of sample. Good precision and reproducibility were claimed, and the accuracy of the method was good, as shown by comparison of the results with those obtained by ETAAS analysis along with a recovery study. An LOD of 0.037 μg/l was reported. Wine samples (six) had Cd contents in the range not determined to 2.14 μg/l.

Application of GFAAS, using Zeeman background correction, to the determination of lead and cadmium content of a large number of red and white wines (60) showed no statistically significant differences (Kim, 2004). The Pb content and Cd content ranges were 5–87 μg/l and <0.1–3.0 μg/l, respectively. All samples were microwave digested in concentrated HNO_3 and matrix modifiers were ammonium dihydrogen phosphate and magnesium nitrate.

Ajtony *et al.* (2009) have analyzed the As, Cd, Cu and Pb in wine simultaneously using both direct injection of samples and digestion based sample pretreatment methods (adding HNO_3 and/or H_2O_2). The direct injection method was optimized with the application of the chemical modifiers $Pd(NO_3)_2$ and $Mg(NO_3)_2$, giving better reproducibility and allowing the optimal 600 °C and 2200 °C temperatures for pyrolysis and atomization, respectively. A multielement graphite furnace atomic absorption spectrometer (GFAAS), with a transversally heated graphite atomizer (THGA) (as opposed to the more usual end heated graphite atomizer), end capped tubes and integrated graphite platforms (IGPs) was used for the analyses. Zeeman effect background correction was used. The detection limits (LoDs) were found to be 5.0, 0.03, 1.2, and 0.8 μg/l for As, Cd, Cu, and Pb, respectively, and the characteristic mass data were 24 pg, 1.3 pg, 13 pg and 35 pg for As, Cd, Cu and Pb, respectively. For the direct injection method, recoveries were 96–102% for all the metals except arsenic, where a compromise recovery of 45–85% was achieved. For the 35 red and white wine samples studied, the highest metal contents were observed for Cu ranging (20–640 μg/l), then for Pb (6–90 μg/l) and Cd (0.05–16.5 μg/l). Arsenic levels were below the LoD for all samples.

Other than ETAAS (GFAAS) methods or FAAS methods using treated or preconcentrated samples, like those described above, recent atomic spectroscopic determinations of lead, cadmium and other toxic heavy metals in less alcoholic beverages have tended to use more specialized methods that require little or no sample pretreatment or methods that enhance the sensitivity of FAAS toward the analyte (Dessuy *et al.*, 2008; Schiavo *et al.*, 2008; Elçi *et al.*, 2009).

An example of the latter kind can be found in the report of Elçi *et al.* (2009). Here, a flow injection hydride generation (FI-HG) system linked to a flame atomic absorption spectrometer with a flame quartz atomizer was developed for direct determination of lead in wine and rum samples. Samples were acidified to 0.40% (v:v) HCl for wine and to 0.30% (v:v) HCl for rum, which were then mixed on line with 3% (m:v) $K_3Fe(CN)_6$ solution in 0.03% (v:v) HCl prior to reaction with 0.2% (m:v) alkaline $NaBH_4$ solution. Sensitivity of FAAS was increased by the conversion of lead in the sample to volatile lead hyride (PbH_4), via reaction with potassium ferricyanide ($K_3Fe(CN)_6$) (as oxidant) and sodium tetrahydroborate ($NaBH_4$) (as reductant). The Pb contents of a rum and two different red wine samples determined by this method agreed with those obtained by an established ICP-MS method. A detection limit of 0.16 μg/l was achieved for a sample volume of 170 μl, which compares favorably with LoDs from other FAAS methods.

Another example is given by the direct determination of arsenic in beers by ETAAS with deuterium lamp background compensation (Husáková *et al.*, 2007). Here, the use of a Pd modifier, the atomization from platform and insertion of a cooling step before atomization resulted in a 40% increase of sensitivity in peak height measurements. Previously, Arsenic in foodstuffs was determined by much more time consuming and labor intensive AAS methods that involved ashing of samples or the use of hydride generation. Several modifiers were tested, but Pd was the best for the removal of phosphate interferences and it was able to stabilize As at charring temperatures of 1500 °C. Also, addition of the modifier to standards as well as samples made possible the use of matrix free standard solutions for the accurate analysis of diluted degassed beer samples. The accuracy of this method was checked by the comparison of results with those obtained from microwave digested samples measured by an inductively coupled plasma orthogonal acceleration time of flight mass spectrometer (ICP-oa-TOFMS) method. The accuracy was also confirmed by the analysis of certified reference samples. The arsenic content of beers (seven samples) was found to be 30.0–37.8 μg/l, well below the maximum limit of 200 μg/l set by the Czech government.

Thermospray flame furnace atomic absorption spectrometry (TS-FF-AAS) was evaluated for direct determination (without preliminary acid digestion of the sample) of Cu, Cd and Pb in wines and grape juices (Schiavo *et al.*, 2008). The main advantage of TS-FF-AAS was a significant increase in sensitivity. By using a nickel tube associated with a ceramic capillary, the sample was introduced into the flame, where the residence time of the atomic cloud in the atomizer was increased, thus improving the formation of analyte atoms and sensitivity. A sample volume of 150 μl was introduced into a heated nickel tube at a flow rate of 0.54 ml/min

and 0.14 M HNO_3 was used as sample carrier flowing at 2.5 ml/min. Recoveries, LoDs, accuracy (checked with addition recovery methods) and reproducibility compared favorably with the results of previous literature FAAS and GFAAS methods. Furthermore, the optimized method allowed a sample throughput of 45 per hour.

Dessuy *et al.* (2008) have developed a method for the determination of lead in wine by ETAAS without any sample preparation and using calibration against aqueous standards. Several matrix modifiers were tested, but addition of the equivalent of 7.5 μg Pd to the sample gave optimum results. The data obtained for seven wines using this method and a standard acid digestion procedure were compared, where a Student *t*-test did not show any significant difference. Atomization in a transversally heated filter atomizer (THFA) was compared with atomization in a conventional transversally heated platform furnace. The former proved to give higher sensitivity, lowering both the characteristic mass, LoD and reproducibility for aqueous solutions (injection volume of 20 μl). The Pb content found in seven arbitrarily chosen white and red wines was 6–60 μg/l.

Although Thalium compounds are very minor inorganic components of alcoholic drinks, the element is of interest because of its high toxicity (as either Tl^+ or Tl^{3+}). The problem is that the natural thallium contents of beverages are lower than detection limits claimed by both hydride generation AAS (HG-AAS) and electrothermal AAS (ETAAS). An extraction/preconcentration electrothermal atomic absorption spectroscopy (ETAAS) method has been developed for the determination of Tl in wine (Cvetković *et al.*, 2002). The wine sample was decomposed with a mixture of nitric acid and hydrogen peroxide and both Tl(I) and Tl(III) were extracted from 0.5 M KI solution into *iso*-butyl methyl ketone (IBMK). This procedure gave a 50-fold preconcentration and allowed the determination of 0.05 μg/l of Tl in wine. Tartaric acid, silver nitrate and ammonium tetrachloropalladate were used as matrix modifiers to give thermal stability to the highly volatile iodide complexes of Tl. The results using two different ETAAS instruments for this procedure were compared and it appeared that the behavior of the volatile species in the graphite tube during the temperature program depends not only on the chemical and thermal properties of the compound, but also on the tube geometry, internal gas flow, platform type, etc. It was thus recommended that careful optimization of the instrumental parameters is carried out. The Bulgarian and Macedonian wine samples (three) had Tl contents of 0.1–0.8 μg/l.

Both FAAS and GFAAS have been used to estimate a range of metals (other than or including Cd and Pb) in alcoholic beverages. Generally, both techniques are most effective for the analysis of beer or wine (where there are complex organic matrices) when matrix modifiers are employed, but often, distilled spirits can be analyzed without additives. Copper, iron, manganese and zinc in Portuguese wines were determined by FAAS using sequential injection analysis (Costa *et al.*, 2000). Selecting volume samples of up to 750 μl allowed analysis of all the metals above trace levels, but the assay of Cu at trace levels (<0.20 mg/l) required the conversion of free Cu^{2+} to the chelate complex with diethylammonium-*N*,*N*-diethyldithiocarbamate, its retention on and then elution from a C_{18} column coupled to the system. In this case a 2 ml sample allowed the determination of Cu between 0.05 and 0.50 mg/l. Although results using this method compared well with those from reference methods at that time, nowadays there are similar methods with higher sensitivities.

Jurado *et al.* (2007) and Moutsatsou *et al.* (2003) were able to analyze aniseed spirits (Section 3.5.6) and Greek anis type beverage distillery wastes (respectively) without sample pretreatment (other than dilution) for a range of metals, including Pb and Cd, using GFAAS with deuterium background correction and FAAS, respectively. In the case of aniseed spirits, samples were diluted in a dilute aqueous-ethanolic solution of nitric acid. Copper was present in the range 6-473 μg/l, whereas Pb and Cd were present at concentrations less than 6 and 1.4 μg/l, respectively. The high Cu levels were thought to arise, at least in part, from the use of copper stills. The distillation process is well known to give wastes that are high in both toxic organic and metallic residues (see, for example, Sections 3.2.5 and 3.5.3) and consequently disposal of large quantities of waste pose serious environmental problems. The main source of metals in the distillery wastes was the bronze pot stills commonly used for distillation, but another source was the botanicals (aniseed, fennel, star

anise, etc.) (Section 3.5.6) added to the pomace before distillation. Likewise Adam *et al.* (2002) used GFAAS to determine a range of metals in Scotch whisky using 10 μl of sample with neither sample pretreatment nor the use of matrix modifiers.

Direct determination of Cu, Mn, Pb and Zn in Brazilian beers was carried out using thermospray flame furnace AAS (Nascentes *et al.*, 2005). By introducing a sample volume of 300 μl into the hot Ni tube at a flow rate of 0.4 ml/min using 0.14 M nitric acid solution or air as carrier, the sensitivity was increased, leading to low LoDs compared with a standard GFAAS method, which also required the use of a matrix modifier. The beers were directly analyzed after ultrasonic degasification. Results were in agreement with those obtained by GFAAS, at a 95% confidence level.

The determination of iron, manganese and zinc in alcoholic beverages by AAS has received considerable attention, partly because of potential health hazards and partly because of the involvement of Fe and Mn in certain spoilage processes. Cerdeira *et al.* (2001) used sequential injection analysis (SIA) with FAAS for the sequential determination of Fe(III) and total Fe in Portuguese table wines. The sampling rate was 18 samples per hour. The determination of Fe(III) was based on the extraction of the complex formed between Fe(III) and thiocyanate into methyl *iso*-butyl ketone using an on line continuous liquid–liquid extraction device. Following this, the determination of total iron was carried out by sending a small sample aliquot (83–333 μl) directly to the nebulizer. The injection of about 1.8 ml of sample enabled the determination of Fe(III) ranging between 0.10–6.00 mg/l, whereas injection of sample volumes of up to 333 μl enabled the assay of total Fe in the range 0.25–5.00 mg/l. The results compared well with those obtained by reference methods. The speciation of Fe in the nine wine samples was generally somewhat in favor of Fe^{2+} and there appeared to be no correlation of Fe^{2+}/F^{3+} and wine color.

Riganakos and Veltsistas (2003) used FAAS (direct method and an $HNO_3/HClO_4$ digest method) to measure the total Fe content of 36 Greek wines, as a comparison with colorimetric methods for the determination of Fe^{2+} and Fe^{3+}. Agreement between the methods was good, the wines having a range of total Fe between 1.0 and 10.0 mg/l.

More recent determinations of iron in wine have also used FAAS (Ferreira *et al.*, 2008; dos Santos *et al.*, 2009). Manganese and iron in wine were determined by fast sequential FAAS, using the reference element technique to correct for matrix effects (Ferreira *et al.*, 2008). Cobalt, silver, nickel and indium were tested as references, but cobalt and indium at concentrations of 2 and 10 mg/l (respectively) were best for quantification of manganese and iron, respectively, which could be determined with quantification limits of 27 and 40 μg/l, respectively. The content of manganese in 16 tested wines varied from 0.78 to 2.89 mg/l and that of iron from 0.88 to 9.22 mg/l. The analytical results agreed (at the 95% confidence level) with those obtained by ICPOES after complete mineralization of the samples using acid digestion.

A different approach to the analysis of Fe and Mn in wine was recently suggested by dos Santos *et al.* (2009). Here, photo-oxidation with UV radiation/H_2O_2 was used as a sample pretreatment for the determination of iron and manganese in wines FAAS. The method was optimized according to pH, concentration of the buffer solution, concentrated hydrogen peroxide volume and UV irradiation time. The evaluation of sample degradation was monitored by measuring the absorbance at the maximum wavelength of red wine (530 nm). This allowed the determination of Fe and Mn with LoDs of 30 and 22 μg/l, respectively, for a 5 ml volume of digested sample. Accuracy was determined by standard addition/recovery tests and the results were in agreement (at the 95% confidence limit) with those obtained by a standard FAAS acid digestion procedure. Application of this method to six wines found Fe and Mn concentrations of 1.58–2.77 mg/l and 1.30–1.91 mg/l, respectively.

Occasionally, organic compounds in samples can be determined indirectly by AAS. For example, tannins in wines were estimated by FAAS, using a continuous precipitation system (Yebra *et al.*, 1995). Tannins were precipitated by injecting copper acetate complex into the sample, and the unprecipitated copper was determined by atomic absorption spectrometry. The detection limit was 0.7 pg/ml. The results were consistently

lower than those for the same samples obtained by the official Folin–Ciocalteu method (Section 4.4.3), but the latter method is often considered to overestimate total phenolic content.

More recently, total thiol contents of wines have been determined by a method that uses a commercially available flow injection cold vapor mercury analyzer (based on vapor generation of Hg° by $NaBH_4$ reduction) and atomic absorption detection (Bramanti *et al.*, 2008). Instrumental detection limits are as low as 2.5 pg, permitting sample dilution, therefore, minimizing interferences occurring with complex real wine matrices. The results from this method were compared with those from an HPLC method, where agreement was found to be good. Total thiol contents of wine (eight samples) were found to be in the range 11.0–28.2 μmol/l.

Other investigations of the iron, manganese and other metal contents of alcoholic drinks have focused on the partitioning of the metal species in the sample and hence involve fractionation of the sample before AAS analysis (Karadjova *et al.*, 2002; Paleogolos *et al.*, 2002; Pohl, 2007; Pohl, 2009; Pohl and Prusisz, 2009). Karadjova *et al.* (2002) used FAAS to determine Fe, Cu and Zn in different fractions of eight Macedonian and Turkish red and white wines. The charge of the metal species was established using cation and anion exchange separation based on solid phase extraction, XAD-8 resin was used for the separation of wine polyphenols in complexes with wine proteins and polysaccharides and Dowex ion exchange resins were used for the separation of cationic and anionic species of the three metals. The results indicated that almost 30% Fe, about 45% Cu and about 15% Zn are present in wine complexed with polyphenols and proteins, with only a few % of these metals bound to the carbohydrate fraction. The remainder of the metals were present as unbound ('free' or 'labile') cationic species, with Fe(II) being more abundant than Fe(III), as suggested by other studies.

Threefold fractionation of Mn and Zn species in beer was carried out using a column of hydrophobic adsorbing Amberlite XAD7 resin connected in series with a column containing a strong cation exchanger, Dowex 50Wx4 (Pohl and Prusisz, 2007). The fractions (containing hydrophobically bound metal, cation forms and residual neutral or anionic species) were digested in concentrated HNO_3 and H_2O_2 and then subjected to analysis by FAAS. For both Mn and Zn, the major form of the metal in the majority of beers was as cationic forms, but significant fractions of the total metal contents exist as polyphenolic complexes and low molecular mass, hydrophilic, neutral or negatively charged species. A nonionic adsorbing resin Amberlite XAD-16 and a strong cation exchange resin Dowex 50W-x8-200 linked in series was used for the fractionation of Mn in wine and its subsequent analysis by FAAS (Pohl, 2009). The major form of Mn in six wines was found to be the cation form.

A similar tandem arrangement of columns (Amberlite XAD-7HP and Dowex 50W-x8-200) was used to study the fractionation of Fe species in wine (Pohl and Prusisz, 2009). This time the fractions were polyphenol-bound Fe, cationic Fe and neutral/anionic complexes of Fe with organic acids. An Fe(III) single element standard solution made up in a medium imitating wine, containing citrate, tartrate, Ca^{2+}, K^+, Mg^{2+} and Na^+ was used as standard working solution for the calibration of Fe in wine. The big majority of Fe in the wines was present as cationic forms: hydrated cations or cations complexed with hydroxyacids and amino acids.

Since it is known that simple metal ions and complex cations with low molecular weight organic compounds are readily absorbed in the intestines, the above studies suggest that a high percentage of Fe, Mn and Zn in alcoholic beverages (as cationic forms) is readily bioavailable.

A FAAS method using a nonionic surfactant to trap polyphenol bound iron has been used to determine bound and free iron in 10 Greek commercial and homemade wines (Paleogolos *et al.*, 2002). The method makes use of precipitation of polyphenols and bound iron in the micelles of the surfactant. After centrifugation, the surfactant rich phase containing the tannin bound iron was directly aspirated into the nebulizer of an FAA spectrometer after its uptake with a methanolic solution of HCl, KCl and 8-hydroxyquinoline. The supernatant was subjected to the same procedure in the presence of ammonium pyrrolidine dithiocarbamate (APDC), which chelates free iron. The total iron content was also determined by conventional methods for comparison;

agreement between the two methods was good. Less than 10% of the iron in commercial wines was in tannin bound form, whereas this figure for the two domestic wines rose to 23.8% and 30.2%.

Atomic absorption spectroscopy has been used to determine the metal profiles of beers, wines and spirits for discrimination amongst samples and the determination of origin or type, by chemometric analysis (Peña *et al.*, 1999; Bellido-Milla *et al.*, 2000; Álvarez *et al.*, 2007; Moreno *et al.*, 2007). As far as wines are concerned, minerals are very useful composition elements for discrimination according to geographical origin, since the mineral profile should bear a direct relationship with the composition of the soil, although climatic factors and agricultural practices can also affect the mineral profile. The situation is a rather more difficult for beers, because it is likely that many beers are brewed using cereals that may have been be grown in different locations, although some brewers tend to buy their malted cereals from particular maltsters, who in turn tend to buy their grains from particular farmers.

Peña *et al.*, 1999 used FAAS data for Fe, Co, Mn, Ni and Zn, along with product data to discriminate between red wines of the same grape varieties from Ribeira Sacra and Valdeorras regions (Galicia, Spain) (39 samples in total), using principal component analysis (PCA), linear discriminant analysis (LDA) and K nearest neighbors (K-NN). Atomic emission spectroscopy was used to determine K, Li, Na and Rb. The method was able to discriminate between samples, but with a little overlap, suggesting that improvement could be made by use of a bigger reference set.

Atomic emission spectroscopy (AES) is often used in conjunction with inductively coupled plasma (ICP), gas chromatography or high performance liquid chromatography in the determination of a wide range of elements in alcoholic drinks, including nonmetals (such as B, C, Cl, P, S, Se, Si etc.), which are often difficult to determine by other atomic spectroscopy methods. An atomic emission spectrometer linked to a gas chromatograph has proved to be a highly sensitive and selective instrument (GC-AED) for the determination of certain nonmetals (such as Cl, S and Se) in organic compounds. The organic compounds are atomized in the AED and characteristic emission lines for the elements of interest are used for detection and quantification.

For example, the determination of organosulfur and organoselenium compounds in wine has been achieved by GC-AED (Swan, 2000; Campillo *et al.*, 2009). Swan (2000) analyzed red wines for dimethyl sulfide (DMS) and its precursors using this technique. Free DMS present in wines was purged from diluted samples to an adsorbent composed of gold coated glass wool. This was thermally desorbed to a valving and cryogenic trap system coupled to the GC-AED instrument. Permeated methylethylsulfide (MES) was used as an internal standard because the detector showed a compound independent response (at 181 nm) for sulfur from short chain alkylated sulfur compounds. There was no interference from SO_2 because of the high specificity of gold for sulfides. Release of DMS from precursors caused by the addition of aqueous NaOH depended on the concentration of NaOH and hence could only be estimated.

GC with a microwave induced plasma AED has been used to determine eight volatile organic sulfur and selenium compounds in beers, wines and spirits, using headspace SPME (carboxen/polydimethylsiloxane fiber) (Section 4.2.4) to extract and preconcentrate the analytes before GC-AED analysis (Campillo *et al.*, 2009). The scavenger gases in the AED were hydrogen and oxygen, and S and Se were determined simultaneously at 191 nm and 196 nm, respectively. Ethyl methyl sulfide and isopropyl disulfide were used as internal standards. Detection limits ranged from 8 ng/l to 40 ng/l, depending on the compound and the beverage sample analyzed, with a fiber exposure time of 20 min at ambient temperature. The optimized method was successfully applied to 40 different samples, where organosulfur compounds DMS, DMDS and/or methyl propyl sulfide (MPS) were found in half of them, detection levels being in the range 0.04–152 ng/l.

Possibly the most important development in this area was the application of HPLC-ICPAES to the simultaneous determination of alcohols, carbohydrates, carboxylic acids and metals in a range of foodstuffs, including wine (Paredes *et al.*, 2006). Using cation exchange chromatography, chromatograms were obtained by monitoring the carbon emission signal at 193.09 nm. A relatively high RF power (1350 W), with a high

outer gas flow rate (15 l/min) and low intermediate and nebulizer gas flow rates (0.2 and 0.6 l/min, respectively were used in the ICP-AES detector. Five sugars and polyols (fructose, glucose, lactose, sorbitol and sucrose), five carboxylic acids (acetic, citric, lactic, malic and tartaric), three alcohols (ethanol, methanol and glycerol) and seven metals (Al, Ba, Cu, Fe, Mn, Ni and Zn) could be determined in a single chromatographic run. The quantification of these species was in good agreement with results from other methods (HPLC-UV, FI and ICPMS, for example) and LoDs compared favorably with those of existing methods.

Other examples include the determination of 2,4,6-trichloroanisole (TCA) and haloanisoles in wines and cork stoppers (Campillo *et al*., 2004; 2008). Here the organohalogen components were atomized in the AED and were detected and quantified (simultaneously) using the 478 nm and 479 nm emission lines for Br and Cl, respectively.

For the analysis of TCA (Campillo *et al*., 2004), oxygen at 20 psi was used as reagent gas (scavenger gas), whereas oxygen at 5 psi was used for the analysis of haloanisoles (Campillo *et al*., 2008). The LoD for TCA was 25 pg/g for cork and 5ng/l for wine, whereas the LoDs for haloanisoles in wine were in the range 1.2–18.5 ng/l, illustrating the high sensitivity of this method compared with many other methods that have been used to estimate halophenols and haloanisoles in cork and wine. Campillo *et al.* used a purge and trap method of analyte preconcentration for TCA analysis (2004) and SPME for haloanisole analysis (2008).

Dietary intake of silicon is important mainly for the maintenance of bone mineral density. As the element (as silicates) is prevalent in the husk of cereal grains, it is probably this source that gives beer its relatively high silicon content compared with most other alcoholic beverages. Recently, ICP-AES has been used in the determination of Si in a wide range range of beers, produced by a variety of breweries using different cereals or combinations of cereals (barley, rice, sorghum, wheat) (Casey and Bamforth, 2010). The Si content was 6.4–56.5 mg/l, with the highest values generally being associated with the beers of smaller independent breweries, which usually use higher proportions of malted barley in their grist bills (barley husks have high levels of silica). Analysis of the change in Si content during a typical trial brew indicated that much of that element is lost in the spent grains, but significant levels are extracted into the wort and survive into the finished beer. A nitric acid, hydrogen peroxide, hydrofluoric acid digestion was required for ICP-AES analysis of Si in solid samples.

ICP-AES is particularly useful in the determination of metals in alcoholic beverages or in the determination of authenticity (in conjunction with statistical analysis) as the following examples demonstrate.

ICP-OES (AES), ^{1}H NMR and HPLC (ion chromatography) data combined with chemometrics was used to classify 33 red and white wines from three regions of Slovenia and three regions of Apulia (Italy) (Brescia *et al.*, 2003). AES analysis was performed on Al, B, Ba, Cu, Fe, Mn and Zn after mineralization (ashing) of the samples and digesting the ashes in 70% aqueous HNO_3 and 40% aqueous H_2O_2. Differentiation was possible between wines from Slovenia and Apulia, but was unsatisfactory for wines in different regions, probably because of small geoclimatic conditions.

Wines produced at different origins in the Western Cape area (96 samples) were differentiated on the basis of their elemental composition using ICP-AES and stepwise discriminant analysis, canonical discriminant analysis and linear discriminant analysis (Minnaar *et al.*, 2005). Seven grape varieties were used to make the various wines, so classification accuracy was 38–75% for red wines, 68% for Chardonnay white wines and 93% and 100% for Sauvignon Blanc and Chenin Blanc white wines, respectively.

More recently, Álvarez *et al.* (2007) used ICP-AES to study the mineral profile of Montilla-Moriles fino wines (50 samples), according to their content of Zn, P, Mn, Fe, Mg, Cu, Ca, Al, Sr, Ba, Na and K. Samples were digested in H_2O_2, followed by HNO_3 before analysis. The initial results indicated that mineral data sets were non-normally distributed and so nonparametric statistics (median, interquartile range) were applied. The interrelation of element couples was studied using the Spearman nonparametric sample correlation method. Correlations were established between P/Mg, P/Ca, Ca/Al, Ca/Na and Al/Na, thus allowing discimination between Montilla-Moriles wines and similar wines from other regions.

ICP-AES (as well as GFAAS) was used to study the metal content (Al, Ba, Cu, Fe, Mn, Sr, Zn, Ca, K, Na and Mg; and Ni, Pb, respectively) of 54 commercial wines from the Canary Islands (Moreno *et al.*, 2007). Samples were processed by dry ashing followed by solution with 5% nitric acid. Supervised learning pattern recognition procedures were applied and linear discriminant analysis (LDA) led to about 90% of correct classification of wines from two regions in the Canary Islands. Probabilistic neural networks gave better results; sensitivities and specificities higher than 95% for the two classes.

FAAS was used to determine Al, Ca, Cu, Fe, Mg, Mn and Zn in a range of beers, including ales, lagers, stouts and wheat beers (Bellido-Milla *et al.*, 2000). Na and K were determined by FAES. A sample preparation method for AAS, based on treatment with HNO_3 and H_2O_2 in a microwave oven, was developed for this study. UV-visible molecular absorption spectra were used as an index for the organic matter content, and along with conductivity and pH data were used in chemometric analysis. Analysis of variance (ANOVA) showed statistically significant differences among the four types of beers studied were found for Fe, Cu, Mn, Mg, Ca and Na content, conductivity and molecular absorption spectrum. ANOVA also revealed differences between canned and bottled beers, the former having higher Cr and Cu contents. Linear discriminant analysis (LDA) proved capable of grouping the samples correctly, although there was overlap between ales and stouts.

The 16 most abundant metals in 68 distilled spirits from a number of different countries were determined by ICP-AES and the results were analyzed using canonical discriminant analysis and classification binary tree methods in order to establish which of the analyzed metals present the best diagnostic parameters for the determination of authenticity of traditional Cypriot zivania (Kokkinofta *et al.*, 2003). It was found that Cu, Mg and Zn concentrations provided promising parameters for differentiation of zivania from various other spirits, including ouzo, tsipoura, tsikoudia (all Greek), arak, brandy, gin, grappa and vodka.

On the other hand, calcium, copper, iron, magnesium, potassium, sodium, strontium, sulfur and zinc levels were found to be the most discriminant elements, determined by ICP-AES, in the differentiation between silver, gold and aged mescal and Tequila (Section 3.5.5) (Ceballos-Magaña *et al.*, 2009). The application of probalistic neural networks to the data allowed 100% success, thereby providing a reliable method for the authetication of origin and detection of fraud, which is a serious problem regarding Tequila in particular.

4.4.5 Mass Spectrometry

Mass spectrometry (MS) has long been useful in wine analysis as a sensitive mode of detection for GC and HPLC separations, as GC-MS (Section 4.3.2) and LC-MS (Section 4.3.3). In this way, components can be identified by digital comparison of their mass spectra with those in mass spectral libraries (either proprietory ones, like the Wiley or NIST libraries, or in house libraries). Also, new compounds can often be identified from their fragmentation patterns, although other spectroscopic analysis (especially NMR) can be of great help, as in the identification of new pigments in port wine. Similarly, tandem mass spectrometry (especially MS-MS) is very useful at unraveling the structures of more complex components, such as condensed polyphenols. Because of rapid scanning abilities, quadrupole (Q) and ion trap (QIT) mass spectrometers are probably still the most widely used types in GC-MS and LC-MS, despite their disadvantages regarding low resolution and low m/z limits. The reader is referred to Lambert *et al.* (1998), Silverstein *et al.* (2005) and Lee (1998) for general accounts on mass spectrometry and to Smith (2004) and de Hoffmann and Stroobant (2002) for more specialist texts.

For GC-MS and LC-MS, information regarding the chromatographic and interface aspects will be found in Sections 4.3.2 and 4.3.3, respectively, along with a brief account of MS aspects in some cases. The present will be more or less confined to aspects of mass spectrometry.

A schematic layout of a mass spectrometer is shown in Figure 4.4.27, where it can be seen that the essential parts are the ion source, the analyzer, the detector and the computer. Ion sources and analyzers are discussed

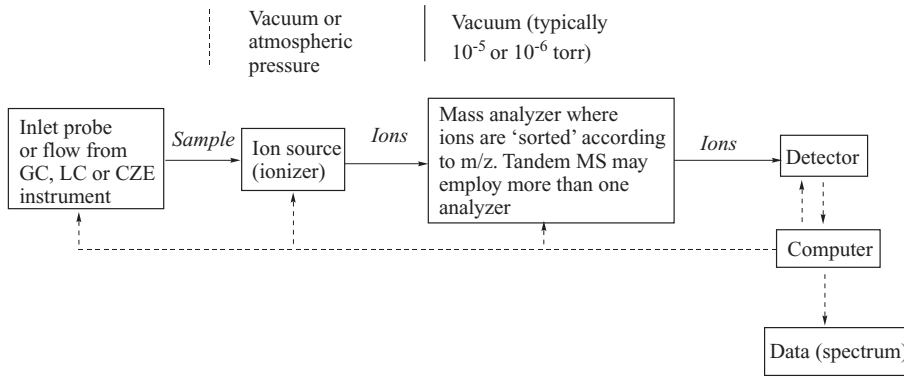


Figure 4.4.27 *Essential features of a mass spectrometer*

in the next paragraphs. The computer and data system are very important in modern MS instruments. The computer not only processes the mass spectral output from the detector, but also controls each section of the instrument in both real experiments and in 'tuning.'

Ionization

This is fundamental to all MS methods: the substance being studied needs to be ionized and its ions subsequently selected (analyzed) and detected. Because ion selection and detection is carried out at high vacuum (10^{-5}–10^{-6} torr; 10^{-3} torr for ion trap analyzers), ionization must occur in the gas phase. Either the substance must be vaporized prior to its ionization (as in EI and CI) or it is vaporized and ionized at the same time (as in ESI).

Electron Ionization (EI)

This is the oldest ionization technique still in use with commercial mass spectrometers; it is still used in GC-MS, but not LC-MS. Electrons are emitted from a hot filament and are accelerated through an electric field of potential difference typically 70 V, after which they have energies of around 70 eV. Fast electrons graze the analyte molecules (which must be in the gas phase) and cause ionization, usually to form molecular radical cations, but sometimes radical anions may be formed by electron capture.

Electrospray Ionization (ESI)

This mode of ionization is most commonly used with HPLC (including capillary column HPLC) and CZE, using mixed aqueous organic solvents (e.g. H_2O/MeCN) and (sometimes) added (volatile) buffer salts, acids or bases to aid ionization. The HPLC or CZE eluent containing the sample is converted to an aerosol by the action of a nebulizing gas and a high voltage applied to the capillary nebulizer tube (Figure 4.4.28). Although ESI is a 'soft' ionization technique (it gives a high abundance of molecular ions), some fragmentation often occurs, especially at higher voltages. In cases where fragmentation needs to be suppressed, ESI can be performed at lower capillary voltages.

An outline mechanism of positive ion production in an electrospray ion source is shown in Figure 4.4.29. The tiny droplets that make up the aerosol contain many protonated solvent and analyte molecules (as well

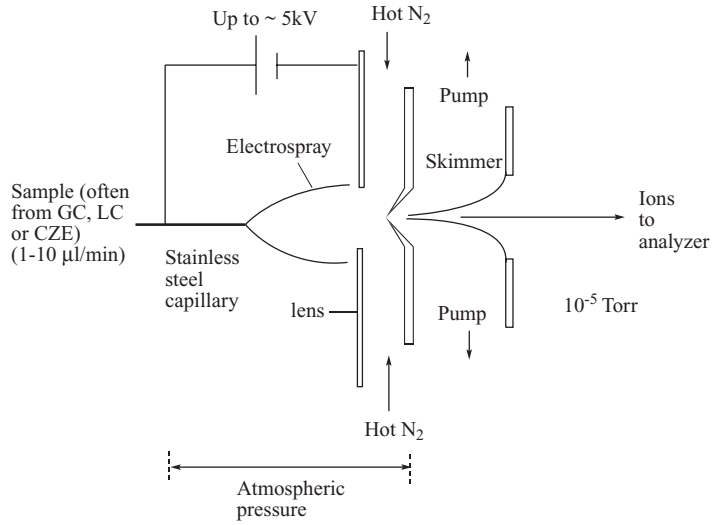


Figure 4.4.28 *Schematic diagram of electrospray ion source. Electospray is a plume of ionized solvent droplets, containing the analyte*

as a great many unprotonated, neutral molecules). A stream of hot gas rapidly vaporizes large numbers of neutral (mainly solvent) molecules, until the droplets become too small for the number of ions they contain. They then explode, releasing their ions, which enter the high vacuum region where any remaining neutral molecules are pumped to waste. The ions are subsequently focused and accelerated (and often gated) using skimmers and octapole lenses into the analyzer space.

Multiple charged ions are formed if there are many ionizable sites in the molecule, as in peptides and proteins, so that the formula masses of large molecules can be determined by ESI – another big advantage over EI. Most analyzers have limits on the size of m/z that can be measured with acceptable accuracy. For example, if a sample molecule has M = 10 000, it would be difficult to measure its m/z value if the ion was merely M^+ or MH^+, but if it has 20 ionizable groups it can form $[M + 20H]^{20+}$ in ESIMS, with an m/z value of 10 020/20 = 501, which is readily measured by a quadrupole or ion trap analyzer.

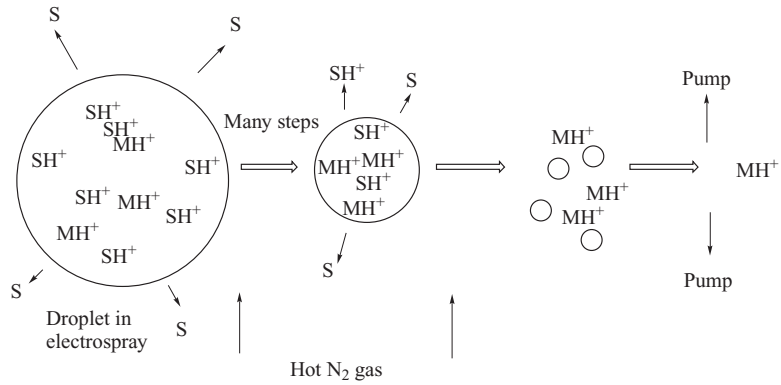


Figure 4.4.29 *Simplified scheme for formation of ions by ESI. Operation here is in positive ion mode*

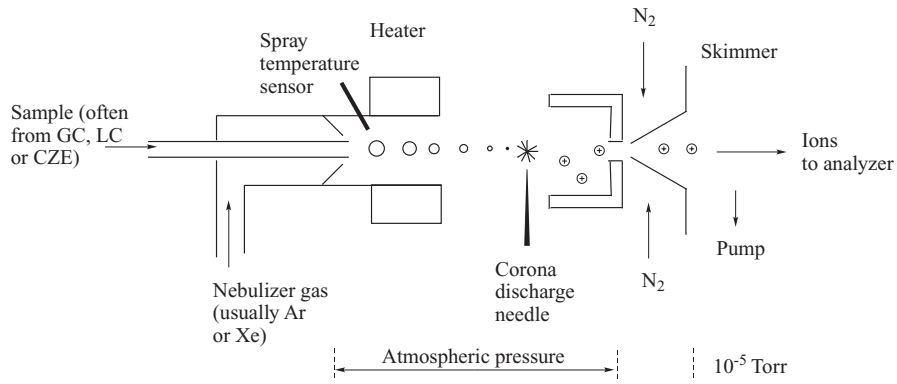


Figure 4.4.30 *Schematic diagram of an APCI source. Chemical ionization of analytes occurs in the micro droplets due to corona discharge*

Atmospheric Pressure Chemical Ionization (APCI)

This method (again popular in LC-MS) is similar to CI (it uses ion–molecule reactions), except that it operates at atmospheric pressure and the primary ions are produced by corona discharge on the eluent spray emerging from the HPLC capillary (Figure 4.4.30).

Analyzers

Mass spectrometers are usually classified according to the nature of their analyzers, where the ions are sorted, irrespective of the type of ion source employed. The two categories are:

- *Dispersive Instruments.* These are capable of separating gas phase ions according to their m/z ratios
- *Nondispersive Instruments.* These detect the presence of ions of selected m/z ratios by a resonance technique, without actually separating them.

The most common analyzers are: (a) magnetic sectors (B), (b) electric sectors (E), (c) electric + magnetic sector (double focus), (d) quadrupoles (Q), (e) ion trap (QIT), (f) time of flight (TOF) and (g) ion cyclotron resonance (ICR). All are dispersive, except (g). Nowadays, many mass spectrometry experiments on alcoholic beverages are carried out using a mass spectrometer that is attached to the outlet capillary of a GC, HPLC or CZE instrument. In these cases, the most widely used analyzers are quadrupole and ion trap, but TOF analyzers are sometimes used, either coupled to a quadrupole in a QTOF tandem mass spectrometer or for specific analysis of macromolecules using matrix assisted laser ionization (MALDI).

Quadrupole Analyzers (Q)

A quadrupole mass analyzer is made up of a set of four exactly straight and parallel metal rods, as shown in Figure 4.4.31. These are cylindrical (though the ideal shape is hyperbolic). Diametrically opposed rods are connected together electrically and both pairs are linked to a direct source of potential (V_{DC}) and an alternating radiofrequency source (V_{RF}). For the two pairs of rods, the DC voltages (V_{DC}) are of equal amplitude and opposite polarity, whereas the RF voltages (V_{RF}) are of equal amplitude and are 180° out of phase. Optimally, $V_{DC}/V_{RF} \leq 0.168$, where V_{DC} is typically 0–200 V and V_{RF} is typically 0–1200 V (peak to peak).

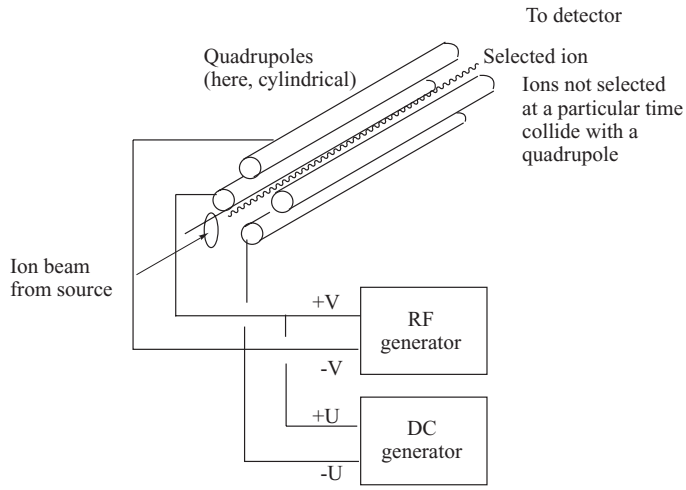


Figure 4.4.31 *Simplified diagram of a quadrupole analyzer*

The combination of DC and RF has no effect on the forward (z) motion of the ions, but instead they are subjected to complex sideways (x and y) motions, due to an electric potential (F) resulting from varying electric fields $+(U + V\cos\,\omega t)$ and $-(U + V\cos\,\omega t)$ and given by Equation 4.4.20.

$$F = \left(\frac{x^2 - y^2}{r^2}\right)(U + V\cos\omega t) \tag{4.4.20}$$

where r is the internal radius, ω is the frequency of the RF field (rad/s) and t is time, U is the DC voltage and V is the RF voltage.

For a fixed m/z value and fixed value of r, there is only a very narrow range of voltages (frequency is usually fixed) for which a stable ion trajectory is available (i.e. a trajectory that allows the ion through the quadrupolar space to the detector). This is called the resonance condition. Otherwise the ions eventually strike one of the rods. Ions of different m/z values can be allowed through to the detector successively very rapidly, so that scanning speeds of 6000 amu/s are routine. High precision engineering and placing of the rods ensures a unit mass resolution for up to 2000 amu.

Ion Trap Analyzers (QIT)

Strictly, these are quadrupole ion traps (hence the abbreviation QIT), since four charged electrodes are used to trap ions (Figure 4.4.32). Some QIT analyzers employ only an RF voltage of fixed frequency but of variable voltage, others allow the application of a DC voltage as well, like quadrupole analyzers. In concept, a QIT analyzer resembles a Q analyzer bent back on itself to form a closed loop, so that ions entering the space between the electrodes are trapped in three dimensional trajectories which are prevented from excessive motion (ions repel) by the presence of helium in the trap at *ca.* 10^{-3} Torr. A resonance condition exists for each m/z for a particular value of V, but a slight increase in V will cause the trajectory to become unstable and the ion is ejected out of the ion trap space to the detector. Alternatively, if an RF voltage at the secular frequency of a particular ion is applied to the end caps (i.e. in the z direction), the ion will come into resonance and its oscillations will be so large that it will be ejected from the trap in the z direction. Like quadrupole

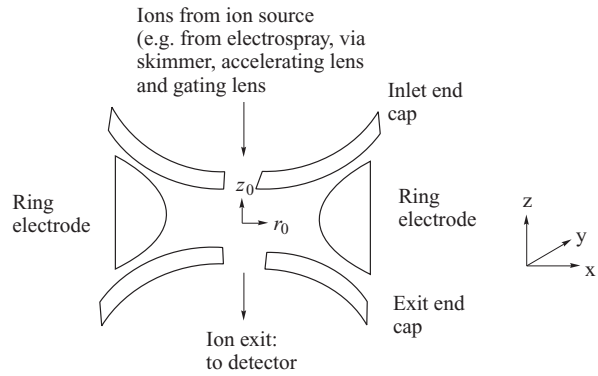


Figure 4.4.32 *Schematic view of an ion trap analyzer. Not to scale*

analyzers, scanning is very rapid. Tandem MS experiments (of the 'in time' type) can be carried using QIT analyzers by collision with helium atoms, which are always present. Fragmentation can be improved by excitation of selected ions at their secular frequency.

Tandem Mass Spectrometry

Sometimes known as MS/MS (or MS^n), tandem MS refers to instruments in which at least two independent stages of m/z analysis are used. This can be done either with the same analyzer (MS/MS 'in time') or using additional analyzers (MS/MS 'in space'). The most common use of tandem MS is in structure determination. Ions produced in one space or at one time undergo fragmentation (usually by collision induced decomposition (CID) with an inert gas), whence the fragment ions are analyzed in a different space or time. CID can be carried out at various energies, using various gases at various pressures, thus controlling fragmentation pathways and maximizing the information available.

The practical limit to the number of analyzers used in MS/MS in space experiments is three or four (MS^3/MS^4). Typical in space tandem MS arrangements are QqQ, QqTOF, BEqQ, BqQ. Tandem MS of the 'in time' type can be performed using quadrupole ion trap (QIT), ion cyclotron resonance (ICR) and time of flight (TOF) analyzers. The practical limit to the number of determinations that can be carried out using in time MS/MS is eight (MS^8).

The most common type of MS/MS instrument is the 'triple quad,' with three quadrupole analyzers, although only two are used for mass spectrometry (see next): its symbol is QqQ. The most usual kind of MS/MS experiment, known as 'product ion scan' is described next in relation to this type. The first quadrupole (Q) is used to select ions (precursor ions) of a particular m/z value. The middle one (q) is used for excitation (usually collisional activation) of these ions. The last quadrupole (Q) analyzes the fragment ions produced by decomposition of the precursor ions in the middle quadrupole space. Product ion scan and other MS/MS experimental types are outlined in Figure 4.4.33.

Mass spectrometry has two major uses in the analysis of alcoholic beverages:

- Identification of components from molecular weight and fragmentation data. Here it is usually combined with GC, LC or capillary electrophoresis, but sometimes direct inlet MS is used and NMR often provides support.
- Isotope ratio mass spectrometry (IRMS). Biological and geographical history influence isotope ratios and hence their determination can provide evidence of origin.

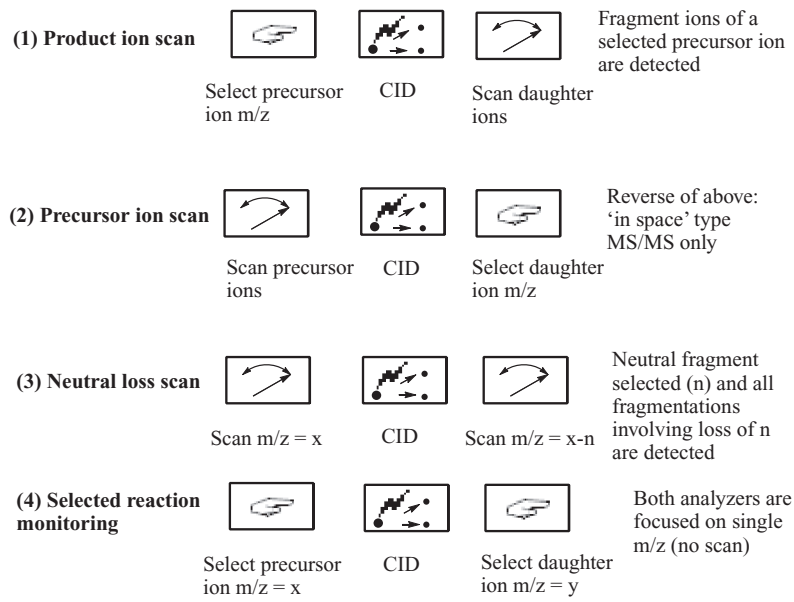


Figure 4.4.33 *Select and scan sequences for tandem MS. These are typical sequences used with QqQ and other tandem MS techniques, with (1) being the most common. (4) is analogous with SIM mode in GC-MS*

Identification and Quantification of Components

The biggest area of application of mass spectrometry (particularly tandem MS) is in the study of polyphenols in fruits and wines, where the big majority of analyses are conducted using LC-MS (Section 4.3.3). Earlier studies used mostly single analyzer MS instruments and many relied on NMR for unambiguous structure determination of new polyphenols. Identity of known polyphenols was achieved by comparing fragmentation with those of standards and/or those in the literature. More recent MS studies often involve tandem instruments, where QqQ and QIT configurations are the most common. Product ion scanning, neutral loss scan and selected reaction monitoring are the most usual modes (Figure 4.4.33), usually MS^2, but occasionally MS^3. Mass spectrometers with QTOF configurations are gaining in popularity, because when operating in product ion scan mode, accurate masses of daughter ions can be used for fragment identification and hence can be a great help in elucidation of the structure of the molecular ion. The application of tandem mass spectrometry to the study of flavonoid phenols has been extensively reviewed (March and Brodbelt, 2008), although there are relatively few examples involving alcoholic beverages or their raw materials.

Direct inlet MS experiments, using a medical syringe infusion pump for example, are comparatively rare in this field, but procyanidin polymers in pear skins (Guyot *et al.*, 1996b), oligomeric anthocyanins in grape skins (Vidal *et al.*, 2004a) and polyphenol standards (Wolfender *et al.*, 1998) have been studied in this manner. The negative ion ESI spectra of pear skins extracted with 0.25% (v:v) aqueous acetic:methanol (75:25) (the acid aided ionization) showed a family of polymeric epicatechin-based procyanidins (Figure 4.4.34) up to a polymerization degree of 17 (Guyot *et al.*, 1996b). The suggestion of epicatechin, rather than catechin monomeric units, was deduced from thioacidolysis experiments. Epicatechin units of mass 290 lose two protons for every interflavan linkage, so that ions at m/z 1153.0, 1441.0 and 2305.5, for example, are consistent with $(M - H)^-$ of tetrameric, pentameric and octameric ions (respectively), for example. Similarly, ions with multiple negative charges were identified; for example the ion at m/z 1663.2 was unambiguously

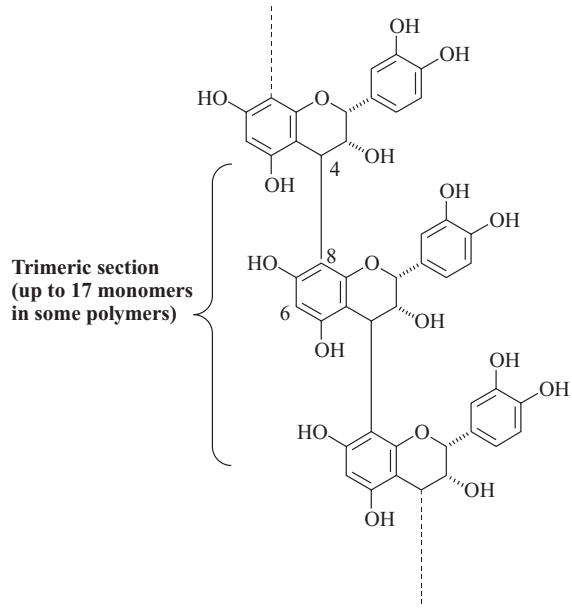


Figure 4.4.34 *General structure of epicatechin based polymeric proanthocyanidins. The existence of 4→6 linkages cannot be excluded. MS alone cannot distinguish between these possibilities. Guyot* et al. *(1996b)*

assigned to $[17\text{-mer} - 3H]^{3-}$. Also, enlargement of the spectrum peaks revealed isotopic clusters that were compared with computer calculated isotopic distribution diagrams for particular molecular formulas. Thus the isotope distribution of the peak at m/z 1585.0 corresponded to that of a molecular formula $C_{165}H_{132}O_{66}$; a doubly charged undecamer ($[11\text{-mer} - 2H]^{2-}$).

Similarly, direct introduction of a multilayer counter current chromatography fraction of a black grape skin extract into the mass spectrometer, using ESI, showed this fraction was composed almost entirely of oligomers, in support of the results of LC-MS analysis, color-bleaching tests with SO_2 and thiolysis (Vidal *et al.*, 2004a). The oligomers were direct condensation products extending as far as trimers, although it was unclear whether the anthocyanin units were linked by a carbon-carbon bond (as in B type proanthocyanidins) or by a carbon–carbon bond and an ether bond as in A type oligomers (see Figure 5.8.7, Section 5.8.6). The terminal unit was in the flavylium form, but the extension units would be in the flavene form for the B type oligomers and the flavan form for the A type oligomers.

The majority of LC-MS studies on polyphenols use electrospray ion sources, although isorhamnetin glycosides were detected and characterized in apple extracts using negative ion APCI (Schieber *et al.*, 2002). In this study, collision induced dissociation spectra were obtained at 20 eV, using xenon as the collision gas (9.1×10^{-4} Torr). Dissociation of a pseudomolecular ion ($[M - H]^-$) at m/z 477 gave a peak at m/z 315, which was assigned to isorhamnetin (aglycone). Isorhamnetin and rhamnetin could be differentiated by performing MS^2 and MS^3 product ion scans on the pure standards and comparing the results with those of LC-MS analysis: CID of isorhamnetin yields an ion at m/z 300 as the most abundant, whereas rhamnetin gave an intense fragment at m/z 165. The presence of isorhamnetin 3-*O* -glucoside was confirmed in apple extracts of the Brettacher variety on the basis of retention time, UV spectra and MS analyses. Tandem MS was able to assign a component that coeluted with quercetin 3-rhamnoside as isorhamnetin 3-*O*-galactoside.

Phenolic profiles of commercial apple juices and the juice of crushed whole eating apples and German cider apples, were determined using LC-MS with negative ion ESI and a triple quadrupole (QqQ) MSD (Kahle *et al.*, 2005). MS^2 experiments were performed at a collision energy of 20–40 eV with argon at 2.0×10^{-3} Torr pressure serving as the collision gas. Model solutions of pure reference compounds were used for identification via MS^2 and/or retention times. The pseudomolecular ion was chosen for either low energy or high energy CID fragmentation studies, according to identity. Thus, collision energies of 15–20 eV were used for chlorogenic acid and other hydroxycinnamic acids, 27–35 eV for dihydrochalcones, 20–30 eV for flavan-3-ols (including dimeric procyanidins) and 26–40 eV for flavonols.

In general, quadrupole (Q) tends to be used rather more than ion trap (QIT) MS instrumentation in the LC-MS analysis of beverage polyphenols (despite the capability of performing MS^3 and MS^4 experiments with QIT), but ion trap MSD is common and has been used to study the phenolic components of colored fruit extracts (González Paramás *et al.*, 2006; Sadilova *et al.*, 2006; Seeram, 2006a; 2006b) and wine (Salas *et al.*, 2004) and also for the detection and characterization of phenolic oxidation products of cider apple juice (variety: Kermerrien) (Bernillon *et al.*, 2004). In the latter study, negative ion ESI spectra were acquired using full scan (m/z 50–2000) and selected ion monitoring (SIM). Monomeric phenols and one dimeric proanthocyanidin (B_2) were identified using HPLC retention times of pure standards. The major polyphenols in unoxidized juice were caffeoylquinic acid, procyanidin B_2, (–)-epicatechin, *p*-coumaroylquinic acid, phloretin xyloglucoside and phlorizdin, as confirmed by their pseudomolecular ions ($[M - H]^-$) at m/z 353, 577, 289, 337, 565 and 435, respectively. Caffeoylquinic acid (CQA) plays a central role in the mechanism of oxidation of cider apple juice; study of oxidized apple juice in the SIM mode at m/z = 641, 705 and 929, showed seven chromatographic peaks corresponding to CQA-catechin dimers, two peaks corresponding to CQA dimers and six peaks corresponding to CQA-procyanidin B_2 dimers. Oxidation increased the number of polymeric proanthocyanidins, as indicated by the broadening and intensification of the unresolved baselines of the HPLC-UV chromatograms.

More recently, Lin and Harnly (2008) used LC-ESI-MS (as well as HPLC-DAD) to characterize the phenolic compounds (flavones, flavonols, flavan-3-ols, hydroxycinnamic acids, hydroxybenzoic acids and others) in the skin extracts of 16 pear varieties. Aglycones in the hydrolyzed skin extracts were also studied. ESI was conducted in both negative and positive modes and at high and low fragmentation voltages. In order to study less concentrated components in the hydrolyzed skin extracts, negative and positive selective ion monitoring detection was used at m/z 271/269 (for trihydroxyflavones), 287/285 (for tetrahydroxyflavone), 303/301 (for pentahydroxyflavone) and 317/315 (for tetrahydroxymethoxyflavone). As a result of this work, phenolic profiles were constructed for 16 pear varieties, whose subclasses (Asian, Yali, fragrant and Bartlett) could be grouped according to principal component analysis of the pear skin phenol data. Pears of the Bartlett group (*P. communis* L.) contained higher levels of isorhamnetin glycosides (including malonyl forms) and lesser amounts of quercetin glycosides, whereas Asian pears contained arbutin and chlorogenic acid, with only traces of flavonoids. Yali pears were richer in 3,5-dicaffeoylquinic acid (rather than chlorogenic acid) and had significant caffeic acid content. Fragrant pears had significant rutin, quercetin 3-*O*-2″-xylosyl-6″-rhamnosylglucoside and isorhamnetin 3-*O*-rutinoside contents.

All the above examples focused on negative ion ESI (and in one case APCI), since the mass spectra of polyphenols generally show more intense peaks in this mode, as the phenolic OH groups are easily ionized. Anthocyanins, on the other hand, are best studied in positive ion mode, because at fruit and wine pH they exist largely in the cationic form. In the following examples of the LC-MS determination of phenolic compounds in red or purple colored fruit or red wine, positive ionization modes are generally used for anthocyanins (and derivatives) (Burns *et al.*, 2003; Dugo *et al.*, 2003; 2004; Salas *et al.*, 2004; Vidal *et al.*, 2004b; Tian *et al.*, 2005; González-Paramás *et al.*, 2006; Sadilova *et al.*, 2006; Seeram *et al.*, 2006a; 2006b; Morata *et al.*, 2007) and negative ionization modes for polyphenols, (Vivas de Gaulejac *et al.*, 2001; Urpí-Sardà *et al.*, 2005; Seeram *et al.*, 2006a; 2006b; Lin and Harnly, 2007), respectively.

LC-MS (using +ESI and –ESI and a QIT analyzer) and HPLC-DAD was used to identify phenolic compounds in strawberries (Seeram *et al.*, 2006a). Here differentiation between ellagic acid and quercetin ($[M-H]^-$ = 301 amu for both aglycones) was achieved by MS^2; the former gives daughter ions at m/z 257 and 229, whilst the latter yields fragments at m/z 179 and 151. Tandem MS was also used to characterize complex hydrolyzable tannins, such as galloyl-bis-hexahydroxydiphenic acid (MS m/z = 935.0; MS^2 m/z = 898, 633, 463, 301, 257, 229) (molecular weight 936). Seeram *et al.* (2006b) also used a similar instrumentation to characterize the phenolic compounds in blackberry, black raspberry, blueberry, cranberry, red raspberry and strawberry extracts. MS^2 fragmentation was carried out at 50% energy and zoom scan analysis was used to determine the charge state of some of the ellagitannin components, which are more common in the *Rubus* species (red raspberry especially) and strawberry (*Fragaria ananassa*). As in many other cases, identities of the components were deduced by matching their molecular ions obtained from LC-MS and LC-MS^2 with literature data for phenolic compounds of small colorful berries (e.g. Maatta-Riihinen *et al.*, 2003; 2004; Wu and Prior, 2005; Zadernowski *et al.*, 2005).

LC-MS using positive ion electrospray has been used to characterize the anthocyanins of Silician blood oranges (Dugo *et al.*, 2003), Cabernet Sauvignon wines and red wines from hybrids (Burns *et al.*, 2003), black raspberry (Tian *et al.*, 2005), thermally degraded black carrot, elderberry and strawberry extracts (Sadilova *et al.*, 2006) and red wines (Dugo *et al.*, 2004). Furthermore, similar techniques have been used to study vitisins A and B, and their vinylphenol adducts in red wine (Section 2.9.2) (Morata *et al.*, 2007), flavanol-anthocyanin adducts in red wine (Salas *et al.*, 2004), flavanol-anthocyanin condensed pigments (González-Paramás *et al.*, 2006).

Determination of complete anthocyanin profiles by LC-MS has been demonstrated to be a more reliable means of differentiating between wines of Cabernet Sauvignon and those from hybrid grapes (without tasting them), rather than use the ratio of acetylated to *p*-coumaroylated derivatives of nine characteristic anthocyanins (Burns *et al.*, 2003). Thus examination of full anthocyanin profiles may lead to detection of adulteration of Cabernet Sauvignon wines.

Anthocyanin adducts and condensation products have received considerable attention from LC-MS. Morata *et al.* (2007) used positive ESI and a single quadrupole analyzer to study anthocyanin–pyruvic acid and anthocyanin–acetaldehyde adducts (vitisin A and B, and their coumaroylated derivatives) (Section 2.10.7) in red wine. The compounds were identified by their molecular ions and their major fragments, which are produced by loss of the sugar moiety, as shown in Figure 4.4.35. Using a QIT mass detector, González-Paramás *et al.* (2006) showed that two minor pigments in the first part of HPLC chromatograms of black grape skin extracts were flavanol–anthocyanin condensation products. Their molecular ions (m/z = 751 and 781) yielded MS^2 fragments that indicated the loss of a hexose moiety (m/z = 589 and 619). MS^3 fragments suggest loss of water (m/z = 571 and 601), loss of $C_6H_6O_3$ (m/z = 463 and 493), flavanol RDA fission (m/z = 437 and 467), partial loss of flavanol (at m/z = 343 and 373) and loss of a flavanol unit (m/z = 301 and 331). The condensation products from this data, were shown to be (epi)catechin-peonidin 3-glucoside and (epi)catechin-malvidin 3-glucoside (Figure 4.4.36). This work was in general agreement with earlier work of Salas *et al.* (2004) on flavanol-anthocyanin pigments in wine.

Although most LC-MS^n studies of phenolic compounds have used product ion scan, Tian *et al.* (2005) used selected reaction monitoring (Figure 4.4.36)(along with selected ion monitoring) to determine the anthocyanins in freeze dried black raspberry juice extracts.

SRM was carried out by measurement of m/z 449→287 for cyanidin 3-glycoside, m/z 581→287 for cyanidin 3-sambubioside, m/z 595→287 for cyanidin 3-rutinoside and m/z 727→287 for cyanidin 3-(2G-xyloylrutinoside). Cyanidin 3-arabinoside (m/z 419→287) was used as an internal standard during extraction and analysis. Although, the majority of this work was carried out using ESI in the positive ion mode, negative ion LC-ESI/MS showed the pseudomolecular ions to be of the type $[M-2H]^-$ and $[\text{aglycone}-2H]^-$. Presumably, the more usual $[M-H]^-$ are formed, but are not detected because of the positive charge residing on anthocyanidins and anthocyanins.

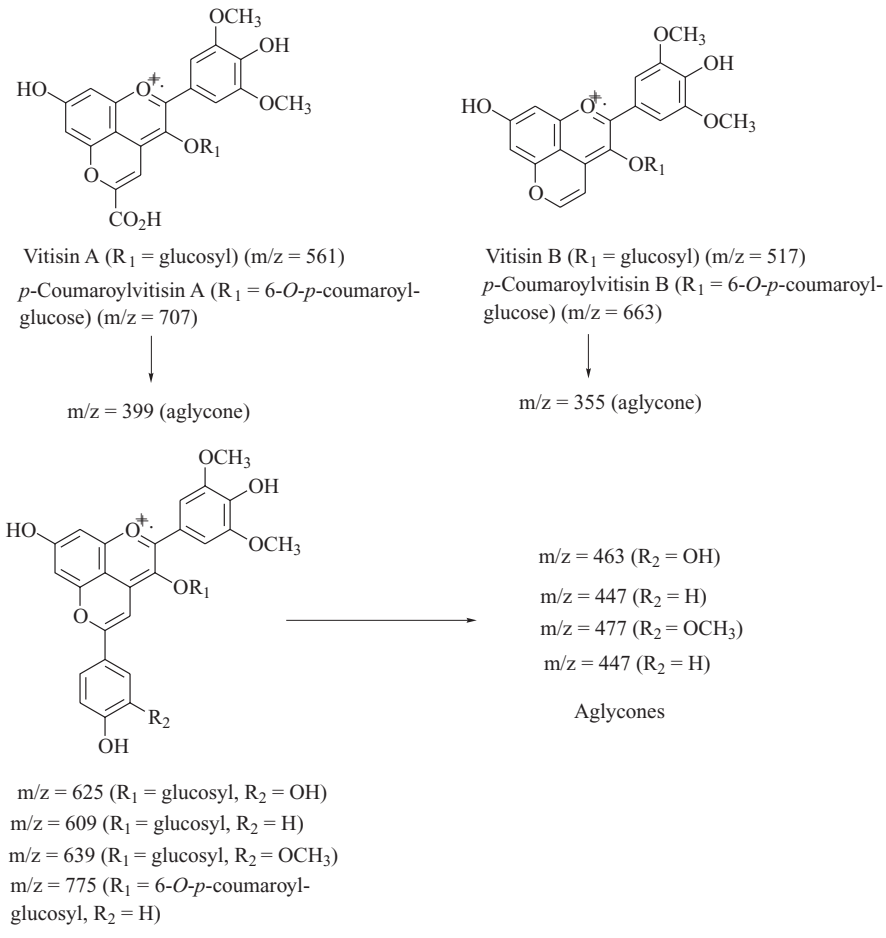


Figure 4.4.35 *Fragmentation of vitisins A and B,* p-*coumaroylvitisins A and B, and their 4-vinylphenol adducts*

Using multiple standard reaction monitoring and negative ESI, Urpí-Sardà *et al.* (2005) were able to identify and quantify resveratrol metabolites in human low density lipoprotein (LDL) for the first time after moderate consumption of red wine, thus giving support to the theory of dietary phenols preventing oxidation of LDL and hence minimizing development of atherosclerosis (Section 5.8.9). Five transitions were monitored for each analysis: resveratrol (m/z 227→185), resveratrol glucosides (389→227), resveratrol glucuronides (403→227) and resveratrol sulfates (307→285). Taxifolin (m/z 303→285) was used as an internal standard and dwell time for each transition was 350 ms with a 5 ms pause between mass ranges. Although several metabolites were found in LDL, the highest concentrations were of *trans*-resveratrol-3-*O*-glucuronide (up to 278.32 pmol/mg of LDL protein), which is known to be synthesized in the liver from dietary *trans*-resveratrol (Tang *et al.*, 2003).

LC-MS has been applied to the study of reactions involving phenolic compounds, as in the thermal degradation of anthocyanins (Sadilova *et al.*, 2006) and the oxidation of red wine (Vivas *et al.*, 2001). In the first mentioned study, MS^2 and MS^3 product ion scans with a QIT analyzer were used to identify the decomposition products of anthocyanins in black carrot, elderberry and strawberry. Upon heating, Cyanidin 3-glucoside ($M^+ = 449$) gave protocatechuic acid (MS^3: m/z (MH^+) 155→111→111) and phloroglucinaldehyde

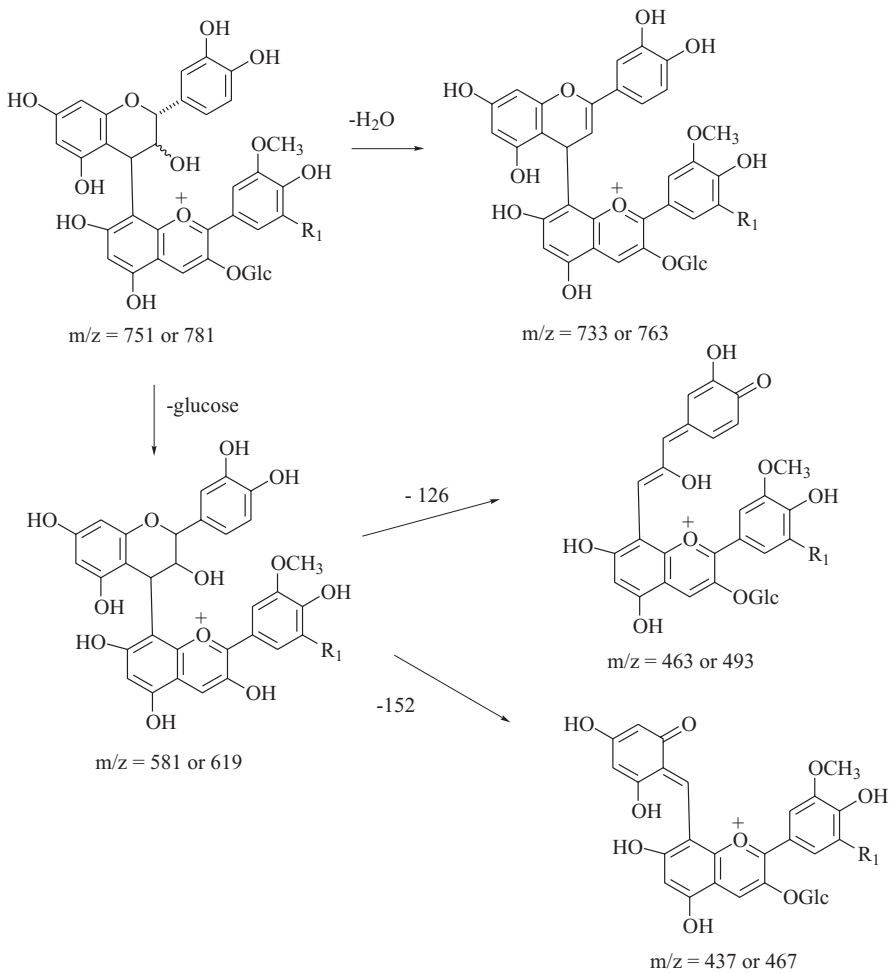


Figure 4.4.36 *Tandem MS fragmentation scheme for flavanol-anthocyanin grape skin pigments. R1 = H or OCH_3 (Gonzalez-Paramas* et al.*, 2006; Salas* et al.*, 2004)*

(MS^2: m/z 155 (MH^+)→127), whereas pelargonidin 3-glucoside ($M^+ = 433$) gave phloroglucinaldehyde and 4-hydroxybenzoic acid (MS^3: m/z 139 (MH^+)→95→95). Also, adducts between thermal decomposition products (e.g. 4-hydroxybenzoic acid) and an original anthocyanidin (e.g. pelargonidin 3-glucoside) (MS^3: m/z 553 (M+)→391→297) were tentatively assigned (e.g. M = 553 ≡ 433 + 138 – 18). In the latter study, low voltage negative ESI was used to identify the quinonic forms of monomeric flavan-3-ols and dimeric procyanidins with regard to their oxidation in red wine. Enzymic oxidation (using laccase or tyrosinase) and chemical oxidation (in the presence of Cu^{2+}or Fe^{2+}) were used as model reactions on pure phenolic compounds. The quinonic forms themselves were unstable, rapidly polymerizing and precipitating, so they were also studied as their stabilized sulfones, produced by reaction with benzenesulfinic acid. Quinonic forms of flavan-3-ols (m/z = 289; sulfonated forms, 429), but not procyanidins, were found in five year old red wine. Interestingly, LC-MS analysis of oxidized model solutions of procyanidin B_2 showed that enzymic oxidation produced just one quinone ring (monosulfonated m/z = 717), whereas chemical oxidation

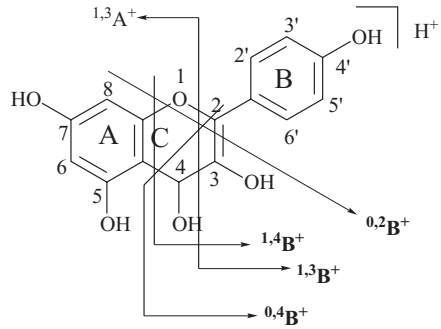


Figure 4.4.37 *Fragmentation (positive ionization mode) nomenclature for flavones, flavanones and isoflavones, illustrated for kaempferol. March and Brodbelt (2008); Ma* et al. *(1997)*

gave two products; one as for enzymic oxidation and the other with two quinonic functions (disulfonated m/z = 855.9).

As seen in the two examples above (Figures 4.4.35 and 4.4.36), anthocyanins and their adducts, and dimeric condensation products with flavanols fragment via a variety of pathways. Likewise, flavonoid aglycone pseudomolecular ions follow many fragmentation routes, as indicated in Figure 4.4.37, which also shows the fragmentation nomenclature commonly used to describe the fragmentation of flavones, flavanones and isoflavones (March and Brodbelt, 2008; Ma *et al.*, 1997).

The astringency of many alcoholic beverages with high polyphenol content (such as red wine) is thought to occur by the precipitation of protein–polyphenol complexes, the proteins originating from the lubricating salivary proteins in the mouth, including PRPs (proline-rich proteins) of the protid and submandibular glands. These proteins are characterized by high proline content and repetitive Pro-Gln-Gly-Pro-Pro sequences. Energy resolved mass spectrometry (ERMS) has been used to study interactions between various flavonoids (monomeric and dimeric) and synthetic segments of PRPs, particularly $IB7_{14}$ (^{1}SerProProProProGlyGlyLysProGlnGlyProProProGlnGlyGly^{14}). DE_{50} values (obtained by monitoring the additional energy needed to separate the partners of a complex) represent the relative affinity of flavonoids for the peptide in the gas phase and present data suggest flavonoid aglycones form weaker 1:1 complexes than the various glycosides (March and Brodbelt, 2008). This data showed epicatechin to be more effective than catechin in binding to $IB7_{14}$, in agreement with sensory analysis, which attributes greater astringency to epicatechin (Thorngate and Noble, 1995; Kallithraka *et al.*, 1997). Also, the data shows procyanidin B_3 (a catechin dimer) to have a much higher affinity for $IB7_{14}$ than catechin, again in agreement with sensory analysis regarding the higher astringency of oligomers over monomers (Simon *et al.*, 2003; Vidal *et al.*, 2003; Vidal *et al.*, 2004b; Preys *et al.*, 2006).

In practice, tandem MS has shown that flavonoid-$IB7_{14}$ complexes with stoichiometries of 1:1 to 4:1, even up to 16:1 for flavan-3-ol- $IB7_{14}$ complexes, suggesting stacking of the flavan-3-ol units, as well as flavanol–peptide interactions.

Other important components of alcoholic beverages have been studied by mass spectrometry. Volatile components (such as aroma compounds) have been determined mainly by GC-MS and are discussed in Section 4.3.2, whereas less volatile constituents are have been studied mostly by LC-MS and discussed here and in Section 4.3.3. These include pentacyclic triterpenes (Arramon *et al.*, 2003), chlorophyll catabolites (Müller *et al.*, 2007; Mendes-Pinto *et al.*, 2005), carotenoids (Mendes-Pinto *et al.*, 2005), proteins (Marangon *et al.*, 2009; Wigand, 2009), Maillard intermediates (Silva Ferreira *et al.*, 2007) and proteins (Marangon *et al.*, 2009; Wigand *et al.*, 2009).

Negative ion electrospray using selected ion monitoring was used to identify pentacyclic triterpenoids in wine and brandy (Armagnac), which are derived from oak casks during the maturation period (Arramon *et al.*, 2003). A cone voltage in the ion source of 30 eV or 75 eV was used, the latter to encourage fragmentation of the pseudomolecular ions ($[M - H]^-$). Four major triterpenoids were characterized: 2α,3β,19α-trihydroxyolean-12-ene-24,28-dioic acid (m/z = 517) and its 28-β-D-glucopyranoside (m/z = 679), 2α,3β,19α, 23-tetrahydroxyolean-12-ene-24,28-dioic acid (m/z = 533) and its 28-β-D-glucopyranoside (m/z = 695). Fragmentation of the two glycosides yielded main daughter ions at m/z 517 and 533, corresponding to the two aglycones. Interestingly, for the two aglycones, there were abundant high mass peaks at m/z 1035 and 1067, corresponding to dimeric quasimolecular ions ($[2M - H]^-$).

Nonfluorescent chlorophyll catabolites (NCCs) (degradation products) in the skins and flesh of apples and pears have been characterized by positive ion ESI MS (Müller *et al.*, 2007). Two major catabolites from pear were found to have accurately measured pseudomolecular ions ($[M + H]^+$) at m/z 807.341 and 645.292, which corresponded to $C_{41}H_{50}N_4O_{13}$ (m/z calc.) = 807.345) and $C_{35}H_{40}N_4O_8$ (m/z calc.) = 645.292), respectively, the former being the glucoside of the latter (see Figure 5.11.1, Section 5.11.2).

Chlorophyll, chlorophyll derived compounds and carotenoids in grapes and Port wine have also been characterized by positive ESI MS (Mendes-Pinto *et al.*, 2005). Eight carotenoids and six chlorophyll derived components isolated from Port wine grapes were discovered by LC-MS. Many of these survived the conversion into wine and identified compounds in Port wine included (with m/z) pheophorbide b (607), (all-*E*) lutein ($M^+ - H_2O$ = 551), (13*Z* or 13′*Z*)-lutein ($M^+ - H_2O$ = 551), pheophytin b (885), pheophytin a (871), (all-*E*)-β-carotene (537) and (13*Z*)-β-carotene (537). Several unknown compounds were classified as either chlorophyll degradation products or carotenoids and several compounds were identified by comparison of UV spectra of standards, but not by MS. Comparison of the HPLC profiles of grape juice and wine showed that wine had higher levels of chlorophyll degradation compounds and no chlorophyll. Also, aged Port wines had lower total carotenoid contents than younger wines, some of the carotenoids presumably having been converted to norisoprenoid flavor components during the aging process.

Application of both APCI and ESI (both in negative and positive ion modes) to the identification of Maillard reaction intermediates in oxidatively aged (which in many cases, is equivalent to spoilage) wine has been carried out by Silva Ferreira *et al.* (2007). The focus here was on certain keto acid and dicarbonyl intermediates, which were analyzed as their quinoxalinol/quinoxaline derivatives. Identification of key intermediates was carried out using product ion scan MS^2, based on fragmentation of pseudomolecular ions, as follows (for negative ion mode): phenylpyruvic acid (m/z 163→91); oxobutyric acid (m/z 100.8→57); ketoglutaric acid (145→101); methylthiobutyric acid (m/z 163→91), optimizing the operational parameters (including collision energy) for maximum intensity of pseudomolecular ions (see Section 4.3.3 for details of LC-MS and Section 2.6.2 for details of the Maillard reaction).

Although proteins are minor components of wine, they can influence wine quality by affecting haze formation, so for this reason alone they are worthy of investigation. The usual method of analysis is to prepare the sample – usually by dialysis and lyophilization and other treatments (Wigand *et al.*, 2009) or by separation into fractions using chromatography (Marangon *et al.*, 2009) – and then to separate the proteins first using SDS-PAGE (Section 4.6.1). The bands from SDS-PAGE separation, after reduction and alkylation, are then subjected to MS analysis, usually via HPLC. In a recent study (Marangon *et al.*, 2009), positive ion electrospray was used to produce ions that were analyzed using a QTOF system. The TOF-MS was set to scan m/z 370–1600 (1.0 s) and the three most abundant multiply charged ions (counts >50) in the scan were sequentially subjected to MS^2 analysis, where spectra were accumulated for 2 s (m/z 100–1600). The resulting mass data were matched against data base searches with a fragment mass tolerance of 0.6 Da, allowance of one missed cleavage per peptide and a number of variable modifications.

In another recent study (Wigand *et al.*, 2009), tryptic peptides were analyzed using positive mode ESI with a NanoLockSpray source, with the lock mass channel being sampled every 30 s. The instrument was

run in data directed acquisition mode, with the three most intense peaks selected for MS^2 fragmentation analysis and fragmentation of the precursor ion being achieved by collision with argon atoms. Collision energy was varied between 15 and 35 eV, depending on the precursor ion mass and charge, and the TOF analyzer integration time was 1 s, with an interscan delay of 0.1 s. The MS data were processed and searched using software and databases containing all known protein sequences from *V. vinifera*, *S. cerevisiae*, known possible contaminants (e.g. trypsin, human keratins) and typical fining agents (e.g. casein, collagens, etc.). The maximum mass error was 15 ppm for precursor ions and 30 ppm for fragment ions, and as in the previous study, one missed cleavage per peptide and certain variable modifications were allowed. All identified peptide sequences were verified by manual interpretation of the fragment spectra.

Tandem MS (again mostly via LC-MS) has been applied extensively to the analysis of pesticide residues in crops, food and beverages, including wine and other alcoholic beverages (Budde, 2004; Picó *et al.*, 2004). ESI and APCI are the major ionization techniques and many analyses are performed using QqQ analyzers, where all modes of MS^2 are used, with product ion scan and neutral loss scan perhaps being the most common. However, the QIT analyzer is particularly sensitive and its MS^n capability makes it especially attractive for analysis of residues, despite less reliable quantification for difficult matrices. So called hybrid instruments, such as Waters/Applied Biosystems QSTAR XL (QTOF), have gained in favor in this area of study, partly as a result of increased availability and increased performance since their inception at the turn in the 1990s. They offer big advantages of accurate mass determination of product ions and very high sensitivity for product ion scan. For example, determination of two insecticides (buprofezin and hexythiazox) in fruit skins used LC-QTOF, with lowest calibration levels of 0.075 and 0.01 mg/kg, respectively (Grimalt *et al.*, 2007). Up to 21 identification points (IPs) were earned by the QTOF system at the highest insecticide level (5mg/kg), and there were 11–21 IPs at the 0.1 and 1 mg/kg levels, thus demonstrating the very high potential of QTOF for identification purposes.

APCI in positive ion mode was used to determine a number of fungicide residues in wine grapes, since one of the fungicides (bitertanol) did not give a signal in ESI and APCI in general was 25–100 times more sensitive for these particular compounds (Juan-García *et al.*, 2004). Full scan LC-MS chromatograms were obtained by scanning from m/z 80–340 (0.68 s). Time scheduled selected ion monitoring of the most abundant ions of each compound was performed as follows (in order of retention times): carboxin (m/z 236, 143), flutriafol (302), pyrimethanil (200), triadimefon (294, 197), tebuconazole (308) and bitertanol (338, 269).

Mycotoxins in foodstuffs, arising via fungal infection, constitutes another area of application of mass spectrometry (again mostly by LC-MS). Deoxynivalenol (DON) and T-2 toxin are just two mycotoxins found in cereal and cereal products that are produced when the crops are infected with *Fusarium* species (Section 5.11.4). Modern mass detectors, such as Finnigan Surveyor MSQ types are able to collect scanning data and SIM data from the same run, thus allowing simultaneous quantitative and qualitative survey data to be obtained from one run (Moncur, 2004). SIM was based on m/z 319 for DON and 489 for T-2 toxin. The ionization method was positive electrospray, giving pseudomolecular ions of the type $(M + Na)^+$. Similarly, a Thermo Scientific TSQ Discovery Max instrument (QqQ type) using heated ESI in positive ion mode was able to determine 12 mycotoxins extracted from food matrices, via LC-MS (Huls *et al.*, 2004). Selected reaction monitoring (SRM) was used, with collision energies being optimized (22–42 eV) (like other MS conditions) using auto tune software. Two product ions were measured for each mycotoxin, one a quantifier ion, the other a qualifier ion, so that identity of the analytes could be confirmed from the ion ratios. The SRM details were (precursor ion, quantifier ion and qualifier ion m/z values): aflatoxin B1 (313, 241, 285); aflatoxin B2 (315, 259, 287); aflatoxin G1 (329, 243, 283); aflatoxin G2 (331, 245, 275); fumonisin B1 (722, 334, 352); fumonisin B2 (706, 336, 318); ochratoxin (404, 239, 221); zearalenon (319, 187, 185); deoxynivanenol (297, 249, 231); diacetoxyscripenol (367, 307, 289). See Figure 5.11.3 in Section 5.11.4 for the structures of some of these mycotoxins.

Inductively coupled plasma-mass spectrometry (ICP-MS) is a highly sensitive technique capable of determining a range of metals and some nonmetals below the picogram level. The fundamentals of ICP are outlined in Section 4.4.4. Like most spectroscopic methods, it can be linked with a chromatographic method, particularly HPLC, thus providing a very sensitive detector. Another advantage of using an ICP-MS detector is its mass selectivity; peaks can be identified and quantified for a particular element even if coelution with other components (containing different elements) is occurring. Often, a quadrupole MS instrument is combined with the ICP source, but TOF and double focusing magnetic sector instruments have also been used.

There are many reports of the application of ICP-MS to the determination of metals and other species in wine. For example, the technique has been used to measure isotope ratios for the determination of origin (Almeida and Vasconselos, 2001), to determine multiple elements for discrimination of origin (Perez-Trujillo *et al.*, 2003; Taylor *et al.*, 2003), anisoles (Gómez-Ariza *et al.*, 2005), lead (Myors *et al.*, 2005) and arsenic species (Wangkarn and Pergantis, 2000).

The Sr isotope ratio $^{87}Sr/^{86}Sr$ in wines (10 samples) was determined by ICP-MS in order to distinguish the wines, which were from a total of six different regions (Almeida and Vasconselos, 2001). After sample preparation by UV irradiation and the removal of Rb interference by a cation exchange process, the sample was evaporated to near dryness, digested in conc. HNO_3, evaporated again to near dryness and the residue was dissolved in dilute aqueous HNO_3 for ICP-MS analysis. After corrections for instrumental and sample bias were applied, the results were able to distinguish between the wines.

Mineral profiles determined by ICP-MS have also been used to characterize wines according to origin. Thirty-nine trace and ultratrace elements (including several rare earth metals) were analyzed in 153 samples of wine from the Canary Islands (Perez-Trujillo *et al.*, 2003). It was found that, using LDA and back propagation artificial neural networks (BP-ANN), it was possible to classify the wines according to the island of origin: the wines of La Palma, Lanzarote and El-Hierro could be distinguished according to their Sr, Rb and Pb content, respectively.

Likewise, simultaneous determination of 34 trace elements in 95 Canadian wines by ICP-MS and treatment of the results with discriminant analysis allowed 100% accuracy using 10 of these elements in the classification of according to origin: Okanagan Valley (British Columbia) and Niagara Peninsular (Ontario) (Taylor *et al.*, 2003). Once again, the Sr level was found to be a good indicator of likely origin. In the ICP part of the method, a high RF power (1500 W) was used to maintain a stable plasma in the presence of ethanol in the samples, even though the samples had been diluted 1:1 with 0.2 M aqueous HNO_3 before analysis. Because of the higher than normal power used, a plasma gas flow rate of 14 l/min was used. In order to determine precision and accuracy, five water reference materials were matrix matched to the wine samples.

In the analysis of important wine contaminants such as Pb, reference methods around the world need to give interconsistent results. The Comité Consultatif pour le Quantité de Matière (CCQM) is an international organization that evaluates primary methods and measurement techniques of high accuracy by making international intercomparisons in many measurement areas. An exact matching isotope dilution mass spectrometric (IDMS) method using ICP has been applied to the determination of Pb in wine (Myors *et al.*, 2005). Isotopic levels (^{206}Pb and ^{208}Pb) in the samples, after digestion in conc. HNO_3, were measured using a magnetic sector instrument at low resolution (300) in order to approximate simultaneous determination. Mass bias correction was made using standard isotope ratio reference materials. Exact matching IDMS is an iterative process in which the initial estimated concentration of the analyte is subjected to iteration and is refined to allow improved matching of the analyte and calibration standard in the next iteration. This process is continued until there is no significant difference between results.

As with other spectroscopic methods, ICP-MS has been used to investigate speciation of elements in samples. Narrow bore ion paired RPHPLC-ICP-MS was used to separate four arsenic species (arsenite, dimethylarsinic acid, monomethylarsonic acid and arsenate), which were determined as As by the quadrupole MS instrument operating in the single ion monitering mode (m/z = 75) (Wangkarn and Pergantis, 2000).

Arsenite at trace levels was the only As species found in eight different wines. The ion pairing agent tetrabutylammonium hydroxide was used to improve the HPLC resolution.

A combination of SPME-GC (Section 4.2.4) with ECD and ICP-MS detectors was used to determine haloanisoles (contaminants that give rise to an off odor in wine known as cork taint) at low levels (ng/l) in wines, using a chemometric method to obtain the highest sensitivity for all analytes simultaneously (Gómez-Ariza *et al.*, 2005). The haloanisoles were analyzed in the mass spectrometer as ^{35}Cl and ^{79}Br after ionization in the plasma with 8.8% oxygen added to the plasma gas to ease the formation of ions.

Isotope Ratio Mass Spectrometry (IRMS)

This technique involves measuring the isotope ratio (typically $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{18}O/^{16}O$) of an analyte that has been converted to a simple gas prior to analysis. This gas (typically, CO, CO or H_2O) must be isotopically representative of the original analyte. The method relies on the fact that the isotope ratios of carbon, hydrogen, nitrogen and oxygen in natural products, such as grape or wine components (e.g. sugars, ethanol and water), depend upon their biological and geographical origins and are influenced by processing conditions, such as additions of sugar, water or the use of must concentration methods. Thus IRMS can be used to check authenticity and to detect fraud. In particular, ethanol and wine water isotopic parameters have been used since the 1980s to check wine authenticity and wine isotopic measurement is now an official EC method that can be used by all wine producing member states of the European Union (EC, 1990; 1997). In general, D/H ratios are measured by SNIF NMR (Section 4.4.1), whereas $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios are determined by IRMS, sometimes involving combustion or pyrolysis of more complex components to simple gases, as mentioned above.

In practice, for the determination of $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios, gas chromatography-isotope ratio mass spectrometry (GC-IRMS) is often used, with on line combustion/pyrolysis units (GC-C/P-IRMS) for the production of simple gases (e.g. CO_2 from ethanol by combustion, CO from ethanol by pyrolysis) (Aguilar-Cisneros *et al.*, 2002). Alternatively, the IRMS instrument can be linked to an elemental analyzer and equipped with a dual gas inlet system from a combustor/pyrolyzer so that IRMS data can be checked against elementary analysis data (Guyon *et al.*, 2006). In all cases, daily system stability checks need to be carried out by measuring reference samples (e.g. ethanol) with known isotope ratios. Also stability checks on working references gases (e.g. CO) need to be performed regularly by measurement of IAEA standards (VPDB, VSMOW, IAEA-CO-9, etc.).

The areas most investigated by IRMS are:

- Addition of exogenous sugar to grape must before or after fermentation
- Dilution of must or wine with water
- Addition of ethanol derived from extraneous sources
- Addition of exogenous glycerol to wines
- Use of exogenous CO_2 for sparkling wines.

Nowadays, for sugar enrichment analysis, isotopic data of the controlled samples are compared to reference points in data banks held by each EU member state (EC, 2000). Data bank maintenance and data collection are supervized by BEVABS, part of the European Commission.

Photosynthesis (the conversion of CO_2 and H_2O in green plants into essential biomolecules, including sugars) occurs by two routes, according to how CO_2 is incorporated into the plant metabolism ('fixed'): the C_3 pathway (the Calvin cycle, via 3-ribulose diphosphate) and the C_4 pathway (the Hatch–Slack pathway, via oxaloacetate). Grapevines and sugar beets use the C_3 pathway, whereas maize ('corn') and sugar cane use

the C_4 pathway. The carbon isotope ratio is defined by Equation 4.4.21.

$$\delta^{13}C\,(‰) = 10^3 \left\{ \frac{R_s}{R_{std}} - 1 \right\} \tag{4.4.21}$$

Here R_s and R_{std} are the $^{13}C/^{12}C$ ratios of the sample and standard, respectively. The references are international standard ratios; for example, VPDB for $^{13}C/^{12}C$ and VSMOW for $^{18}O/^{16}O$, although in many cases, reference gases of known isotopic ratios are used (working standards) and then related to international standards (International Atomic Energy Agency (IAEA) standards).

For components of C_3 plants, $\delta^{13}C$ lies in the range –22‰ to –33‰, whereas for C_4 plant products it is in the range –10‰ to –20‰. Using such methods, it is possible to detect prefermentation addition of cane or 'corn' sugar in still wines (Košir *et al.*, 2001; Ogrinc *et al.*, 2003) and sparkling wines (Martinelli *et al.*, 2003) (including Champagne) by measurement of $\delta^{13}C$ in ethanol and CO_2, respectively. It was also possible to detect added beet sugar in sparkling wines, but not in still wines; for these, measurement of δ^2H is needed and is best done in conjunction with NMR (Ogrinc *et al.*, 2003). Hence IRMS has been useful in the detection of illegal additions of sugar, before fermentation (where the process is called 'chaptalization,' after its inventor, Jean Chaptal – Napoleon Buonaparte's minister of agriculture) and to the finished wine (see Ogrinc *et al.*, 2003 and references therein). However, it should be noted that addition of sugar is often legal, but only in clearly defined situations and in strictly regulated ways, which differ considerably from region to region, and from country to country.

In a study of the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios in the ethanol of samples of commercial and authentic tequila, using GC-C/P-IRMS (Aguilar-Cisneros *et al.*, 2002), it was found that $\delta^{13}C_{VPDB}$ and $\delta^{18}O_{VSMOW}$ data for authentic samples were much more homogeneous, especially the latter, which varied between +22.1 and +22.8‰ for 100% pure Agave tequilas and between +20.8 and 21.7‰ for mixed tequilas (for definitions of Tequila see Section 3.5.5). Most commercial Tequila evidently had a considerable content of exogenous ethanol, probably derived from grain neutral spirits. However, IRMS was unable to distinguish between white, rested and aged categories of authentic 100% agave and mixed Tequilas. The $^{13}C/^{12}C$ isotope ratios were measured in CO_2 produced by combustion of ethanol in an Al_2O_3/Cu/Ni/Pt oxidative reactor and $^{18}O/^{16}O$ ratios were determined in CO produced by pyrolysis of ethanol in an Al_2O_3/Ni/Pt reactor, using a 1% H_2-He auxilliary gas.

In a study of $^{13}C/^{12}C$ isotope ratios in sparkling wines, a needle device with two valves and a connector to a vacuum line was used to obtain samples of CO_2 from the headspace above the wine in unopened bottles of Cava, Granvás, Aguja (semi-sparkling) and gasified wines (González-Martin *et al.*, 1997). The standard $^{13}C/^{12}C$ ratio was that of the international PDB standard. For the Cavas, values of $\delta^{13}C$ ($‰_{PDB}$) ranged between –9.48 and –21.17‰, indicating the presence of CO_2 from two sources: added cane sugar and nutural grape sugar. The lower the numerical values reflect greater the amounts of CO_2 derived from added sugar. The Granvás and Aguja samples had similar values of $\delta^{13}C$, but the gasified wines had generally much higher numerical values (between –28.24 and –39.71‰, much closer to $\delta^{13}C$ values associated with commercial 'food quality' CO_2 (derived from fossil fuel combustion) (from –20.56 to –36.22‰, with most close to 30‰).

The above study analyzed CO_2 in the wine headspace, but a later report describes $\delta^{13}C$ values for the wine and the CO_2 bubbles produced during secondary fermentation for 75 sparkling wines from around the world (Martinelli *et al.*, 2003). Lower numerical values of $\delta^{13}C$ for American, Australian and Brazilian wines (–15.8 to –20.5‰) (n = 28), suggested use of maize or cane sugar in the manufacture of these wines, whereas Argentine, Chilean and European sparkling wines had average $\delta^{13}C$ values of –26.1 ± 1.6‰ (n = 5) and –25.5 ± 1.2‰ (n = 12), respectively, suggesting either less chaptalization or the use of beet sugar. The isotopic composition of the CO_2 bubbles followed similar trends. Thus, the average $\delta^{13}C$ (CO_2) value

for most Brazilian and Argentine wines was –10.8 ± 1.2‰ (n = 23), whereas the average value for most Chilean and European wines was –22.0 ± 1.2‰ (n = 13), suggesting the use of sugar cane (or maize sugar) and beet sugar, respectively, in the promotion of secondary fermentation.

More recently, reports have been made on the influence of enoligical practices on isotope ratios, particularly the newer practices of reverse osmosis (RO) and high vacuum evaporation (HVE) to enrich the must prior to fermentation (Guyon *et al.*, 2006). In the European Union, EC regulation 1493/99 (EC, 1999) states that concentration of must by use of RO or HVE should not exceed 20% and the subsequent increase in alcohol content should not exceed 2%. Ethanol obtained from laboratory fermented wines by distillation was subjected to $^{13}C/^{12}C$ isotope ratio measurement using IRMS and based on CO_2 released upon ethanol combustion. Water $^{18}O/^{16}O$ ratios were measured in CO_2 previously equilibrated with wine or the extracted water. The $\delta^{13}C$ and $\delta^{18}O$ ratio values were given in reference to VPDB and VSMOW, respectively. Measurements were carried out in duplicate with the difference between the two measurements not exceeding 0.1‰ or 0.2‰ for $\delta^{13}C$ and $\delta^{18}O$, respectively. A reference sample was analyzed after every eight samples to control instrument drift. The isotopic ratios of musts and laboratory fermented wines from control musts were compared with those of wines fermented from RO- or HVE-enriched musts. No isotopic fractionation (at sugar or water level) was found for the RO wine, but the HVE process increased wine $\delta^{18}O$ with regard to the initial must.

Determination of $\delta^{18}O$ values for wine water has been used for the assignment of wine origin and as evidence of aldulteration by addition of water to wine. However, data from authentic references wines for the evaluation of measured $\delta^{18}O$ values are not always available and there were no empirical (or model) relationships that give independent estimates of $\delta^{18}O$ for wine water until Hermann and Voerkelius (2009) used a model based on the meteoerological parameters relative humidity and mean temperature for this purpose. The performance of this model was tested on measurement of wine water $\delta^{18}O$ for 775 German wines from six vintages, where differences between computed and mean measured values were $\leq 0.4\%$.

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4.5

Electrochemical Methods

Electrochemical (or electroanalytical) methods depend on the analytes in solution having electrochemical properties; either they are ionized in solution or can be oxidized (lose electrons) or reduced (gain electrons) at electrode surfaces, so that interaction occurs between the analytes and an external measuring electric circuit via the electrodes. It is important that the sample solution containing the analytes is electrically conducting; electrolytes such as potassium chloride or sulfate ('background electrolytes') can be added to boost the conductivity.

Conductance is the simplest electrochemical property of a solution – it is a measure of the inverse of the electrical resistance to the passage of an electric current through a solution. It is not so much used in the analysis of alcoholic beverages, apart from the minicontact test for assessing the tartrate stability of wines (Ribéreau-Gayon *et al.*, 2000), and ion chromatography (Section 4.3.3), which normally uses a conductometric detector. Also, in brewing, there are conductometric methods (e.g. EBC, 1998) for the estimation of the α-acid content of hops and hop products, based on the decrease in conductivity occurring via precipitation of lead ions by bittering substances.

In the minicontact test, the wine is kept at 0–0.1 °C with 4 g/l of potassium hydrogen tartrate added over a maximum period of 18 min. The result of the test is the decrease in the conductivity (in μS/cm) of the wine after this treatment. In experiments on the tartrate stability of Sherry, it was shown that a value of 10 μS/cm was the upper limit for a range of Sherries kept at +4 °C and –4 °C; a wine with test result of 10 μS/cm or less would not form a sediment under these conditions (Gómez Benítez *et al.*, 2003).

Conductance measurements can be used to check the absence of ions in purified water or change in conductance can be monitored during the course of a reaction in which the number of ions in solution changes, or a change in ion mobility occurs.

Potentiometry and voltammetry, in their various forms, are the electrochemical techniques that have wide application to the analysis of alcoholic drinks and other foodstuffs. Potentiometric methods are passive – they measure the solution potential as a function of various physicochemical variables, whereas voltametric methods are active (or dynamic) – they monitor redox behavior as a function of dynamic potential changes. The main electrochemical methods used for the determination of certain analytes in alcoholic beverages are summarized in Figure 4.5.1, where the various versions depend both on the physical characteristics of the instrumentation (e.g. on the type of electrode) and on the parameters that are being measured.

Electrochemical methods for the analysis metals in alcoholic drinks possess some notable advantages over other methods, such as atomic spectrometric or chromatographic methods. They are generally fast, sensitive

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

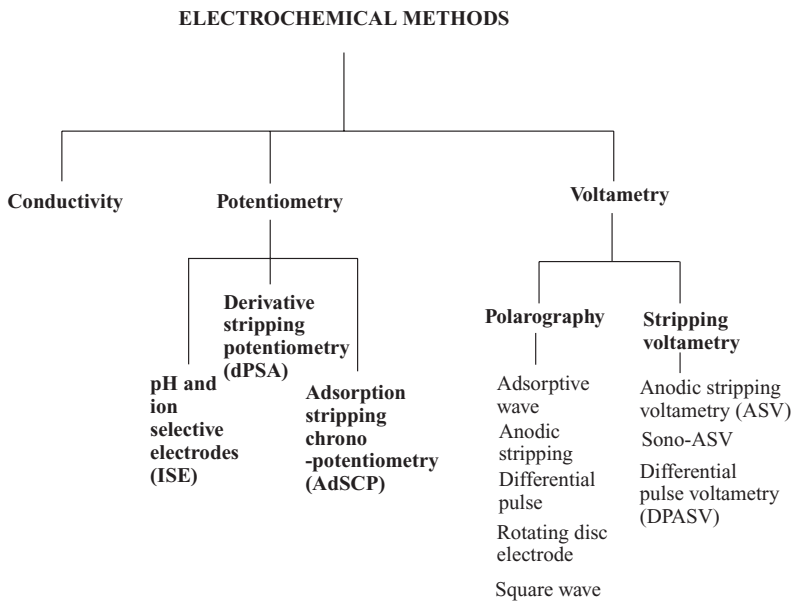

Figure 4.5.1 *Family tree of electrochemical methods used in alcoholic beverage analysis*

and inexpensive, often requiring relatively straightforward instrumentation and manipulation techniques, with little sample pretreatment, other than dilution or matrix modification (additives). Precision and accuracy are often very good and many of the methods are capable of the simultaneous determination of several metals in samples. Although the many variations of electrochemical methods differ in their advantages/challenges, some of them (e.g. potentiometric stripping analysis and related methods) are not affected by high concentrations of organic compounds in the sample and are well suited to the analysis of metals in nondistilled alcoholic beverages, the sample often only requiring slight modification before analysis. On the downside, many of the methods use toxic mercury and mercury (II) compounds and some suffer from interference by other ions (e.g. ion selective electrode methods) or by organic compounds (e.g. some voltammetric methods) in the sample. Also, in some voltammetric experiments, oxidized or reduced analyte can precipitate at the working electrode, thus causing problems by altering the electrode surface and redox characteristics and hence necessitating frequent cleaning of the working electrode. Additionally, potentiometric cells such as ion selective electrodes (ISE) generally need frequent calibration.

Like most chapters of Part 4, the electrochemical analysis chapter assumes a basic undergraduate knowledge of the subject, but an outline of the more important aspects is undertaken during discussion of specific examples relating to alcoholic beverages. Electrochemical methods applied to the analysis of metals in wines and other drinks and foodstuffs have been reviewed by Ibanez *et al.* (2008). For background and further information, the reader is referred to a specialist text on electrochemistry (Brett and Oliveira Brett, 1994), and to a paper on terminology for electrochemical stripping analysis (Fogg and Wang, 1999), as well as to general analytical chemistry texts, such as Skoog *et al.* (1994; 1998) and Tyson (1991).

4.5.1 Potentiometric Methods

Potentiometric methods make use of potential difference caused by redox reactions occurring at the electrodes of an electrochemical cell. These occur at the solid surface of the electrodes via electron transfer between ions

in solution and atoms of the electrode materials [reduction: $M^{n+} + ne^-$ (from electrode) $\rightarrow$ M (at cathode); oxidation: $X^{n-} \rightarrow X + ne^-$ (to electrode, at anode)]. The two electrode systems are connected in the cell via a salt bridge, which is often a concentrated solution of potassium chloride in an agar gel. The bridge provides electrical contact (but not physical contact) between the two electrode systems by allowing transport of K^+ and Cl^- ions across the bridge and at the same time prevents the passage of any other ions.

If the cell is connected to a voltmeter, that instrument will record the net potential difference between the two electrode systems and so all potentiometric measurements are relative. However, if one of the electrodes is a reference electrode, designated as having a potential difference of zero volts, the potential difference of the second electrode system can be can be measured directly from the voltmeter as a 'single electrode potential.' This second electrode is selective to particular ions (M^{n+}/M or X^{n-}/X) and is sometimes known as an ion sensitive electrode, whereas the reference electrode is ion insensitive, being separated from contact with the solution containing M^{n+} or X^{n-} ions. The standard hydrogen electrode (SHE) designated zero potential is that in which H_2 gas at a pressure of 1 atm and temperature of 20 °C is in contact with a 1.228 M solution of H^+ ions (provided by hydrochloric acid and also at 20 °C), via bubbling over a platinum electrode coated with platinum black (finely divided Pt) that dips in the solution.

A much more convenient standard electrode is the saturated calomel electrode (SCE), which has a potential of –0.242 V with respect to the SHE. In practice, a number of reference electrode systems are used, their actual potentials being known accurately with respect to the SHE.

Electrode potentials are related to the concentration (strictly the activity) of ions in solution by the Nernst equation (Equation 4.5.1).

$$E = E^0 \pm \frac{RT}{nF} \ln [X] \tag{4.5.1}$$

where E^0 is the potential (in V) under standard concentration and temperature conditions, R is the gas constant, T is the temperature (in K) and n is the ionic charge (+ for cation, – for anion).

Converting ln to log (base 10) and inserting appropriate values for R, T (298.15 K or 25.00 °C) and F reduces Equation 4.5.1 to the more convenient Equation 4.5.2.

$$E = E^0 \pm \frac{0.059}{n} \log [X] \tag{4.5.2}$$

The main method of using electrochemical cells for analytical measurements is to change the concentration of the ion being determined, keeping all else constant. For direct measurements, the cell needs to be calibrated with a series of standards of accurately known ionic concentrations and then the potential of unknown solutions of the same ion can be obtained and related to concentration via the calibration curve. Cells have a very wide calibration range or 'dynamic range,' since $E \propto \log$ (concentration); a range of 10^5 or 10^6 is not unusual and the pH electrode has a range of $\sim 10^{13}$.

Ion Selective Electrodes (ISE)

The majority of electrochemical cells are of the ion exchange membrane type, where the membrane acts as an ion sensitive electrode, and a reference electrode (usually Ag/AgCl) plus a potential measuring device (usually a voltmeter) complete the circuit. The reference electrode, say of the Ag/AgCl type, is a strip of Ag immersed in a solution containing both AgCl and the ion of interest (e.g. H^+ from aqueous HCl or Ca^{2+} from aqueous $CaCl_2$), which is also in contact with the inner surface of the membrane. As ion exchange occurs, a potential is set up across the interface between the solution containing the ions of interest and the liquid ion exchanger and even though there are no redox reactions here, the Nernst equation is obeyed. If the

ion exchange material is selective toward exchange of a particular ion the cell is known as an ion selective electrode (ISE).

The membranes used in ISE fall into three broad categories:

- *Liquid membrane* (based ion exchange or ion carrier material, neutral or charged)
- *Solid membrane* (e.g. glass, crystal or solid polymer membrane)
- *Membrane in a special electrode* (gas sensing or enzyme electrode). Typically such a membrane contains an analyte-selective component that is responsible for the recognition process

In liquid membrane electrodes (still the most common type of ISE), liquid organic ion exchangers or ion carriers are supported on inert polymers such as cellulose acetate, or in transparent films of polyvinyl chloride. The binding sites are distributed throughout the matrix, the number and type of which determine the polarity, lipophilicity, transport mechanism and other properties of the membrane.

An example of this type is the potassium sensitive electrode, where the membrane is usually a thin PVC disc impregnated with the macrocyclic antibiotic valinomycin. This compound has a hexagonal ring structure with an internal cavity (rather like a crown ether), which is similar to the K^+ ion in size. It forms host–guest complexes with this ion and preferentially conducts it across the membrane. Unfortunately, like the majority of ISE, it is not 100% selective and can suffer interference from cesium, ammonium and other cations, especially if they are present in the sample at high concentrations. Also, such electrodes usually also suffer from low chemical and physical durability and have rather short working lives. Additionally, like other ion selective electrodes, they need frequent calibration.

Crystalline membranes are made from mono- or polycrystallites of a single substance, such as AgCl (selective for Ag^+/Cl^-) or Ag_2S (selective for Ag^+/S^{2-}), respectively, or of two substances, such as PbS/Ag_2S (selective for Pb^{2+}). They generally have good selectivity, which can be for either the cation or the anion of the membrane forming substance. An example of a crystalline membrane electrode is the fluoride selective electrode, which is based on LaF_3 crystals. Unlike most ISE, the fluoride electrode has only one serious interference, from OH^- ions, but this can be minimized by adjusting the pH of the medium to below 7. Table 4.5.1 gives a list of ion selective electrodes commonly used in alcoholic beverage analysis, along with their major interferences.

Ion selective electrodes have been used to routinely determine many inorganic species in must and wine. For a more specialist use, see, for example, Turbow *et al.*, 2002, where ammonia in must and wine was determined with ISE and an enzymic method. Ammonium selective electrodes are sometimes used (in place

Table 4.5.1 *Some ion selective electrodes used in alcoholic beverage analysis*

Electrode = analyte ion measured	Type	Typical concentration range/M	Main interferences
Ca^{2+}	Liquid membrane	$1–5 \times 10^{-7}$	Fe^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Sr^{2+}
Cl^-	Liquid membrane	$1–5 \times 10^{-6}$	I^-, OH^-, $SO_4{}^{2-}$
Cu^{2+}	Crystalline membrane	$10^{-1}–10^{-8}$	Ag^+, Cd^{2+}, Hg^{2+}
F^-	Crystalline membrane	Saturated–10^{-6}	OH^-
K^+	Liquid membrane	$1–10^{-6}$	Cs^+, $NH_4{}^+$, Tl^+
Pb^{2+}	Crystalline membrane	$1–10^{-6}$	Ag^+, Cd^{2+}, Hg^{2+}
Water hardness (Ca^{2+} and Mg^{2+})	Liquid membrane	$1–6 \times 10^{-6}$	Ba^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Sr^{2+}, Zn^{2+}

of distillation and titration) to determine the ammonium content of the Kjeldahl digest (Section 4.6.3) in the determination of total nitrogen or protein content of fruit juice, wort and alcoholic beverages. See also Couto *et al.* (1998), where a homogeneous crystalline silver double membrane tubular electrode operating with a flow injection unit was able to analyze chloride in wine samples at the rate of 90–200 samples per hour.

Easily the most widely used membrane electrode is the glass electrode or pH electrode. The glass membrane is composed of 63% SiO_2, 28% Li_2O, 5% BaO, 2% La_2O_3 and 2% Cs_2O soaked in a hydrated gel ion exchanger that is highly selective toward hydrogen ions. Schematic diagrams of a glass (pH), a liquid membrane and two solid membrane ion selective electrodes are shown in Figure 4.5.2. Measurement of pH is one of the most common routine measurements in the food and alcoholic beverage industries. Not only is pH itself a useful check on quality, but it can be used, along with other parameters, in chemometric discrimination and authentication of origin studies (see, for example, Sections 4.4.1, 4.4.2 and 4.4.4). Additionally, many methods used for the analysis of alcoholic beverages need the pH of the medium containing the analyte to be carefully adjusted (see, for example, the bovine serum albumin assay of total tannin content, Section 4.4.3) for proper functioning of the method.

Although pH measurement by itself it will not necessarily give a definitive indication of quality, it can give the winemaker or brewer an indication of possible trouble and a guide to what needs to be done to prevent or rectify the problem. For example, low pH (high acidity) is required for biological stability, so musts or wines of high pH need acidification, but too low a pH in must indicates immature grapes and a low wine pH may suggest the presence of too much volatile acidity (Chapter 2.4). Similarly, pH measurement of beer wort

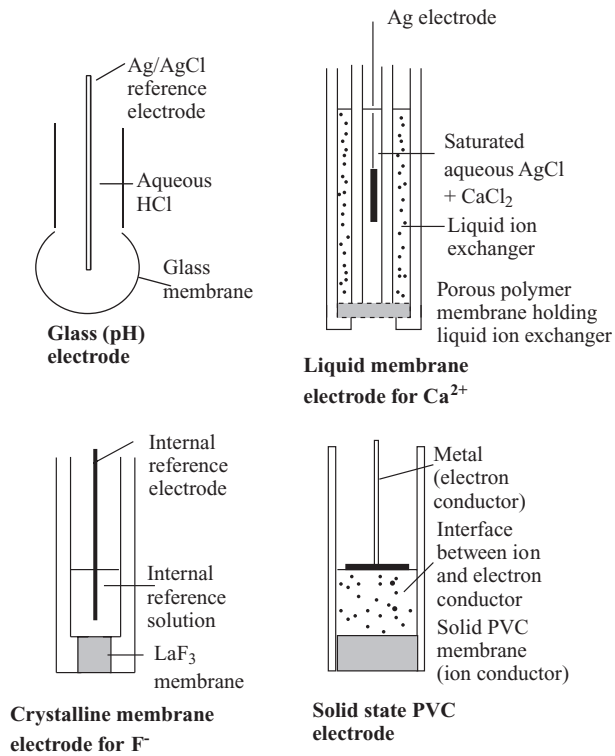

Figure 4.5.2 *Schematic diagrams of common membrane ion selective electrodes. Not drawn to scale*

will indicate whether acidifying salts (such as $CaCl_2$ or $CaSO_4$) or acidifying bacteria (such as *Lactobacillus delbruckii* – see Section 2.6.2) or alkali forming salts (such as $CaCO_3$) need to be added, according to the type of beer being brewed (Section 2.6.2).

Wines and other nondistilled alcoholic beverages, such as beer and cider, are essentially acid–basic buffer solutions, consisting of a number of weak organic and inorganic acids and metal ions. The major acids of wine (in order of decreasing acid strength) are tartaric, malic, lactic and succinic acids; the inorganic acids phosphoric and sulfuric acids are also present at low levels. The major metal ions in wine are ammonium, calcium, potassium and sodium. The superior buffering effect of the unfermented media (e.g. apple juice or grape must) over the corresponding alcoholic drink (e.g. cider or wine) can be seen in the comparison of the pH titration curves, when a strong base is added in portions to medium or wine. The curve for the juice or must is of shallower gradient (Ribéreau-Gayon *et al.*, 2000) than that of the alcoholic beverage, especially around the equivalence point (pH 8–10).

The buffer capacity of a wine or similar beverage is defined, Equation 4.5.3, as the number of base (or acid) equivalents (usually measured in milliequivalents, meq) needed to raise (or lower) the pH by one unit.

$$\beta(\text{buffering capacity}) = \frac{\Delta\,[\text{base}]}{\Delta\,\text{pH}} = \frac{-\Delta\,[\text{acid}]}{\Delta\,\text{pH}} \tag{4.5.3}$$

Although pH, total acidity (TA) and alkalinity of ash (AA) are not related in a simple way (partly because most wine acids are not monoprotic), the approximate Equation 4.5.4 is useful in giving the winemaker an initial idea of the number of milliequivalents of acid or base needed to lower or increase the pH by one unit.

$$\beta = \frac{2.303(\text{TA})(\text{AA})}{(\text{TA}) + (\text{AA})} \tag{4.5.4}$$

For the determination of the total titratable acidity of wine and other beverages, potentiometric titration is often recommended as an alternative method to acid/base titrations with phenolphthalein indicator (Section 4.6.3). The end point is defined as alkali consumption up to pH 7.0 in Europe and the USA or pH 8.2 in Japan.

Hence, a pH meter, with its glass electrode, is an essential piece of equipment for any brewery or winery. The schematic glass electrode shown in Figure 4.5.2 is the conventional type, with a spherical membrane, but versions exist with cylindrical membranes or low surface area membranes that can be used to measure the pH of solid or semi-solid foodstuffs and even the pH of a single grape or fruit. Likewise, hand held battery powered pH meters mean that pH can be measured anywhere in the field, brewery or winery.

Miniaturized and disposable potentiometric electrodes and their application to the analysis of alcoholic drinks (such as in electronic tongues) are dealt with in Section 4.5.3.

Potentiometric Stripping Analysis (PSA)

This technique involves a cycle of events that include electrochemical reduction of cations at the working electrode (cathode) (the enrichment step) and (after a certain time) their chemical oxidation back to cations (the stripping step), as outlined in Figure 4.5.3. A typical PSA instrumentation consists of a glassy carbon working electrode in the electrochemical cell, plated with a mercury film, with an enclosed Ag/AgCl reference electrode and platinum wire counter electrode immersed in a saturated solution of potassium chloride, saturated with silver chloride. This provides an electrical connection to the outer glassy carbon disc of the working electrode via a porous ring. All potentials are measured with respect to the Ag/AgCl (KCl(sat)/AgCl(sat)) reference electrode. The working electrode is coated with a thin mercury film by an electrolysis process, such as of

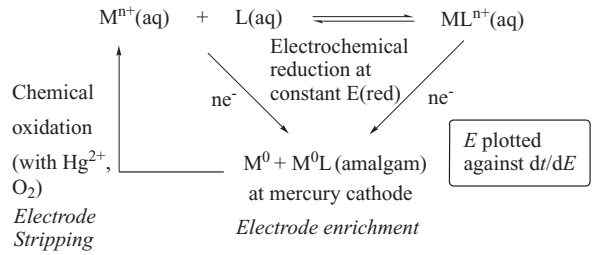

Figure 4.5.3 *Basis of derivative potentiometric stripping analysis (dPSA). Shown for mercury plated glassy carbon working electrode*

a mercury (II) chloride solution (1 g/l) in 1 M hydrochloric acid at –900 V for 1 min against an Ag/AgCl (KCl(sat)/AgCl(sat) reference electrode (Mayer *et al.*, 2003).

A typical method, say for the determination of Cu in wine is as follows. The matrix modifying solution – aqueous HCl and $CaCl_2$, which may also contain the stripping oxidant, such as Hg(II) – and diluted wine sample are mixed in the electrochemical cell. An enrichment potential of –700 mV is used for, say 30 s (although this is dependent on the analyte concentration in the sample under test), with stirring at approximately 2000 rpm and for a further 30 s without stirring. The potentiostat is switched off and the stripping curve recorded over the potential range say –900 to –100 mV. Once stripping had been executed, the electrode is set at a potential of –100 mV for 15 s to clean and restabilize the mercury film.

Often PSA can be carried out on the beverage without sample pretreatment, although matrix modifiers are added to the sample before analysis. For example, HCl and $CaCl_2$ were added to wine samples in order to convert all Cu in the sample to the same species ($CuCl_4^{2-}$) (Green *et al.*, 1997). Matrix modifiers may also be substances that remove interferences, such as potassium metabisulfite added to beer samples in order to oxidize sulfur containing proteins reacting with the thin mercury plating on the working electrode (Mayer *et al.*, 2003). More usually, the sample was merely mixed with hydrochloric acid to lower the pH to ~2 (thereby ensuring the metals are present as free ions) and then filtered (Dugo *et al.*, 2005). In other cases, such as the simultaneous analysis of Cd, Cu and Pb in wine, the samples were digested in concentrated HNO_3 before analysis (Ostapczuk *et al.*, 1997).

Chemical oxidants are often either Hg(II) as in the determination of Cu in wine (Green *et al.*, 1997; Ensafi *et al.*, 2001), the determination of Cu in beer (Mayer *et al.*, 2003) and in the analysis of several metals in Marsala (Dugo *et al.*, 2005), or oxygen as in the simultaneous determination of Cd, Cu and Pb in wine (Ostapczuk *et al.*, 1997) and in the simultaneous analysis of several metals in wine (Salvo *et al.*, 2003).

Mercury film working electrodes have been much used in laboratory potentiometric stripping analyses of alcoholic beverages, but in view of the toxicity of mercury and its compounds, there has been considerable interest in alternative electrodes. In particular, recordable compact disks have been used to make working PSA electrodes and these (CDtrodes) have been used to analyze the Cu content of sugar cane spirits such as cachaça (Section 3.5.2) (Richter *et al.*, 2001). CDs are made of a polycarbonate base on which a thin layer of photosensitive organic dye is deposited. A uniform, mirror-like film (50–100 nm) of very pure gold is fixed on this, followed finally by one, or two layers of protective polymer films. To make CDtrodes, a CD is cut into many slices and the protective films are removed by treatment of each slice with concentrated nitric acid or organic solvents. After washing in a jet of water and drying, a layer of enamel or PVC resin was placed on each strip to determine the electrode area. Electrical contact is supplied by wrapping firmly the bare end of a wire to an electrode with Teflon tape, or by use of a conducting adhesive. These are much cheaper than commercial gold electrodes used for PSA, but give comparable performance and are more versatile (Richter *et al.*, 2001).

A gold working electrode, along with a platinum secondary electrode and a built in Ag/AgCl reference electrode in saturated KCl were used in an automated chronopotentiometric method for the determination of SO_2 in beer (Dvořák *et al.*, 2006). Free and bound SO_2 (Section 2.5.2) were converted to sulfite anions by alkaline hydrolysis of the sample, which was then acidified so that the released SO_2 was separated on line through a semi-permeable membrane and transported to the electrochemical cell by an electrolyte solution. This method had a reproducibility (%RSD) of 8.6% for levels of SO_2 in beer and the results compared well with the official European Brewery Convention method, although the latter method is much slower and more complex.

The stripping step in PSA sometimes suffers interference from organic species present in the test sample, as in the case of alcoholic beverages, especially nondistilled samples. The organic species may alter the surface characteristics of the electrode surface. One way of eliminating this interference is by addition of matrix modifiers such as metabisulfite (Mayer *et al.*, 2003) or complexing agents (Sánchez Misiego *et al.*, 2004). Another way is known as medium exchange PSA. As the name implies, a change of medium from the enrichment to stripping steps is used, sometimes with a series of washes and rinses between the two (Green *et al.*, 1997; Clark and Scollary, 2006). The stripping medium can be water or an aqueous salt solution, such as 4.0 M ammonium acetate (Clark and Scollary, 2006).

However, a regular medium exchange procedure can cause an unpredictable loss of material due to oxidation of analyte in or on the working electrode. To avoid this, the deposition potential can be maintained simultaneously as the exchange solution is introduced into the cell, with stirring (Mikkelsen and Schrøder, 2002).

A version of PSA that is frequently used is derivative potentiometric analysis (dPSA). The stripping potential data are digitally derivatized; E versus t data are converted to E versus dt/dE data, thus presenting a maximum at the point where the conventional PSA curve shows a sharp change of E with t. In this way, resolution and sensitivity are improved. This technique was used to determine Cu in sugar cane spirit (Richter *et al.*, 2001), where the detection limit was found to be 30 ng/l (10 min deposition time) with standard deviation of 1.8% for 20 repetitive measurements using 25 μg/l of Cu with 1 min deposition time. Mayer *et al.* (2003) also used dPSA to determine Cu in beer, where the detection limit was 0.8 μg/l. Dugo *et al.* (2004; 2005) also used derivative potentiometric stripping analysis and derivative adsorption chronopotentiometry to determine Ni^{2+} and a number of metal species, respectively, in alcoholic beverages. The electrochemical parameters for the simultaneous determination of Cd(II), Cu(II), Pb(II) and Zn(II) in Marsala wine (Section 2.10.8) (Dugo *et al.*, 2005) were: –1200 mV electrodeposition potential for 240 s, 0 mV the final acquisition potential, and 120 rpm the agitation speed. For the analysis of Marsala wine, the sample (0.3 ml), ultrapure water (10 ml), Hg(II) (1.0 mg/l) (1.0 ml) and Ga(III) (1.0 mg/l) (0.1 ml) were placed in the electrochemical cell, the Hg(II) being the chemical oxidant. Limits of detection ranged from 0.05 to 0.07 μg/l, reproduciblity was (%RSD) 1.1–2.0% and accuracy expressed in terms of recoveries was 96.8–99.8%.

Another variant of PSA is adsorptive stripping chronopotentiometry (AdSCP), which involves a different enrichment/stripping mechanism rather than different analytical data treatment, as above. The basic mechanism is summarized in Figure 4.5.4. The major differences between PSA and AdSCP are the use of a complexing agent for the analyte and the use of a small stripping current for reduction, rather than a chemical oxidant. Like PSA, glassy carbon mercury film electrodes are frequently used. Adsorptive stripping chronopotentiometry often enables the determination of trace metal concentrations, with a judicious choice of complexing agent and without the need for any sample extraction procedure and preconcentration step that increase the amount of sample handling and may lead to contamination or loss of analyte. However, dilution of the sample prior to analysis minimizes loss of sensitivity toward the analyte caused by the presence of ethanol, sugars and other organic compounds.

AdSCP has been used to determine Ni (II) in many beverages (including wine and spirits) using dimethylglyoxime (DMG) as complexing agent and a glassy carbon mercury film electrode as the working electrode (Dugo *et al.*, 2004). The Ni $(DMG)_2$ complex was adsorbed onto the mercury film of the working electrode

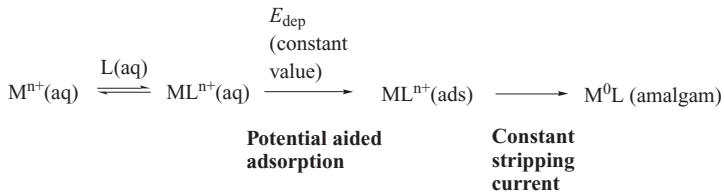

Figure 4.5.4 *Basic mechanism of adsorptive stripping chronopotentiometry*

at an electrolysis potential of –500 mV for 1 min and then reduced by a –5 μA constant cathodic current. The detection limit was 0.2 μg/l at pH 9.5 in ammonia buffer, and the instrumental precision (as %RSD) was 1.5. Accuracy, expressed as recoveries from certified and uncertified matrixes, ranged from 93.0 to 95.5%. Among alcoholic beverages, red wines had the highest Ni (II) content (55.5–105.0 μg/l), whereas white wines, beer and spirits (brandy, vodka and whisky) had 12.0–21.0 μg/l, 10.9–23.4 μg/l and negligible amounts of nickel, respectively.

4.5.2 Voltammetric Methods

Voltammetry is the name that describes a big family of potentiodynamic electrochemical methods, which unlike potentiometric methods, involve changing the working electrode potential at will and measuring the current originating from the system as it responds to the changing potential via redox reactions at the electrode. Linear sweep voltammetry is the simplest form of voltammetric analysis, where the current at a working electrode is measured while the potential between the working electrode and a reference electrode is swept linearly in time. As the analyte undergoes oxidation or reduction, peaks or troughs are observed in the current signal at the working electrode.

Voltammetric methods can be used to study the speciation of metals in alcoholic drinks. Many metals, especially Cu(II) and Zn(II) exist in these beverages as 'free' ions, as labile complexes (those with low formation constants, that easily dissociate) and as tightly bound complexes. In general, metals in the first two of the above forms can be detected using voltammetric methods in samples that need little or no pretreatment (see polarography, later). On the other hand, tightly bound metal concentrations can be found only by substracting the levels of free and labile ions from the total concentration of that ion in the sample. The latter can be found by AAS (Section 4.4.4) or by voltammetric analysis on samples digested in nitric acid.

Apart from the determination of metals in beverages, voltammetry has been used to study redox interactions between different components, such as the sulfur dioxide-ascorbic acid antioxidant system in wine (Bradshaw *et al.*, 2004). By use of square wave voltammetry of ascorbic acid/sulfur dioxide mixtures in model wine solutions and visible spectrophotometric monitoring of the production of yellow catechin polymers under forced oxidative conditions, Bradshaw *et al.* (2004) showed that the presence of ascorbic acid led to enhanced consumption of SO_2 and hence to compromised protection of the wine from browning. This suggested that if winemakers wish to use an ascorbic acid/sulfite mixture to preserve bottled wine from browning, a higher (not lower) amount of sulfite will be needed.

Voltammetric methods have also been used in the indirect analysis of components with relatively high redox potentials, such as ethanol and aliphatic acids, by the use of added oxidants, such as peroxodisulfate ($S_2O_8^{2-}$) (Song *et al.*, 2000) or added reductants such as benzoquinones (Ohtsuki *et al.*, 2001). Polarographic analysis (see later) of the influence of the presence of ethanol on the catalytic current of the sulfate radical anion ($SO_4^{-\bullet}$) was used to assay the ethanol content of distilled spirits (Song *et al.*, 2000). This radical anion

was generated at the electrode surface where it oxidized ethanol to the α-radical, and reduction of this at the electrode produced a polarographic reduction wave. In ethanolic samples in phosphate buffer solution (pH 5.6), in the presence of $K_2S_2O_8$, the first derivative peak current was proportional to ethanol concentration in the range 1×10^{-4}–9×10^{-4} mol/l.

Ohtsuki *et al.* (2001) used a three electrode voltammetric system (plastic formed carbon working electrode, a platinum auxiliary electrode and a standard calomel electrode) to determine the total acid content wines by measuring the reduction prepeak current of 3,5-di-*t*-butyl-1,2-benzoquinone (DBBQ) due to the presence of acids in a nonbuffered model wine solution. The height of the prepeak was proportional to the total acid concentration. The method was tested on 23 wine samples and the results compared well with a reference potentiometric titration method, with the former being superior in sensitivity and consumption of sample.

The most popular method generally is cyclic voltammetry. In a cyclic voltammetry experiment the working electrode potential is ramped linearly versus time. When this potential reaches a set value, it is inverted and inversion can occur several times during the course of a single experiment. The current at the working electrode is plotted versus the applied voltage to give the cyclic voltammogram trace. The usefulness of cyclic voltammetry is highly dependent on the analyte of interest, which has to be redox active within the experimental potential limits. It is also highly desirable for the analyte to display a reversible wave; one in which an analyte is reduced or oxidized on a forward scan and is then reoxidized or rereduced in a predictable way on the return scan.

Cyclic voltammetry was used for the indirect determination of reductones in beer (Section 2.6.2), using the redox dyestuff 2,6-dichlorophenol-indophenol (DCI) (Sobiech *et al.*, 1998). Reductones are antioxidants in beer, originating from hops (bittering substances, polyphenols) and malt (polyphenols, Maillard reaction products). Firstly, a cyclic voltammogram (0–1.1 V at 0.4 V/s) of the sample was recorded and then DCI and ascorbic acid internal standard was added to the sample, where DCI was reduced in quantities that depended on the reductone concentration. Its reduced form was electrochemically reoxidized in the voltammetric experiment, using a constant oxidation working potential of 0.625 V and with no stirring during the reaction of DCI or ascorbic acid with the reductones. After each measurement, the electrode surface was strongly passivated (mainly by DCI and polyphenolic compounds). This necessitated the reactivation of the electrode by use of a square wave generator, which gave a rapid activation at a high redox potential.

Voltammetric methods in general use a three electrode set up: a reference electrode, a working electrode and a counter electrode. Common materials for working electrodes include mercury plated glassy carbon, gold, platinum and silver. The counter electrode is often a platinum wire and the reference electrode is commonly an Ag/AgCl (KCl(sat)/AgCl(sat) electrode or standard calomel electrode. Other working electrode materials have been tested, such as a dental silver amalgam type used for the analysis of heavy metals in wine (Mikkelsen and Schrøder, 2002) and a platinum microdisk electrode used to assay the total acidity of distilled spirits (Baldo *et al.*, 2001). Voltammetry based on the reduction of weak acids at a platinum microelectrode was used for the determination of the total acid content in ethanol–water mixtures of 40–50% (v:v) ethanol content (Baldo *et al.*, 2001). The method was then applied directly to crude samples of distilled spirits (gin, grappa, rum, vodka and whiskey) without sample pretreatment or addition of a supporting electrolyte. The results compared well with those obtained using the AOAC official titrimetric method.

More recently, a titrimetric linear sweep voltammetric analysis of free sulfite in wine samples at a bare glassy carbon electrode has been reported (Scampicchio *et al.*, 2008). The method was based on the reaction of sulfite with Ellman's reagent and voltammetric detection of the equivalence point, either from a plot of electrode current versus concentration or the first derivative of this plot.

Additionally, chemically modified electrodes (CMEs) have been for various analyses (Ijeri and Srivastra, 2000; García *et al.*, 2005; da Silva *et al.*, 2008). Ijeri and Srivastra (2000) used carbon paste electrodes modified with 3% benzo-15-crown-5 crown ether in differential pulse voltammetric experiments to determine Cu(II) in rum. The electrode was constructed by mixing the crown ethers into a graphite powder–paraffin oil

matrix. It gave better voltammetric responses than unmodified electrodes, with a detection limit of 0.05 ppm. By differential pulse anodic stripping voltammetry Cu(II) could be quantified over the range 1 to 100 ppb, with no significant interference from metal ions like Fe(II), Co(II), Mn(II) and Ni(II).

A preactivated glassy polymeric carbon electrode containing a stable electroactive poly(caffeic acid) thin film (containing a quinone moiety), formed by the electrochemical oxidation of caffeic acid (3,4-dihydroxycinnamic acid) (da Silva *et al.*, 2008). The redox properties of the film were investigated at different solution pH values using cyclic and differential pulse voltammetry (DPV), where pH 3.5 was found to give optimum current versus potential curves. The electrode was used in DPV experiments to determine the caffeic acid content of a red wine, the sample being diluted with ammonium acetate buffer (pH 3.5). The concentration determined was 1.08×10^{-5} mol/l, in the range normally associated with red wine, but there was no comparison with established methods for caffeic acid determination reported.

Glassy carbon electrodes modified with films of Prussian Blue (iron hexacyanoferrate) have been used for the determination of free sulfur dioxide in wine (García *et al.*, 2005). The films were electrodeposited and the modified electrodes showed a reversible redox response originating from the oxidation/reduction of iron atoms in the electrode film; it was the Fe(II)-CN-Fe(III)/Fe(III)-CN-Fe(II) redox couple of potential +0.82 V (against SCE) that showed high catalytic activity for the oxidation of sulfite. The detection limit was 5.12 mg/l and there was a precise linear relationship between sulfite concentration and catalytic current. Results for three wines compared well with those from a standard iodometric method.

Stirring or agitation of the solution occurs at some stage in most voltammetric experiments, but during a cyclic voltammetry experiment, the solution is not stirred, resulting in this method's characteristic diffusion controlled peaks. However, stirring the solution between cyclic voltammetry experiments is important so as to supply the electrode surface with fresh analyte for each new experiment. Ultrasound can be used in place of stirring, as in the determination of copper in beer (Agra-Gutierrez *et al.*, 1999) and audible sonication was applied to the analysis of Pb and Zn in wine (Mikkelsen and Schrøder, 2002) by stripping voltammetry. Unlike stirring, which is used only during the deposition step of stripping voltammetry, sonication can be used throughout the entire experiment, and in the case of ultrasound, can be used to clean the working electrode between experiments.

Pulsed techniques, such as normal pulsed, differential pulsed and square wave voltammetry, by monitoring current changes at the working electrode for many analyte redox reactions over a short period of time, are highly sensitive techniques that can used to determine trace amounts of analytes. The peak current is proportional to the analyte concentration. Pulsed voltammetric methods were used to determine Cu in rum (Ijeri and Srivastra, 2000), Pb and Zn in red and white wine (Mikkelsen and Schrøder, 2002), and copper in beer (Agra-Gutierrez *et al.*, 1999)

Stripping Voltammetry

A particularly important group of voltammetric methods in the analysis of alcoholic beverages are the stripping techniques: anodic stripping voltammetry (ASV), cathodic stripping voltammetry CSV) and adsorptive stripping voltammetry (AdSV). These methods have their pulsed and/or differential versions. Like potentiometric stripping (Section 4.5.1), stripping voltammetry involves two steps. The first step is the preconcentration or deposition step, where in ASV, the electrode is held at a suitable negative potential so that the target analyte is reduced onto the electrode surface to form either an amalgam, if using a mercury or mercury coated electrode, or a layer if using a solid electrode. In the second step, the electrode is subjected to a positive potential sweep (in ASV) causing the analyte on or in the electrode to be oxidized, resulting in an anodic current peak that is characteristic of the analyte; its position (E) is related to the analyte identity and its height or area is related to analyte concentration. In favorable circumstances, stripping voltammetric techniques can measure analyte levels down to 10^{-11} M.

Stripping voltammetric methods have been used to determine Cu, Cd, Pb and Zn in wine (Daniele *et al.*, 1989), Cu, Pb and Zn in rum (Barbeira and Stradiotto, 1997), Cu in wine (Wiese and Schwedt, 1997), Cu, Pb and Zn in whiskey, (Barbeira and Stradiotto, 1998), Cu in distilled spirits (Barbeira and Stradiotto, 1999), Cu in beer (Agra-Gutierrez *et al.*, 1999), the analysis of Pb and Zn in wine (Mikkelsen and Schrøder, 2002), Pb and Zn in wine (Mikkelsen *et al.*, 2004), Cu and Zn in wine (Esparza *et al.*, 2007), Pb in wine (Salles *et al.*, 2009) and Cu in tequila (Carreon-Alvarez *et al.*, 2008). The first four reports above describe polarography experiments – using a specific kind of working electrode, known as a hanging mercury drop electrode – and hence will be discussed later.

Mercury thin film and unmodified glassy carbon electrodes were used for the analysis of Cu in beer, by anodic stripping voltammetry with ultrasound agitation, rather than stirring (Agra-Guttierez *et al.*, 1999). Measurable signals were not observed in the absence of ultrasound because of electrode passivation by organic species and lower rates of mass transport. Ultrasound was used for *in situ* cavitational cleaning of the electrode. Results from experiments using either electrode under ultrasound conditions were in excellent agreement with values obtained using atomic absorption spectroscopy, performed by an independent laboratory. Moving on to less hazardous electrode material, a silver–mercury amalgam electrode was used to determine Pb and Zn in several wines and one brandy sample by DPASV, using audible sonication or stirring (Mikkelsen and Schrøder, 2002). Using a similar method, Mikkelsen *at al.* (2004) were able to determine the Pb and Zn contents of 10 wines (red and white), in the ranges 9.7×10^{-8} M–2.4×10^{-7} M for Pb and 2.1×10^{-6}–11.7×10^{-6} M for Zn, with reproducibility (as %RSD) of 3.5% in both cases. The results were verified against atomic absorption spectroscopic results, where correlation was good.

More recent work in stripping voltammetry includes investigations with different electrodes, such as bismuth modified gold microelectrodes (Salles *et al.*, 2009). Bismuth was electrodeposited by an *in situ* procedure in alkaline solution on gold microelectrodes for the determination of Pb(II) in wine square wave anodic stripping voltammetry (SWASV). Even though the deposition time for Pb(II) was relatively high with this electrode, the repeatability of the method was similar to other electrochemical methods, whilst a more extended dynamic concentration range was obtained in comparison with other stripping methods: a linear calibration plot for Pb(II) in the concentration range 40 to 6700 nmol/l was obtained. The detection limit was 12.5 nmol/l.

Recent work has also tended to involve more specialized investigations into speciation and metal–polyphenol or metal–protein complexes. ASV has been used to monitor the fate of Cu and Zn during alcoholic fermentation of wine (Esparza *et al.*, 2007). The Cu concentration dropped from 0.958 to 0.158 m/l and zinc levels decreased from 0.446 to 0.223 mg/l during the course of 32 days of fermentation, presumably as the metal ions formed complexes. Titration results suggested that, on average, natural ligands present in wine produce dissociable 1:1 complexes with Cu and Zn, polyphenols and proteins complexing with Cu(II) and polyphenols with Zn (II). Theoretical calculations suggested that hydrated and chalcone forms of anthocyanins can form viable 1:1 complexes with both Cu and Zn, acting as bidentate ligands, with the remaining four coordination places being occupied by water ligands.

Polarography

This is a special form of voltammetry, where the working electrode is a polarizable dropping mercury electrode (DME), whereas the reference (and also the counter electrode for three electrode systems) is a solid nonpolarizable electrode. The electrochemical response at the DME is determined by combined diffusion/convection mass transport between the analyte and mercury drops regularly falling from a capillary tube. In its simplest form, polarography is a special kind of linear sweep voltammetry where the electrode potential is altered in a linear fashion from its initial to its final potential. Commonly in polarography (e.g. with three electrode systems), the experiment is potentiostatic; it is potential controlled, so that the polarographic curve is a plot of the current passing through the system against the voltage applied to the electrodes, or

on the electrode potential of the dropping electrode. Nowadays, all the variations that apply to voltammetry also apply to polarography, so there is adsorptive wave, anodic stripping, differential pulse, square wave polarography and others.

Despite environmental pressure to curtail the use of mercury, polarography is still a popular technique and has several advantages. Mercury is an effective working electrode because it has a high overpotential toward evolution of hydrogen compared with many other electrode materials and hence many metals can be analyzed, although some metals cannot be analyzed because the positive potential required causes oxidation of mercury. The mercury provides a fresh electrode surface in each drop, thus minimizing contamination caused by adsorption and 'carry over' interferences from previous determinations. This aspect eliminates the need to clean the electrode surface between experiments. Against these advantages, we have the severe toxicity of the metal and the need to use very pure mercury (triple distilled), which is expensive.

Polarography has been widely used in the analysis of alcoholic beverages and indeed it is the oldest electrochemical technique for such purposes. Daniele *et al.* (1989) used DPASV with a mercury microelectrode for the analysis of Cd and Zn in wine at its natural pH, without any sample pretreatment, whereas acidification of the sample with hydrochloric acid to pH 1 allowed simultaneous determination of Cd, Cu, Pb and Zn. Polarographic DPASV was also used to determine Cu in white wine, and in conjunction with ISE analysis and kinetic photometry, the speciation of Cu between ionic, labile and tightly bound forms could be determined (Wiese and Schwedt, 1997). DPASV itself could determine free copper ions and labile Cu(II) species (with complex formation constant $K < 10^{10}$) in nondigested samples. The levels of tightly bound complexes of copper could not be measured in this way, but could be calculated by subtraction of the concentration of ionic and labile species from the total Cu concentration. The latter was determined by DSPASV of concentrated HNO_3/microwave digestions of the samples and by FAAS on the same samples, where agreement was good. In most of the wine samples the dominant form of Cu was as labile species, but tightly bound species were significant in most of the samples and dominant in a few.

Similarly, Barbeira and Stradiotto (1997) used anodic stripping polarography to determine Cu, Pb and Zn in Brazilian sugar cane spirits, without any sample pretreatment or addition of supporting electrolyte. Optimum conditions, using linear sweep ASV were Hg drop size 0.52 mm^2, deposition potential –1.3 V (against Ag/AgCl(sat)), deposition time 10 min., conditioning time 20 s and anodic scan rate 50 mV/s. Detection limits were 0.09, 0.2 and 0.02 mg/l, respectively for Cu, Pb and Zn, considerably lower than the AAS method with which the results matched reasonably well. A similar method was used to analyze the same three metals in whisky, again without any sample pretreatment (Barbeira and Stradiotto, 1998).

Determination of sulfites in alcoholic and other beverages is always of much interest because of the wide use of this useful antioxidant and microbiological stabilizer (Section 2.5.2) and because of the regulations in force that relate to its maximum allowed concentration, due to its possible harmful effects, such as allergenic-like reactions in asthmatics (Section 5.10.2). Several voltammetric methods exist for the analysis of SO_2 and its derivatives in alcoholic beverages. A polarographic method for the determination of free and total sulfur dioxide in beer has been described by Almeida *et al.* (2003). Beer samples were made alkaline, and the aldehyde components were purged from the sample with nitrogen, collected in an electrolyte trapping solution and after derivatization with hydrazine they were determined by polarography. Next, the same sample was strongly acidified and the total SO_2 was purged from the solution, collected and analyzed by polarography, as before. The free SO_2 concentration was calculated from the difference between total SO_2 and aldehyde concentrations. Results were in good agreement with those obtained by the *p*-rosaniline reference method (Section 4.4.3).

Sulfite was determined in wine and fruit juice samples using an indirect amperometric flow injection method (Section 4.6.2) that incorporated a glassy carbon working electrode, a platinum wire auxiliary electrode and an Ag/AgCl (saturated NaCl) reference electrode (Lowinsohn *et al.*, 2004). The current was measured at an

applied potential of 0.0 V, and was proportional to the amount of the Cu^{3+} species chemically generated by the autoxidation of the Cu^{2+} tetraglycine complex induced by sulfite in the presence of traces of Ni^{2+}. The method utilized gas diffusion separation of sulfite (as SO_2) through a PTFE membrane, thereby minimizing matrix interferences. The donor stream at the membrane was deionized water, sample (containing sulfite) and 1 M H_2SO_4, while the acceptor stream consisted of 1mM Cu(II)-tetraglycine solution in a supporting electrolyte solution. The dynamic concentration range was 20–100 mM with a detection limit of 2 mM, with recoveries in the range 92–98%.

Oxygen Electrode

Dissolved oxygen in alcoholic beverages needs careful monitoring, since excessive levels can contribute to a more rapid oxidative deterioration in stored beers (especially) and wines. Probably the most widely used oxygen probe is the 'Clarke oxygen electrode,' which consists of a platinum electrode covered with a polymer membrane (usually Teflon) that is permeable to oxygen, but not to water or electrolytes, and a silver electrode. The poles are connected by a saturated potassium chloride bridge (Figure 4.5.5). Some versions have the platinum electrode in contact with a silver chloride electrode, without the intervening potassium chloride bridge. A constant voltage (~0.6 V) is held across the electrodes so that the Pt electrode is negatively charged (cathode) with respect to the silver electrode (anode). Orbisphere probably produces the best known series of D.O. probes and ancillaries, but battery powered hand held instruments, such as those provided by Hanna, are useful for measuring dissolved oxygen in samples from around the brewery or winery.

When the probe is placed in a sample solution, electrons are donated from the Pt electrode to dissolved oxygen molecules that have permeated the membrane, thus reducing them to hydrogen peroxide, or in the presence of hydrogen ions, to water (Figure 4.5.5). Consequently, electrons flow from the Ag electrode through the electrical circuit to the Pt electrode to regain electrical balance. This current is converted to a voltage that is measured, its magnitude depending on the level of dissolved oxygen. At the same time, chloride ions from the salt bridge combine with Ag^+ to form AgCl at the silver electrode, thus maintaining electrical balance in the probe.

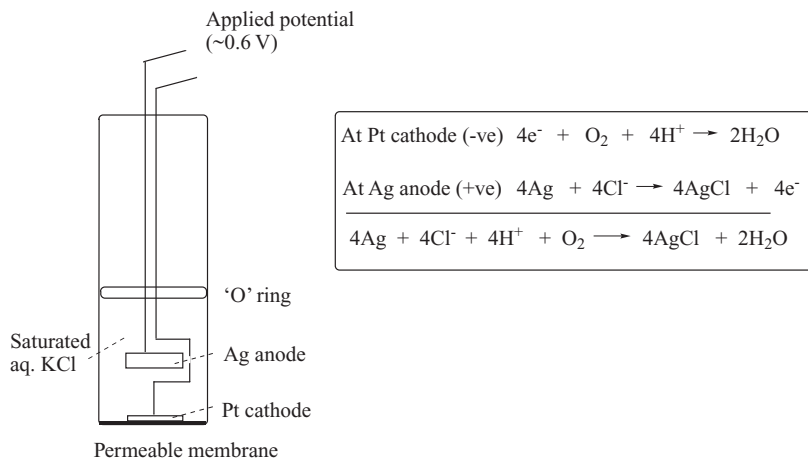

Figure 4.5.5 *Schematic section of a Clarke oxygen electrode and summary of reactions at cathode and anode*

4.5.3 Electrochemical Sensors

A chemical sensor is a device that converts chemical energy (e.g. of redox reactions) to electrical energy or other form of energy, so on this basis the experimental systems discussed in Section 4.5.1 and 4.5.2 (potentiometric methods and voltammetric methods) are sensors. Usually, an analyte recognition process takes place followed by the conversion of chemical information into an electrical signal. Among various classes of chemical sensors, ion selective electrodes (ISE) are one of the most frequently used potentiometric sensors during laboratory analysis as well as in industry, process control, physiological measurements and environmental monitoring.

Discussion here will be limited to electrochemical sensors as miniaturized analytical devices, which can deliver real time and on line information on the presence of specific compounds or ions in complex samples. This includes microelectrode systems developed by photolithographic techniques, giving reduced instrument size, small sample volume requirements, high uniformity and good definition. Also included here are systems based on interdigitated electrodes, which have been used as highly sensitive electrochemical detectors because of their ability to offer large current and higher sensitivity than typical macroelectrodes.

In the analysis of alcoholic beverages and other foodstuffs, electrochemical sensor devices can be divided into two types; those that contain a single electrochemical cell (transducer) that is selective for a single analyte and those that contain an array of nonselective (but cross sensitive) electrochemical cells, as found in electronic tongues and noses. The first type is used to determine the concentration of a specific component, while the second type is used to give a response profile (a 'fingerprint') and, by using chemometric software, can be matched to a sample type, leading to identification of origin or discrimination amongst types. Also, the second kind of sensor may be used to quantify various components (e.g. ethanol, malic acid) and parameters (e.g. pH, °Brix) by training/prediction (calibration/validation) techniques.

Electrochemical sensors use changes in conductometric, potentiometric and voltammetric properties of materials exposed to samples. Sensors of the first variety are often found in electronic noses, of which there are several commercial versions. They typically consist of an array (say 32) of organic polymer based electrically conducting sensors that have adsorptive surfaces, which display changes in electrical resistance on the adsorption of certain components in headspace samples of alcoholic beverages. Such devices have been used to monitor the production of aroma compounds during the fermentation of Muscatel grapes (Pinheiro *et al.*, 2002; 2005) (Figure 4.5.6). In these studies it was found that, without sample pretreatment, the electronic nose could only monitor the formation of ethanol during fermentation. On the other hand, if organophilic pervaporation (with a modified silicone rubber composite membrane) (Section 2.13.3) was used as a concentration sample pretreatment step, the nose was able to detect the aroma compounds, even in the presence of ethanol. Thus under these conditions the nose was able to discriminate between samples and could be used in line to monitor the evolution of aroma compounds during fermentation. The dense, nonporous, hydrophobic membrane separates the upstream side (feed) that contains the feed solution (must/wine) from the downstream side (permeate) containing the compounds to be recovered. The hydrophobic membrane acts as a selective transport barrier, facilitating or preventing sorption and subsequent diffusion of particular components.

Electronic tongues have been devised to discriminate between wine samples and to quantify certain wine parameters, such as ethanol content, pH, shikimic acid content, tartaric acid content and total acidity (Legin *et al.*, 1999). An array of 29 potentiometric sensors that showed poor selectivity, but good cross sensitivity was tested on 22 wines with known parameters (measured by standard methods), mostly from the *V. vinifera* variety Barbera. The sensors were cells with chalcogenide glass and PVC membrane electrodes, including metal ion selective types and ion selective (H^+, Na^+, Cl^-, etc.) types.

Sensor potential values were measured against a conventional Ag/AgCl reference electrode. Partial least squares regression, principal component analysis and self-organising map methods were used to process the results.

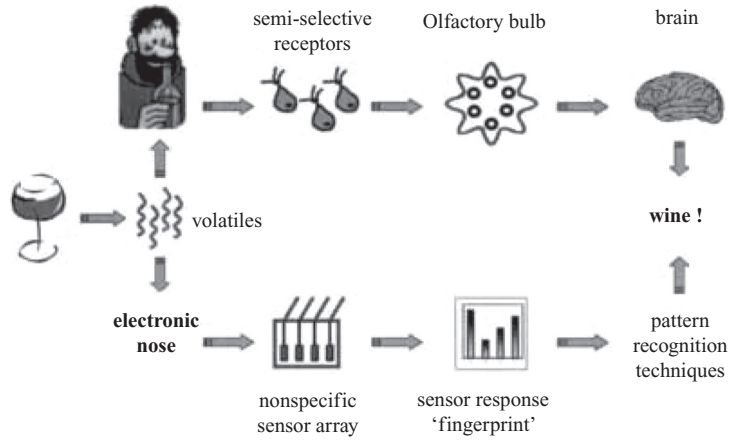

Figure 4.5.6 *Comparison of operation of human olfactory system and electronic nose in identifying wine. Reproduced from Pinheiro* et al. *(2002), with permission of John Wiley & Sons Ltd*

A multisensor system with six ion sensitive field effect transistor (ISFET) sensors monolithically integrated in silicon has been applied as an electronic tongue to discriminate between must and wine samples and to quantify certain must and wine parameters, such as % ABV, °Brix, pH, total acidity and volatile acidity (Moreno i Codinachs *et al.*, 2008). Two sets of multisensors were prepared by depositing ion sensitive membranes on IFSETs: one set with photocurable polymeric membranes sensitive to Ca^{2+}, Cl^{-}, K^{+}, Na^{+}, NH_4^{+} and ions, and the other set with chalcogenide glass membranes sensitive to heavy metal ions (Ag^{+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Tl^{+}). Both multisensor chips contained a pH ISFET sensor. Principal component analysis (PCA) and soft independent modelling class analogy (SIMCA) were used on the data for the pattern recognition and classification of samples and partial least squares (PLS) regression for quantification of the wine parameters.

More recently, an electronic tongue composed of 18 potentiometric chemical sensors was applied to the quantitative analysis of 50 Belgian and Dutch beers of a wide range of styles (Polshin *et al.*, 2010). The main physicochemical parameters (bitterness, CO_2 content, color, ethanol content, pH, polyphenol content, real extract and real fermentation degree) of the same samples were analyzed by standard methods, and relationships between the potentiometric and physicochemical data sets were investigated using canonical correlation analysis. Calibration models with regard to the physicochemical parameters using the electronic tongue were calculated by partial least squares regression. The electronic tongue was capable of prediction of several parameters relating to beer quality, including bitterness, ethanol content, polyphenol content and real extract, with mean relative errors of 10–17%. The lowest error was for bitterness, so in this respect alone, the electronic tongue offered an attractive alternative to the standard spectrophotometric method (Section 4.4.3), being much simpler, more rapid and of similar precision.

An electronic tongue based on pulse voltammetry was used to predict concentrations of bisulfites in wine samples (Labrador *et al.*, 2008). The sensor array consisted of four working electrodes – gold, rhodium, platinum and stainless steel, all of 99.9% purity and of 1 mm diameter – encapsulated into a stainless steel cylinder, which acted as both the body of the ET system and the counter/reference electrode. The ET device was additionally equipped with a self-polishing system, which was used for 30 s between experiments. A white wine whose sulfite content was known from HPLC measurements was used for calibration and 20 wine samples were used as the validation set. Multivariate analysis including cross validation and partial least square (PLS) techniques are applied for data management and prediction model building. Accuracy was good; ascorbic acid and histamine were included in the predictive analysis, but here accuracy was only moderate.

A sensor system using an array of carbon paste electrodes modified with rare earth complexes, including lutetium(III), gadolinium(III) and praseodymium(III) bisphthalocyaninates (Parra *et al.*, 2004). The sensors showed high cross selectivity and responses were evaluated by cyclic voltammetry or square wave voltammetry. The sensor array was tested on six Spanish red wines made from the same grape variety, but of varying ages and geographic origin. The data was analyzed by principal component analysis, where it was found that the sensor array was able to discriminate between the samples, agreeing closely with a PCA analysis of selected red wine taste parameters (e.g. % ABV, dry extract, glycerol content, reducing sugar content, tannin content, tartaric acid content, total acidity and volatile acidity) associated with the tested wines.

Although not an electronic tongue as such, an electrochemical sensor device was used for the very rapid determination (~4 s) of the acidity of juices and alcoholic beverages (Wen *et al.*, 2004). The device actually performed electrochemical titrations ('flash' or 'nanotitration') in the media, with only a small fraction of the sample being titrated (in the immediate vicinity of the electrode), thus leading to an insignificant change in the total acidity. The titration curve was recorded by video and the results were in good agreement with those obtained by standard volumetric titration.

Although ascorbic acid is not a major component of most alcoholic beverages (except perhaps in certain mixers and cocktails) it is sometimes used along with sulfites to prevent premature browning in white wines and it is used extensively in other areas of the food industry. Consequently, there has been considerable interest in developing sensor devices for the electrocatalytic determination of ascorbic acid (Pauliukaite *et al.*, 2005; Nassef *et al.*, 2008). Oxidation of ascorbate to dehydroascorbate is a two electron process that can be catalyzed by a number of reduction processes of electrode modifier substances. This, and the subsequent electrochemical reoxidation of the modifier, constitute the basis of some determinations of ascorbic acid (Figure 4.5.7).

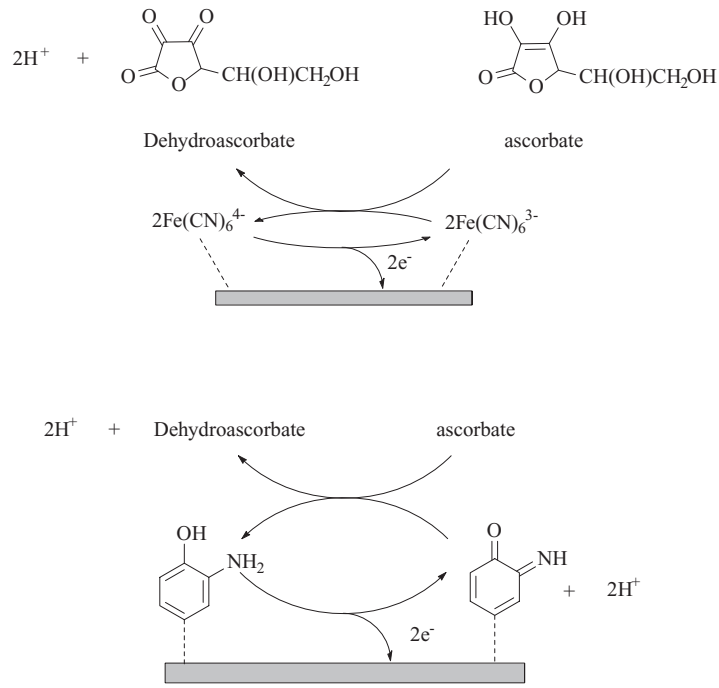

Figure 4.5.7 *Redox coupled systems at modified electrodes used for the electrochemical determination of ascorbic acid. Pauliukaite* et al.*, 2005; Nassef* et al.*, 2008*

Carbon films modified by copper hexacyanoferrate were used as electrodes for detecting ascorbic acid in red and white wines (Pauliukaite *et al.*, 2005). They were made from carbon film resistors, produced from ceramic cylinders (1.5 mm o.d. × 4.0 mm) by pyrolytic deposition of carbon, in such a way that the exposed geometric area was ~0.2 cm^2. Copper hexacyanoferrate was deposited from a solution of 10 mM Cu(II), 10 mM $K_3Fe(CN)_6$ and 100 mM KCl by either chemical (immersion) or electrochemical methods. The chemically deposited films performed best and were used as amperometric sensors at +0.05 V against SCE.

Disposable screen printed electrodes modified with an electrografted *o*-aminophenol film were constructed for the amperometric sensing of ascorbate in various beverages (Nassef *et al.*, 2008), against an Ag/AgCl reference electrode. The film was prepared via the electrochemical reduction of the *in situ* prepared *o*-aminophenol diazonium salt in aqueous solution. The modified electrodes showed pronounced electrocatalytic activity towards the oxidation of ascorbic acid, and the catalytic oxidation peak current was linear with respect to ascorbate concentrations in the range 2–20 μM, with good precision (%RSD = 1.98, n = 8).

Many wines or wine categories are regulated according to their residual sugar content (e.g. Marsala – see Section 2.10.8), which is mostly glucose. Although there are several methods for the analysis of residual glucose and reducing sugars, such as enzymic/colorimetric (Section 4.4.3) and chemical techniques (Section 4.6.3), the use of electrochemical biosensors in this area has resulted in some effective rapid screening methods. Electrochemical biosensors are similar to other sensors, but having at least one biological redox component linked with a physicochemical detector component. They are usually three electrode systems that involve the analyte in a redox enzyme reaction that produces ions at the working electrode. The product ions give rise to a potential, which is subtracted from that of the reference electrode to produce a signal.

Ulasova *et al.* (2003) used Prussian Blue based sensors with a flow injection system (Section 4.6.2) to determine the glucose levels of 10 Italian red and white wines, after a 1:1000 dilution, where agreement with a standard spectrophotometric method was good. The wines had glucose levels in the range 0–60 mg/l. The working electrode was made by depositing a membrane of Nafion®, containing immobilized glucose oxidase on a Prussian Blue modified glassy carbon electrode. An auxiliary electrode made as above, but with bovine serum albumin in place of glucose oxidase, and an Ag/AgCl reference electrode completed the electrochemical cell, which showed a linear response to glucose at an applied potential of 0.0 V in the range 10^{-6}–10^{-3} M.

Biosensors often have two biological redox systems linked, as in one based on platinum chips for the determination of glucose (Alonso Lomillo *et al.*, 2005). Briefly, the biosensor was produced by metallation of (3 × 3 mm) silicon chips, giving a platinum working electrode. Horseradish peroxidase (HRP) and glucose oxidase (GOX) were coimmobilized on this microelectrode by electropolymerization of pyrrole, and hexacyanoferrate or Meldola's blue was used as mediator for electron transfer between the enzymes and electrode (Figure 4.5.8). The sensor was able to quantify glucose in grape juice, with repeatability (%RSD) of 7.1% (n = 4) and so could be used as a tool for the rapid screening of glucose in ripening grapes.

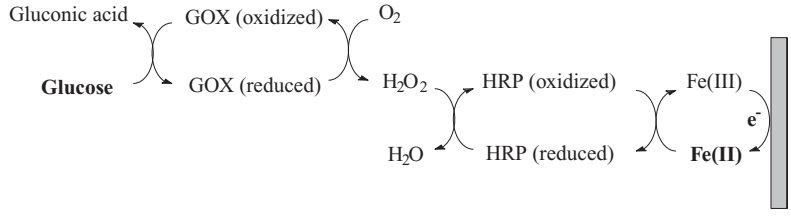

Figure 4.5.8 *Coupled GOX-HRP enzymic reactions and hexacyanoferrate reactions at the working electrode of a glucose biosensor. Alonso Lomilla* et al.*, 2005*

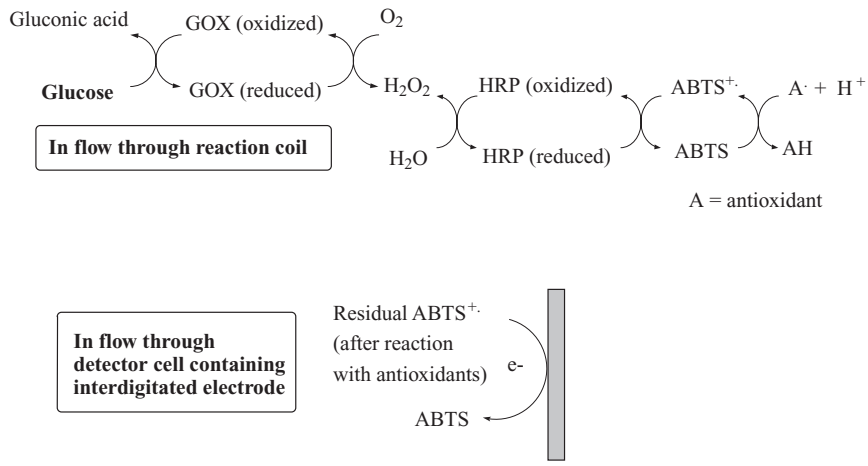

Figure 4.5.9 *Redox reactions associated with biosensor method for the determination of the total antioxidant capacity of alcoholic drinks. Milardovic* et al.*, 2007*

A similar method using flow injection analysis (FIA) was reported for determination of total antioxidant capacity of alcoholic beverages by bienzymatically produced $ABTS^{+\bullet}$ (Section 4.4.3) and an electrochemical detector using an interdigitated gold electrode (IDE) (Milardovic *et al.*, 2007). $ABTS^{+\bullet}$ is produced from ABTS by the glucose oxidase-peroxidase enzyme system immobilized on polyacrylamide gel in the tubular flow through reactor coil. The method is based on electrochemical (biamperometric) measurements using the $ABTS^{+\bullet}$/ABTS redox couple. The biamperometric technique is based on electrochemical detection using two identical working electrodes, upon which is imposed a small potential difference. This indirect method relies on a homogeneous reaction between the analyte and a reversible redox couple as indicator. The reactions involved with this system are shown in Figure 4.5.9. The results, 0.04–0.59 Trolox equivalents for spirits and one liqueur, and 1.23–17.38 Trolox equivalents for wines, were in good agreement with those from a spectroscopic method.

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4.6
Other Methods

4.6.1 Electrophoretic Techniques

The process of electrophoresis can be described as the differential migration of ions by attraction or repulsion in an applied electric field. The simplest form of electrophoresis involves positive (anode) and negative (cathode) electrodes placed in a solution containing ions. When a voltage is applied across the electrodes, anions (negative ions) and cations (positive ions) move through the solution towards the electrode of opposite charge. For general reading, the reader is directed to Westermeier (2005).

Separation by electrophoresis relies on differences in the migration velocity (v) of ions, which is given by Equation 4.6.1.

$$v = \mu_e E \tag{4.6.1}$$

where v is in m/s, μ_e is the electrophoretic mobility (in m^2/V/s) and E is the electric filed strength (in V/m).

The electric field strength is the applied voltage divided by the total capillary length. Electrophoretic mobility expresses the balance of forces acting on each ion; the electrical force in favor of motion and the frictional force against motion. For a particular ion in a given set of conditions, electrophoretic mobility is constant during an electrophoresis experiment.

Electrophoretic mobility is given by Equation 4.6.2.

$$\mu_e = \frac{q}{6\pi\eta r} \tag{4.6.2}$$

where q is the ionic charge, η is the medium viscosity and r is the ionic radius.

At one time, high voltage paper electrophoresis (HVPE) was a fairly popular method for the identification of amino acids and low molecular weight carboxylic acids in fruit juices and alcoholic beverages, but this technique gradually gave way to other electrophoretic methods (especially capillary electrophoresis) and to chromatographic methods. However, recently HVPE has been used to determine the pK_A values and acid–base equilibria of anthocyanins and derivatives such as 5-carboxypyranomalvidin-3-glucoside (vitisin A) in dilute solution (Asenstorfer *et al.*, 2003; 2006; Asenstorfer and Jones, 2007). Paper electrophoresis has been used to study the charge dependent electrophoretic mobility of malvidin-3-glucoside in a variety of buffers over the pH range 1.2 to 10.4 (Asenstorfer *et al.*, 2003). Apparent pK_A values of pK_A (1) 1.76 ± 0.07, pK_A (2)

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

5.36 ± 0.04, and pK_A (3) 8.39 ± 0.07 were determined for malvidin-3-glucoside. It was deduced that the major forms of malvidin-3-glucoside and similar anthocyanins at wine pH (∼3.6) were the neutral hydrated (hemiketal) and quinoidal base forms. In view of these results, coupled with information from UV-visible spectra (Section 4.4.3) and ^{13}C NMR spectra (Section 4.4.1) it was suggested that, at wine pH (3.2–3.8), vitisin A exists as a complex mixture of neutral, anionic, hydrated and nonhydrated species, with the major species being the orange neutral quinonoidal base (Asenstorfer *et al.*, 2006; Asenstorfer and Jones, 2007). See Section 4.4.3 for further discussion on the implications of these results regarding young red wine color.

In these experiments a gel electrophoresis unit was used, with glass fiber paper folded over a glass rod and dipping into two wells containing buffer solutions. A voltage gradient of 7.5 V/cm was applied and the unit was cooled to 15 °C to counter the heat generated. The buffers used were 0.1 M formic/acetic acid (pH 1.0–2.4), 0.1 M citric acid (pH 2.8–7.2), and 0.5 M citric acid/0.5 M sodium pyrophosphate (pH 7.4–10.0).

Oligosaccharides and polysaccharides are important constituents of beer, being present in different proportions depending on production methods and beer style (e.g. ale, lager, light, porter and stout). They cannot be analyzed easily by electrophoretic methods because they are not appreciably ionized, except in very strongly basic media. However, if firstly derivatized with the fluorescent and anionic dyestuff 8-amino-1,3,6-naphthalene trisulfonate, they can be separated and detected with fluorescence assisted carbohydrate electrophoresis (FACE). Oligosaccharides in a wide range of beers were identified via comigration of standards (e.g. oligosaccharides derived from β-glucan, pentosan and purified starch) using such a method (Thomas *et al.*, 2000).

Gel Electrophoresis

Several techniques of this type are regularly applied to the study of macromolecules associated with alcoholic beverages or their raw materials, as well in relation to production processes, including fermentative microorganisms. Polyacrylamide gel electrophoresis (PAGE) is probably the most common analytical technique used to separate and characterize proteins. These, and other macromolecules, dissolved in a buffer solution, are separated by their differential movement through the cross linked gel upon the application of a potential gradient.

Electrophoretic mobility depends on the applied potential difference, the molecular weight and the number of electrical charges on the macromolecule. However, if the sample solution is treated before electrophoresis with sodium dodecyl sulfate (SDS), an anionic detergent, the proteins are denatured into more or less linear molecules containing roughly one absorbed SDS molecule for every two amino acids. The native charge of each protein is hidden by the negative charges of the SDS units, thus the denatured proteins tend to have similar shapes and similar mass to charge ratios. Consequently during SDS-PAGE electrophoresis, proteins are separated largely according to their molecular weights. Proteins with large numbers of disulfide bridge links can be further denatured before electrophoresis by reaction with 2-mercaptoethanol or dithiothreitol. The resulting technique is known as reduced SDS-PAGE.

A solution of acrylamide and bisacrylamide is polymerized, usually in Tris-Cl buffer solution (pH ∼7–9), using ammonium persulfate as an initiator and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) as a free radial stabilizer (promoter). A cross linked gel is formed (acrylamide alone tends to produce linear polymers), the extent of cross linking ('pore size') being determined by both the concentration of acrylamide and the ratio of acrylamide to bisacrylamide. A high acrylamide concentration and a high ratio of bisacrylamide to acrylamide give a highly cross linked (low pore size) polymer, which results in low electrophoretic mobility during electrophoresis. A typical composition for a resolving gel (see below) is 12–16% (w:v) total acrylamide with ∼3–6% of this being bisacrylamide crosslinker. This gives highly cross linked (small pore) gels, typically used as resolving gels for the separation of relatively high molecular weight proteins.

Polyacrylamide gels are polymerized in a gel caster, where the separating (resolving) gel is poured and allowed to polymerize, as described above. Next a thin layer of isopropanol is added, the loading (stacking) gel is poured over this and a comb is placed to create the wells. After the loading gel is polymerized the comb can be removed and the gel is ready for electrophoresis.

The loading gel is a large pore polyacrylamide gel (~4% w:v acrylamide concentration), which is usually prepared in Tris/HCl buffer pH 6.8; 2 pH units lower than that of the common electrophoresis buffer (usually Tris/glycine). These conditions allow SDS-coated proteins to be rapidly concentrated several fold and to form a thin starting zone of less than 20 μm. Sometimes a spacer gel, of pore size intermediate between those of the loading and resolving gels, is used.

The electrophoresis apparatus is set up with cathode buffer covering the gel in the upper negative electrode (cathode) chamber, and with the anode buffer in the lower positive (anode) electrode chamber. The (usually) denatured protein samples are added to the wells at one end of the gel plate and a calibration reference solution containing denatured proteins of known molecular weights is added to a well at the other end. A potential difference is then applied, under which the negatively charged proteins move at different rates down the gel plate toward the anode. Higher voltages give faster migration, but at the cost of poorer resolution: an SDS-PAGE experiment can be expected to take an hour or more. Visualization of the migration progress can be achieved by including bromocresol in the electrophoresis medium.

After a suitable separation has been achieved, the voltage is switched off and the gel plate is removed and usually stained with either Coomassie Brilliant Blue R-250, an anionic dye that binds to proteins nonselectively (although only weakly with some glycoproteins) or a silver nitrate solution, for greater sensitivity.

Many researchers still use the Tris-glycine or Laemmli procedure (1970), described above, that stacks at pH ~6.8 and resolves at pH ~8.3–9.0. However, the latter high pH values promote disulfide bond formation between cysteine residues in the proteins, especially if the protein is rich in such residues, because the pK_A of cysteine residues is 8–9 and because the reducing agent present in the loading buffer does not comigrate with the denatured proteins. To minimize this problem, some researchers carry out the electrophoresis by using a bis-Tris buffer medium of pH ~6.5, which is well below the pK_A of cysteine. The medium also includes sodium bisulfite reducing agent that moves into the gel ahead of the proteins, thus maintaining a reducing environment during electrophoresis. Additionally, acrylamide gels are more stable in lower pH buffers and hence can be made in advance and stored for a considerable time before use.

The most frequently and widely applied form of gel electrophoresis is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), used mainly for the characterization of proteins and their breakdown products (Soares and Sato, 2000; Singh and MacRitchie, 2001; Blanco Gomis *et al.*, 2003; Osman *et al.*, 2003; Liesegang and Stahl, 2005; Gambuti *et al.*, 2006, Cilindre *et al.*, 2007; Hégrová *et al.*, 2009; Marangon *et al.*, 2009; Wigand *et al.*, 2009).

Additionally, gel electrophoretic techniques are used for the identification of plant species and microbial strains via nucleic acid (microsatellite or molecular markers) analysis of plant material, rather than via analysis of native proteins (Viti *et al.*, 2000; Gimble, 2001; Coulon *et al.*, 2006; Larisika *et al.*, 2008; Maestre *et al.*, 2008 and İşçi *et al.*, 2009). Identification of plant species is an important aspect of authentication of wine origin and in the detection of fraud.

A common technique for the analysis of grape vine plant material is restriction fragment length polymorphism (RFLP), whereby the DNA is first extracted from fresh tissue, purified and then amplified by polymerase chain reaction (PCR). The DNA is then cut into restriction fragments using suitable endonucleases, which cut the DNA molecule only at specific base sequences. The restriction fragments are then separated according to length (and molecular weight) by gel electrophoresis, often agarose gel electrophoresis with enhancement by Southern blotting. Alternatively, fragments can be visualized by pre- or posttreatment of the agarose gel with ethidium bromide or silver solutions.

Although such techniques have been applied to must (e.g. see Baleiras-Couto and Eiras-Dias, 2006; Faria *et al.*, 2008), there are problems with DNA analysis in wine (İşçi *et al.*, 2009). Firstly, the levels of DNA in wine are very low, secondly, some degradation occurs during the production process, thirdly, contamination with microbial DNA can occur and fourthly, wine contains many potential inhibitors (e.g. polyphenols and carbohydrates) of the PCR process.

PAGE analysis of proteins is often accompanied by other separation techniques, such as capillary gel electrophoresis (Blanco Gomis *et al.*, 2003) or gel filtration (Osman *et al.*, 2003), and is sometimes carried out on chromatographic fractions (e.g. Soares and Sato, 2000; Singh and MacRitchie, 2001; Osman *et al.*, 2003), as well as extracts. Also, PAGE protein bands, after destaining and possibly other treatments, are frequently subjected to mass spectrometric analysis, such as MALDI-TOF (e.g. Hégrová *et al.*, 2009) or LC-MS (e.g. Marangon *et al.*, 2009; Wigand *et al.*, 2009).

SDS-PAGE can be used to probe native cereal protein structures and their breakdown mechanisms, as in the case of controlled depolymerization of wheat gluten and glutenin subunits by ultrasound (Singh and MacRitchie, 2001). Here, highly pure glutenin (free of gliadins) reduced and unreduced chromatographic fractions were subjected to analysis on 10% SDS-PAGE minigels in a discontinuous (isoelectric focusing) system at pH 6.8, with staining by Coomassie Brilliant Blue R-250. The results (protein profile), coupled with SH content determination, indicated that proteins vary in their ease of breakdown via disulfide (SS) bridge fission and that intramolecular SS bonds are readily reformed in the breakdown of protein subunits.

Interest in protein composition is often in relation to the effects of processing (Osman *et al.*, 2002: 2003), foam promotion or haze formation in beer (Sections 2.6.2 and 2.6.3) (Liesengang and Stahl, 2005), cider (Section 2.8.2) (Blanco Gomis *et al.*, 2003) and wine (Brown *et al.*, 2007; Cilindre *et al.*, 2007) or the 'gushing' phenomenon in beer (Hégrová *et al.*, 2009).

In beer, the major proteins involved in foam production are 'protein Z' (molecular weight $\sim$ 40 kDa) and lipid transfer protein 1 (LTP1) (molecular weight $\sim$ 9.6 kDa), which are normally found in concentrations of 50–200 mg/l and 59–90 mg/l, respectively (Liesengang and Stahl, 2005). SDS-PAGE (and RPHPLC) has shown that beer LPT1, but not barley native LTP1, is hydrolyzed by proteinase A enzymes excreted by yeast during wort fermentation, thus suggesting this protein is modified during malting and or mashing to a more susceptible form (Liesengang and Stahl, 2005). Protein Z was not affected by proteinase A. The gel electrophoresis experiments were carried out on protein fractions, using a standard Laemmli procedure and visualized by Coomassie Brilliant Blue R-250 staining, as well as by an immunoblot technique. Hence, since some the foam promoting protein in beer is degraded by proteinase A and most of this enzyme is released by autolyzed yeast cells, it was suggested that foam quality may be maintained by use of yeasts with low proteinase A activity and conducting the fermentation at low temperatures with rapid separation of the beer from the yeast after fermentation.

On the other hand, SDS-PAGE analysis of barley, wort and beer proteins, using the Laemmli procedure and 'low range' standards (molecular weight 14.4–97.4 kDa), combined with gel filtration has demonstrated the generally similar protein profile of barley malt and beer (Osman *et al.*, 2003), confirming the assumption that beer proteins descend from barley, some unchanged and some modified by processing, as described above. Although most barley proteins are water insoluble, it was found that the process of malting doubled the concentration of soluble proteins (from 20% to 40% of total protein content).

It has also been shown that some proteinases survive the kilning process and are active during mashing, some breaking down insoluble proteins into soluble ones, and others hydrolyzing these further to ammonia, small peptides and amino acids. These are known as free amino nitrogen (FAN). Nondenaturing PAGE (i.e. without SDS and using Tris-HCl buffer of pH 8.5) separation of native barley endoproteases at different points in the malting/kilning process indicated that active endoproteases (as determined by their action on glutelein and haemoglobin substrates) were formed during germination, and 90% of them were still active in the kilned malt (Osman *et al.*, 2002). In this study, Amido Black was used as the staining agent.

Sorghum (*Sorghum bicolor* (L.) Moench) is an important cereal in semi-arid regions of Africa and Asia. It is malted and used to produce a range of alcoholic beverages in both continents, particularly Africa (Section 2.7.2). Recently, SDS-PAGE has been applied to the analysis of proteins at different stages of sorghum germination during the malting process (Correia *et al.*, 2008). Proteins extracted in *tert*-butanol were dried under N_2 and the residues were dissolved in the electrophoretic buffer (SDS, Tris, glycerol and bromophenol blue), and were run under nonreducing and reducing conditions (with added 2-mercaptoethanol) at 170 V for 1.5 h using the Laemmli method, after heating in a boiling water bath for 5 min. The gels were stained with Coomassie Blue R and destained with methanol and acetic acid.

The results indicated an increase in high molecular weight protein aggregates during the first few days of malting, followed by significant degradation by proteases, suggesting that, as with other cereals, germination significantly increases the concentration and availability of proteins, peptides and amino acids.

Certain peptides are produced by barley kernels in response to attack by fungi, such as *Fusarium* spp. (Section 5.11.4). These stress peptides may survive through the brewing processes and their presence in the beer, along with fungal metabolites, is thought to contribute the phenomenon of gushing. Hégrová *et al.* (2009) used SDS-PAGE to show that proteome changes occurred during barley infection, but the basic hordothionin type peptides and LPT1 (important in foam quality) concentrations did not correlate with the degree of gushing.

Blanco Gomis *et al.* (2003) used capillary gel electrophoresis and standard SDS-PAGE to show that Spanish cider contained five major proteins of molecular weight 16–110 kDa. The major protein (16.4 kDa) was known to be involved in haze formation in apple juice and hence it was suggested that the other proteins could contribute to the sensory value of cider – with respect to foam quality, an important quality parameter applied to Spanish cider.

As with beer and cider, proteins are required for good foam (mousse) characteristics of sparkling wines, such as Champagne (Section 2.9.3). It is has been shown that infection of grapes by *Botrytis cinerea* (causing grey mold) adversely influences foam quality (foamability and foam stability) of the resulting wine (Cilindre *et al.*, 2007). SDS-PAGE and two dimensional electrophoresis (2-DE), with immunodetection, showed that *B. cinerea* infected grapes gave wines with a different protein profile to normal wine (i.e. with fewer and different proteins). This suggested that certain grape proteins are proteolized during infection by this fungus and new proteins are synthesized, either by the fungus or by the grape (in response to fungal infection), or are breakdown products of original grape proteins. Loss of good foaming characteristics could be due to a combination of loss of foam promoting proteins and the presence of foam preventing proteins, as a result of *B. cinerea* infection.

Wigand *et al.* (2009) have used SDS-PAGE in conjunction with LC-MS (see Section 4.3.3) to analyze the protein content of wine after enrichment by dialysis and lyophilization. Generally, the method of Laemmli was used, but for comparison of the different wine varieties and homemade 12.5% polyacrylamide gels and for the further identification with mass spectrometry, 10–17.5% gradient polyacrylamide gels were used, each with 3% stacking gels. Protein samples were denatured with 2-mercaptoethanol and applied in a Tris buffer at pH 6.8, containing 4% SDS and bromophenol blue. Coomassie Brilliant Blue R-250 or silver nitrate was used as the stain, although glycoproteins (being insensitive to the above staining methods) were stained with periodic acid-Schiff's reagent (PAS) solution.

In the broader context of improving our understanding of protein haze formation in white wine, Marangon *et al.* (2009) identified Semillon juice proteins by taking five hydrophobic interaction chromatographic (HIC) fractions after RPHPLC separation (see Section 4.3.3) and subjecting them to nonreducing SDS-PAGE, using the method of Laemmli, with Bio-Safe Coomassie or silver stain procedures. All 24 bands of the SDS-PAGE electrophoretogram were subjected to nano LC-MS/MS, where all samples were shown to contain proteins from *V. vinifera*, using plant protein sequence databases. The juice proteins were PR (pathogenisis related) and TL (thaumatin-like) proteins, chitinases, (possibly) ripening related protein, LPT protein isoform1 and

vacuolar invertase 1, G1N1. Semillon wine was treated in the same way, giving a similar protein profile with respect to TL proteins, but had fewer chitinase proteins, indicating that some of these are hydrolyzed by yeast proteases during fermentation. PR and TL type proteins, as well as chitinases are known to be responsible for protein hazes in wine.

Haze protective factors are mannoproteins secreted by yeasts. They prevent protein haze formation in white wines by binding solutes, such as metal ions and polyphenols, which are required for the aggregation of denatured proteins into large particles. Brown *et al.* (2007) used a variety of techniques, including SDA-PAGE to characterize a 6xHis-tagged mannoprotein of molecular weight 178 kDa, which demonstrated haze protection when present in white wine. This offers the possibility of using proteins of this type to prevent haze formation, thereby reducing the need for fining agents such as bentonite (Section 2.9.4), which in the long term are costly and can cause disposal problems.

SDS-PAGE (using the Laemmli procedure on 12% w:v gels and with Coomassie Blue G-250 stain) and gel filtration chromatography have been used to characterize the killer protein (secreted to subdue the activities of other yeasts) of the *S. cerevisiae* strain Y500-4L (Soares and Sato, 2000). The purified killer protein had a molecular weight of 43 kDa, but a band corresponding to 18–20 kDa was found in the PAGE electrophoretogram, suggesting it is a dimer, like other killer proteins.

There has been much interest in the literature on polyphenol–saliva protein interactions, since formation complexes are believed to be responsible for the important sensory attribute known as astringency (see Section 4.7.1). SDS-PAGE (Laemmli procedure, with 14% resolving gel and 4% stacking gel and Coomassie Brilliant Blue R-250 staining), densitometry and sensorial analysis showed that four saliva proteins (amylase, lactoferrin, PRPbg1 and PRPbg2) were best able to distinguish tannin solutions according to different levels of astringency (Gambuti *et al.*, 2006). The Coomassie Blue destaining step (by incubation in 30% acetic acid – methanol or ethanol was omitted) distinguished PRPs, which stained pink-violet, from other proteins, which stained blue. The electrophoretograms showed a greater depletion of free saliva proteins with the more astringent grape seed tannin solution, as opposed to the grape skin tannin solution of Aglianico grapes.

Capillary Zone Electrophoresis (CZE)

This technique, sometimes known simply as capillary electrophoresis (CE) performs electrophoresis in a buffer- filled, narrow bore capillary, normally from 25 to 100 μm in internal diameter (ID), using a potential difference of typically up to 30 kV between the electrodes. The capillary is usually made of fused silica or quartz glass, protected by an external covering of polyimide. A schematic representation of a basic native CZE instrument is shown in Figure 4.6.1. Typically, the detector is situated ~50 cm from the anode. The

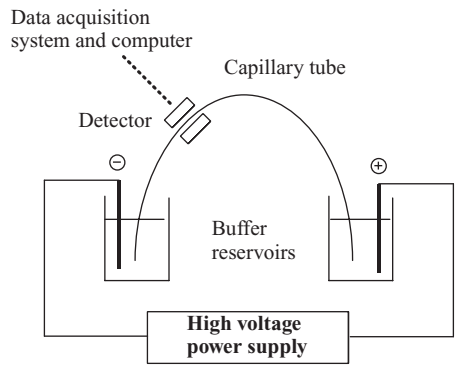


Figure 4.6.1 *Schematic representation of a capillary electrophoresis instrument*

sample may be introduced into the capillary tube by either electrokinetic or hydrodynamic injection. The former is carried by placing one capillary end (with the other capillary end in the cathode reservoir) in the anode buffer reservoir containing the sample and applying the voltage for a few seconds. A plug of buffer with sample moves a little way up the capillary due to electro-osmotic flow (see below). Hydrodynamic injection is carried out using application of pressure or vacuum at the appropriate end of the capillary for a few seconds (no voltage is applied), in which time the pressure differential causes movement of sample and buffer into the capillary. In either case of injection technique, this is repeated with the sample capillary end in a vial of pure water, which is then moved to an anode buffer reservoir containing no sample and the experiment is performed.

The mechanism of separation depends upon the existence of a double layer of counter ions (cations) to balance the overall charge of ionized silanol groups on the surface of the silica or quartz capillary wall (Figure 4.6.2). Under the influence of an applied potential gradient, the outer (diffuse) layer of counter ions migrates in the direction of the electrode of opposite polarity (the cathode) carrying its solvation shell, containing neutral analytes, and the rest of the medium, along with it. This migration is known as the electro-osmotic flow (EOF) (Figure 4.6.2). Ionic analytes will move at different rates, according to their charge densities, solvation spheres and masses, and hence will be sequentially monitored as they pass by the detector (CZE has a similar range of detector types to HPLC). Even most anions migrate in the same direction, since the EOF rate is greater than their reverse migration rates.

Since samples are normally introduced at the anode and EOF moves in the direction of the cathode, cations have positive effective electrophoretic mobility, neutral species have zero mobility and anions have negative mobility. In other words, cations migrate faster than the EOF, neutrals migrate with the same velocity as the EOF and anions migrate more slowly than the EOF. Mesityl oxide is often used to measure the EOF under the experimental conditions, according to buffer type, concentration, pH and temperature.

Cations have the lowest migration times, since their speed is augmented by the electro-osmotic flow. Anions have the highest migration times because although electrically attracted towards the anode, they are dragged towards the cathode by the EOF. In between the two extremes, neutral components migrate with the EOF, but are unresolved by the native CE technique. Cations with the highest charge densities (e.g. Al^{3+}) migrate first, followed by cations with lower charge densities (e.g. Na^+). Conversely, anions with lower charge densities (e.g. Br^- or RCO_2^-) migrate earlier than those with greater charge densities (e.g. F^- or SO_4^{2-}).

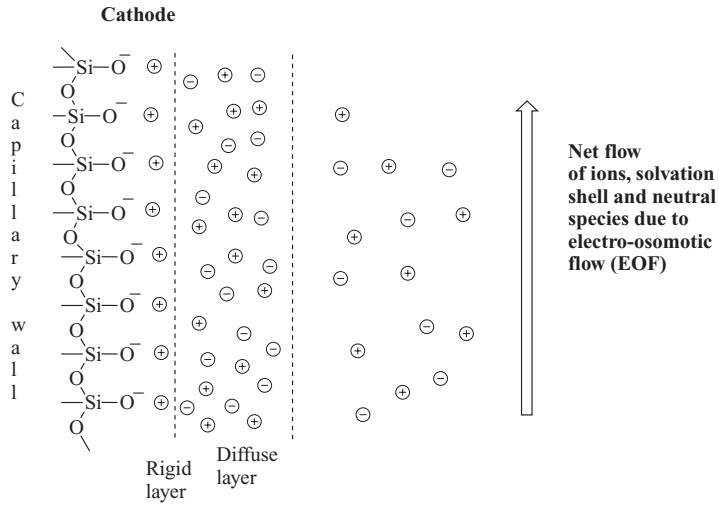


Figure 4.6.2 *Stern's model of double layer and EOF*

It is possible to change the charge density of many ions by adjustment of the buffer medium pH, thus influencing their relative migration rates. Also, EOF modifiers, such as cationic surfactants cetyltrimethylammonium bromide (CTAB) or hexadimethrin bromide (HDB), can be used to reverse the direction of the EOF, so making analysis of anions easier. On flushing the capillary with cationic modifier solution, the additive is physically adsorbed onto the capillary surface by ionic interactions, thus providing a dynamic, but reproducible coating. Such a technique is often used in the CE analysis of carboxylate and other anions in alcoholic beverages.

The selection of a background electrolyte (BGE) in capillary electrophoresis is a very important – for good resolution and high sensitivity, the mobility of BGE should be similar to that of the analytes, and where indirect UV detection is used, the BGE should have high molar absorptivity. As in HPLC, UV-visible detectors are popular, but many ionic analytes do not absorb strongly in the more readily accessible near UV region of the electromagnetic spectrum (say 190–300 nm). These analytes can be determined indirectly by adding chromophore containing counter ions to the sample. The counter ions form UV or visible light absorbing ion pairs with the analytes, which migrate at different velocities during electrophoresis.

Capillary electrophoresis has been widely applied to the determination of organic acids in juices and alcoholic drinks, the analytes generally being studied as their anions at relatively high pH. Indeed, in the case of organic acids, there are more CE reports than HPLC reports in the literature over the last decade (Mato *et al.*, 2005). Most of the CE reports involve the use of an EOF modifier or a coated capillary (Castiñeira *et al.*, 2000; Saavedra and Barbas, 2003; Esteves *et al.*, 2004; Simonet *et al.*, 2004; Bianchi *et al.*, 2005; Mardones *et al.*, 2005; Cortacero-Ramírez *et al.*, 2005; Mato *et al.*; 2007; Peres *et al.*, 2009), rather than native CE (Castiñeira *et al.*, 2000). The organic acids are generally separated as anions at pH ∼6.4–9.2, but there is at least one report on their separation as derivatives (Santalad *et al.*, 2007). There is also a report that uses zone electrophoresis on a poly(methyl methacrylate) chip, with integrated conductivity detection (Masár *et al.*, 2005a).

Generally, running negative potentials of 14–30 kV have been used for CZE analysis of acids in juice and alcoholic beverages (Mato *et al.*, 2007). Reproducibilities (determined as %RSD) are usually less than 3%, with recoveries generally in the range 90.0–103.0% and LoDs between 0.04 mg/l and 25 mg/l, thus comparing well with HPLC. The majority of reports deal with the simultaneous determination of a number of acids.

Castiñeira *et al.* (2000) examined the capillary electrophoretic analysis of acids in wine, using both uncoated and myristyltrimethylammonium bromide (MTAB) coated capillaries and a variety of background electrolytes (BGEs). Detection was by direct or indirect UV spectroscopy, depending on the BGE. The optimum conditions, electrolyte containing containing 3 mM phosphate (BGE) and 0.5 mM MTAB (as EOF modifier) at pH 6.5, allowed the determination of acetic, malic, lactic, succinic and tartaric acids were determined simultaneously in ∼6 min, with precision better than 1% and limits of detection in the range 0.015–0.054 mg/l.

There were some problems relating to poor peak shape, lack of sensitivity and limited ranges of linear detector response associated with some of the earlier CE determinations of short chain organic acids of wine. A background electrolyte of 2,6-pyridinedicarboxylic acid, along with EOF modifier cetyltrimethylammonium bromide (CTAB), have been found to be useful in the CZE analysis of low molecular weight wine acids (de Villiers *et al.*, 2003; Esteves *et al.*, 2004). De Villiers *et al.* (2003) used 7.5 mM pyridine dicarboxylic acid (PDC) as BGE, containing 0.5 mM CTAB as well as 0.5 mM EDTA at pH 5.60 to determine the six most important acids in 20 red and white South African wines. The samples required no pretreatment other than ×80 dilution (and addition of formic acid internal standard) and the analytes were detected indirectly at 350 nm (due to the presence of PDC as the BGE), with a reference wavelength of 210 nm. EDTA was present to improve the peak shape and quantification of citric acid. Limitations regarding linearity were shown to be the result of a lower sample pH than that of the BGE. Esteves *et al.* (2004) used a similar running electrolyte (5 mM PDC with 5 mM CTAB, but no EDTA, at pH 5.6) to determine five organic acids in 21 red and white

Port wines. Peres *et al.* (2009), on the other hand, preferred to use 10 mM 3,5-dinitrobenzoic acid (DNB) as background electrolyte at pH 3.6 (containing 0.2 mM CTAB EOF flow reverser). DNB was considered to be a good BGE because of its similar mobility to the analytes at pH 3.6 and also because it was a good chromophore, it allowed indirect detection of six acids at 254 nm in 23 Brazilian wines.

Direct UV detection at 185 nm was chosen by Mato *et al.* (2007) for the CE analysis of six organic acids in grape juice and wines. For this, the electrolyte was aqueous 7.5 mM NaH_2PO_4 and 2.5 mM Na_2HPO_4, with added 2.5 mM tetradecyltrimethylammonium hydroxide (TTAOH) as electro osmotic flow modifier and 0.24 mM $CaCl_2$ as selectivity modifier, at pH 6.40. Using this running electrolyte and a running potential of –25 kV, it was possible to determine six acids in less than 3 min. Likewise, direct UV detection (at 210 nm) was chosen for the analysis of 19 low molecular weight organic acids in seven beer samples (Cortacero-Ramírez *et al.*, 2005). The running buffer was 50 mM sodium phosphate in water:propan-2-ol (3:1) with added hexadimetrine bromide (HDB; 0.001%) as EOF modifier; this concentration caused a fast anodic electro-osmotic flow. The 19 acids could be determined in 22 min, with reproducibilities (%RSD) of 2.4–5.9% and LoDs of 0.04–3.3 mg/l.

Analysis of the shikimic acid content of Chilean red wines by CZE was undertaken by Mardones *et al.* (2005) in order to assess this as a means of varietal differentiation. Two methods were used, one at pH 7.5 using *p*-aminobenzoic acid for indirect detection of the analyte at 260 nm and the other at pH 7.0 with direct detection at 213 nm. In both cases the buffer system was bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane (Bis-Tris), with added $CaCl_2$ or LiCl and trimethyl(tetradecyl)ammoniumbromide (TTAB) as EOF modifier. Cabernet Sauvignon wines were found to have a higher shikimate content than other varietal wines from Chile (Merlot and Carmenère). Interestingly, Carmenère has all but disappeared from the Bordeaux area, where it once was an important red wine variety. It generally produced high quality wine, and possibly following the initiative of the New World (particularly Chile), it may experience a modest revival in France (2009).

Rather than using an EOF modifier in the electrolyte solution, the internal capillary walls can be coated with a modifier, such as polyacrylamide or HDB (Saavedra and Barbas, 2003; Bianchi *et al.*, 2005). Eight organic acids, as well as nitrate and sulfite were determined in Rioja red and white wines using a polyacrylamide coated capillary with 200 mM phosphate buffer as BGE (pH 7.5), using direct UV detection at 214 nm (Saavedra and Barbas, 2003). Bianchi *et al.* (2005) used a dynamically coated capillary, although the coating required a fairly lengthy process and had to repeated after 10 determinations. A new fused silica capillary was treated by rinsing at high pressure (20 psi) with 0.5 M NaOH for 20 min, with water for 5 min, followed with 0.1 M hydrochloric acid for 20 min. This procedure was followed by a second treatment with water for 5 min, 0.1 M sodium hydroxide for 20 min, and water for 5 min. Aqueous HDB (0.1% (w:v)) was then flushed through the capillary at 20 psi for 5 min. Using this capillary with a running electrolyte consisting of 35% (v:v) methanol in a solution of 22 mM benzoic acid at pH 6.10 adjusted with 1.0 M Tris-base buffer, determination of acetic, malic, lactic, succinic and tartaric acids in 12 Italian wines was carried out in less than 4 min for each sample.

Derivatization (Section 4.3.3) is not often used these days in either HPLC or CZE analysis of organic acids, but such a method was used to good effect in the analysis of as many as 11 acids in grape wines, indigenous Thai wines, fruit juices, vegetable juices and beer (Santalad *et al.*, 2007). Preanalysis derivatization was carried out with 2-nitrophenylhydrazine (2-NPH) in the presence of *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC.HCl). The running buffer was 30 mM borate in water:acetonitrile (9:1). Results were comparable with those in the literature and, in terms of repeatability, accuracy and LoDs, the method was similar to other CZE methods.

Zone electrophoresis, using a miniaturized chip based system (lab on a chip), has been applied to the analysis of 22 inorganic and organic acids in Slovakian wines of independently known composition (Masár *et al.*, 2005a). The chip was poly (methylmethacrylate) (PMMA) of 115 mm length with channels for injection,

separation and waste, and with two integrated platinum conductivity detectors. Careful control of pH (5.6 and 5.9), using Bis-Tris or Bis-Tris/Bis-Tris propane, 2-[*N*-morpholino]ethanesulfonic acid as carrier ion and PVA as EOF supressor and ionic strength, as well as the use of cyclodextrin complexing agents, allowed the resolution of 22 acids (oxalate and fluoride comigrated).

Capillary electrophoresis has also been much used in the determination of phenolic substances in alcoholic drinks and their raw materials. Much of the interest has been inspired by reports of health benefits, particularly with regard to antioxidant action (Chapter 5.8). There are reports on flavan-3-ols and nonflavonoid phenols in white wines (Andrade *et al.*, 2001), nonflavonoid phenols in wine (Dobiášová *et al.*, 2002; Spanilá *et al.*, 2005; Saenz-Navajas *et al.*, 2009), flavonoid phenols in wine (Wang and Huang, 2004), in apple juice and cider (Peng *et al.*, 2005), in grape skins (Priego Capote *et al.*, 2007), anthocyanins (and derivatives) in wine (Saenz-Lopez *et al.*, 2003; Asenstorfer *et al.*, 2003; 2006; Asenstorfer and Jones, 2007; Saenz-Navajas *et al.*, 2009), in grape skins (Priego Capote *et al.*, 2007), wood derived phenolic aldehydes (Panossian *et al.*, 2001) and total phenolic contents of wine (Minussi *et al.*, 2003; Pazourek *et al.*, 2005). Because many phenolic compounds are present in comparatively low concentrations in alcoholic beverages and their raw materials, some kind of preanalysis focusing (e.g. using solvent extraction or solid phase extraction) is generally required.

Nonflavonoid and flavonoid phenols can be well resolved by native CZE (cathodically driven migration, using uncoated fused silica capillaries and applied voltages of *ca.* +20 kV) using sodium tetraborate buffers (pH ~9–10) as running electrolyte (e.g. Andrade *et al.*, 2001; Dobiášová *et al*., 2002; Spanilá *et al.*, 2005), sometimes with added organic solvent, such as 10% methanol (Saenz-Navajas *et al.*, 2009). Generally, the phenols are detected by UV absorption at 305 nm, or if a diode array detector (DAD) at more than one wavelength.

Tyrosol, a number of benzoic acid and hydroxycinnamic acid type phenols and (–)-epicatechin were determined by CZE after solvent extraction from some Portuguese and Spanish white wines of the *V. vinifera* varieties Albariño, Alvarinho and Loureiro (Andrade *et al.*, 2001). The electropherograms were considered to be rather superior to reversed phase HPLC chromatograms of the same samples, having better resolution, better peak shape and shorter experimental time. The variety Loureiro had a greater total amount of these phenols than Alvarinho.

More recently, reactions between individual hydroxycinnamic acids (caffeic, *p*-coumaric and ferulic acids) alone and in the presence of malvidin 3-glucoside in model wine solutions were studied over a period of six months storage using CZE with UV detection (Saenz-Navajas *et al.*, 2009). The running buffer was 50 mM sodium tetraborate buffer solution (pH 9.4) containing 10% methanol (v:v) as modifier and, unusually, the capillary temperature was kept at 10 °C. LC-MS was used to confirm the structures of product compounds detected by CZE. A number of derivatives corresponding to self-condensation products of the hydroxycinnamic acids and cocondensation products of hydroxycinnamic acids and malvidin 3-glucoside were detected and given tentative structures.

Both diasteroisomers of the stilbene phenols *cis*- and *trans*-resveratrol were determined in red and white wines (Dobiášová *et al*., 2002; Spanilá *et al.*, 2005). Solid phase extraction (SPE – see Section 4.2.4) with ODS columns was used to focus the stilbenes prior to analysis by CZE. For both Alsace (Dobiášová *et al*., 2002) and Australian wines (Spanilá *et al.*, 2005), the *tran*s isomer was generally more abundant and total resveratrols were higher in red wines (up to 5.61 mg/l for Alsace Pinot Noir and 6.27 mg/l for New South Wales Cabernet-Shiraz).

Resveratrols were determined in 47 Czech and Australian red and white wines by CZE after preconcentration by SPE (Pazourek *et al.*, 2005). The running buffer was 25 mM sodium tetraborate (pH 9.38) at 25 °C and the applied potential was +20 kV. Mesityl oxide (0.1%) was used as an OEF marker. Detection was by UV absorption at 305 nm. The electropherogram data were used as fingerprints, which were subjected to a chemometric treatment known as artificial neural networks. This allowed the prediction of vine variety and vintage within the limits of the tested wines (eight varieties and three vintages) with greater than 90% success.

Flavonoid phenols (Section 5.8.6), distributed widely throughout the plant kingdom, have been determined by CZE in wine (Andrade *et al.*, 2001; Wang and Huang, 2004), apples and cider (Peng *et al.,* 2005) and grape skins (Priego Capote *et al.*, 2007). Wang and Huang (2004) studied flavones and flavonols in wine using a running buffer of 35 mM borax (pH 8.9) with applied field strength of 240 V/cm on a fused capillary of 70 cm (effective length 45 cm) × 75 μm (≡ 16.8 kV applied voltage). The flavonols quercetin, kaempferol and myristin, determined by UV detection, were the most abundant of nine flavonoid phenols in the wine sample, where CZE percentage recovery and reproducibility (as %RSD) ranges were 90.1–99.8 and 0.43–5.68, respectively.

The chalcone phloridzin, along with chlorogenic acid (a hydroxycinnamic acid derivative), the flavan-3-ol (–)-epicatechin and the flavone myricetin were determined in commercial apple juice and Chinese ciders by CE with amperometric (voltammetric) detection (Peng *et al.*, 2005). The running buffer was 50 mM borate buffer (pH 8.7) and using an applied voltage of 18 kV, the analytes could be separated within 20 min in a 75 cm length capillary, with good accuracy (recoveries 95–98%) and precision (%RSD 1.8–3.6).

All the above studies used either solvent extraction or SPE for preconcentration of sample and most of the CE experiments were run with purely aqueous buffers. In contrast, a superheated ethanol-water extraction (120 °C, 80 bar) procedure followed by centrifugation was used to extract phenolic compounds from pomace grape skins to be determined by CE using an aqueous/organic buffer with both UV and fluorescence detection (Priego Capote *et al.*, 2007). Sodium tetraborate (50 mM) with 10% methanol (pH 8.4) solution was used as background electrolyte with an applied potential of 25 kV to separate 10 polyphenolic compounds (kaempferol, myricetin and quercetin, (+)-catechin, (–)-epicatechin, resveratrol and four anthocyanins) within 10 min. Intraday variability of results ranged from 3.49 to 6.27%. Pomace grape skins are those remaining after fermenting and pressing red wine (Section 2.9.2). They were high in malvidin 3-glucoside, delphinidin 3-glucoside, kaempferol, myricetin, quercetin and resveratrol, but low in other anthocyanins and the flavan-3-ols, those compounds probably having been leached from the skins in the winemaking process.

Anthocyanins in wines have been determined by CZE with UV-visible detection, using sodium tetraborate buffer background electrolyte with 15% methanol at an applied potential of 25 kV (Saenz-Lopez *et al.*, 2003). Reproducibility (%RSD <8%) and accuracy (% recoveries: 94–107%) were comparable with those of solvent gradient HPLC methods, although CZE experimental time was less and there was no need for solvent gradient.

The above research was largely concerned with the phenolic compounds originating from raw materials (e.g. grapes or apples) and perhaps some produced from these during fermentation and storage. Phenolic compounds that originate from wood make important contributions to the mouthfeel of many alcoholic drinks. Wood maturation derived aromatic phenolic aldehydes, coniferaldehyde, sinapaldehyde, syringaldehyde and vanillin, have been determined in brandy and wine by CZE using an uncoated silica capillary, 50 mM borate buffer (pH 9.3) and diode array detection (DAD) at 348, 362, 404 and 422 nm (Panossian *et al.*, 2001). Detection limits were 0.1425–0.275 μg/l.

Preservatives play an important role in some alcoholic drinks, notably wine, but because of potential health problems, their use is strictly regulated (Section 5.10.2) and hence regular determination is necessary. Sulfites in wine have been determined by CZE (Masár *et al.*, 2005b) and sorbate in wine has likewise been analyzed by CZE (Dobiášová *et al.*, 2002; Huang *et al.*, 2005). Sorbic acid has been determined in wine by CZE directly, without a pretreatment step, with a good precision and a detection limit of 0.03 mg/l (Dobiášová *et al*., 2002). Alsace white wines were found to have sorbic acid levels of 7.4–185.8 mg/l.

In-sample hydrogen peroxide oxidation of total sulfite in wine at high pH (∼10) to sulfate with the separation and quantification of the latter anion by zone electrophoresis (ZE) on line coupled with isotachophoresis (ITP) on a column coupling chip, with integrated conductivity detection (Masár *et al.*, 2005b). The ITP-ZE separations were carried out in a hydrodynamically closed separation compartment of the chip at pH 3.5–4.0

in an electrolyte consisting of chloride or citrate, β-alanine, Bis-Tris propane buffer with suppressed electroosmotic flow due to added methyl hydroxyethylcellulose. ITP extensively eliminated the matrix, allowing high accuracy (recovery: 99–100%) and a detection limit of 90 μg /l.

Again, inspired largely by possible health hazards and existing regulations, biogenic amines in alcoholic beverages have received some attention from CE analysts (Jayarajah *et al.*, 2007; Cortacero-Ramírez *et al.*, 2007).

A portable microfabricated capillary electrophoresis instrument (lab on a chip) was used for the determination of biogenic amines, particularly histamine and tyramine, in a variety of wines and sake (Jayarajah *et al.*, 2007). The analytes were determined with fluorescamine labels on their primary amine groups and analyzed on a microfabricated glass CE device containing 21.4 cm long separation channels. Samples were injected by applying –2.5 kV at the waste reservoir and earthing the sample and cathode reservoirs, while floating the anode during the injection period (10 s). The sample and waste reservoirs were then held at –2.65 kV, the anode at 0 V, and the cathode at –15 kV during CE runs. Tyramine was found mainly in red wines (<1–3.4 mg/l), which had histamine levels of 1.8–19 mg/l. The highest levels of histamine (20–40 mg/l) were found in sake. Analysis of a wine undergoing a typical winemaking process indicated that the biogenic amines were formed during both alcoholic and malolactic fermentation.

Conventional CZE was used for the simultaneous determination of 10 biogenic amines in beer samples, using laser induced fluorescence detection (Cortacero-Ramírez *et al.*, 2007). Sample amines were first derivatized with fluorescein isothiocyanate (FITC), filtered and then subjected to CZE using an uncoated capillary with an applied potential of 30 kV and 50 mM sodium borate and 20% acetone electrolyte at pH 9.3. It was possible to analyze biogenic amines in brewing process samples and in beer samples in less than 30 min, obtaining detection limits in the range 0.3–11.9 μg/l.

CE has been used in the analysis of a number of other components of alcoholic beverages, such as the fungal contaminant ochratoxin A (González-Peñas *et al.*, 2006), and pesticide residues (Rodríguez *et al.*, 2003).

The presence of ochratoxin A in wine due to contamination of grapes (mainly by *Aspergillus spp.*, see Section 5.11.4) is a problem and since the EU has established a maximum level of tolerance in wine of 2 ng/ml, its regular determination in wine is important. Ochratoxin A (extracted from samples in chloroform) was determined in a number of fortified wines by CZE using fused silica capillary with a 'bubble cell' of 48.5 cm total length (40 cm effective length) and 50 μm i.d. at 10 °C (González-Peñas *et al.*, 2006). The electrolyte was sodium tetraborate (10 mM) (pH 9.3) and the applied potential was 20 kV. UV detection was used and fixed at 380 nm. Although the method compared well with an established HPLC-fluorescence detection method in terms of accuracy and precision, it was considered unsuitable for routine analysis of real samples because of its high detection limit; a situation that could be rectified by using a fluorescence detector.

CZE offers several advantages over the most popular chromatographic method of wine analysis (HPLC), amongst which are greater speed, higher resolution and lower solvent usage. However, two limitations of CZE (in its native form, as described above) are its inability to resolve uncharged analytes and its inability to resolve ionic analytes of similar electrophoretic mobilities. These two limitations can be eliminated by the introduction of a second mode of separation – partition – either by the addition of a small amount of surfactant (which forms micelles) or by using a capillary that is packed with a typical HPLC reversed phase. The first of these modifications is known as micellar electrokinetic chromatography (MEKC), whereas the second is called capillary electrochromatography (CEC).

Micellar Electrokinetic Capillary Chromatography (MECC OR MEKC)

MEKC is a mode of electrokinetic chromatography in which a surfactant, such as sodium dodecylsulfate (SDS), is added (at concentrations that favor micelle formation) to the electrolyte solution. The separation

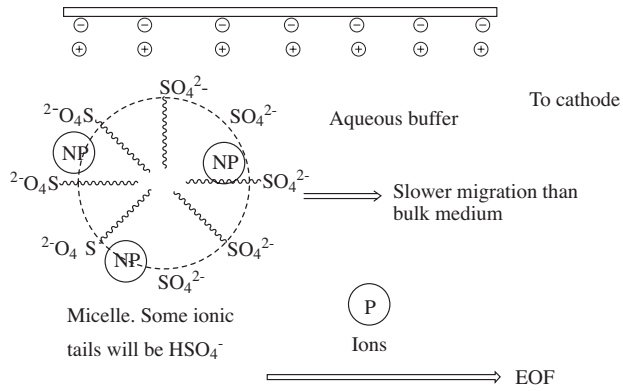


Figure 4.6.3 *Simplified mechanism of separation of neutral analytes by MEKC. NP = nonpolar analyte, P = polar analyte. Ions differentially interact with micelles*

principle of MEKC is based on a differential partition of both ionic and particularly neutral analytes between the micelles and the solvent as the micelles migrate toward the cathode with the electro-osmotic flow – more slowly than the bulk of liquid (Figure 4.6.3). The micelles thus constitute a pseudo or moving stationary phase: hydrophobic (nonpolar) neutral molecules are closely associated with the hydrophobic interiors of the micelles and migrate with the micelles, whereas hydrophilic (polar) neutral molecules associate only weakly the micelles and migrate more quickly, at about the same rate as the bulk flow.

Capillary Electrochromatography (CEC)

This technique is essentially a hybrid of CE and HPLC; it can be regarded as capillary HPLC in which flow of mobile phase is caused by electro-osmotic drive, rather than by pump action (Figure 4.6.4). The stationary phases, mostly particulate silica based reversed phases of the C_{18} and C_8 type, are similar to those of HPLC. It has most of the advantages of reversed phase HPLC, but with higher efficiencies, because of the longer columns (up to 100 cm) with smaller particles (down to 0.5 μm). Efficiencies of 700 000 or more plates per meter are not uncommon. Resolution may also be improved, because of the flat 'plug' flow through the stationary phase produced by the EOF, as opposed to parabolic flow produced by pump action. Finally, because no pump is used, there is no back pressure problem, and very small stationary phase particles can be used. On the negative side, packing of capillaries with stationary phase material requires much skill and consequently reproducibility of CEC column performance has been problematic.

Five common food preservatives (benzoic acid, butyl paraben, methyl paraben, propyl paraben and sorbic acid) in foodstuffs, including Taiwanese wine, were analyzed by CEC using a methacrylate ester based monolithic capillary, rather than a particulate silica based column (Huang *et al.*, 2004). Optimal separation of the five preservatives was obtained within 7.0 min with a mobile phase composed of aqueous phosphate buffer: acetonitrile (35:65 v:v) at pH 3.0.

4.6.2 Flow Injection Techniques

Flow injection analysis (FIA) is basically an automated sample and reagent handling technique. It was developed in the 1970s to cut down the manual handling of solutions, to minimize the amount of materials

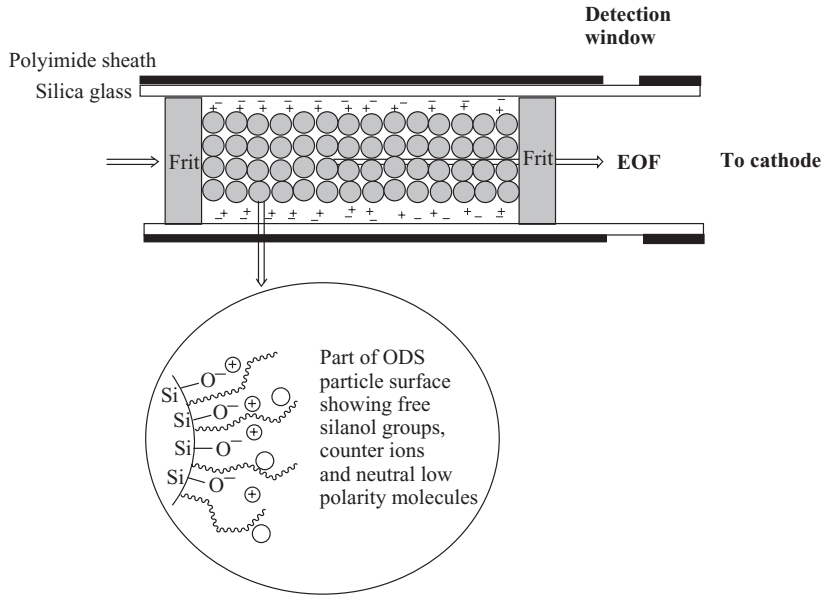


Figure 4.6.4 *Principle of capillary electrochromatograhy. Not drawn to scale*

handled (and therefore the amount of waste) and to increase sample throughput. It was originally devised for serial assay of large numbers of samples, as in quality control, but since the method allows automated handling of sample and reagent solutions under strictly controlled reaction conditions, its versatility made it suitable for a wide range of situations. Nowadays, FIA has been applied to the study of live cells, monitoring chemical processes in real time, biotechnology, immunoassays, including antibody/antigen reactions, and the determination of a wide range of components in alcoholic beverages and other foodstuffs.

The original technique (Figure 4.6.5) is still probably the most widely utilized. In its simplest form, the sample is injected into a flowing carrier stream of reagent, although this can be performed the other way around (inverse flow injection analysis). As the injected zone moves downstream, the sample solution disperses into the reagent steam, where reaction occurs and the product is formed, which now has a desired physical parameter for detection purposes (e.g. an UV-visible chromophore, a fluorophore or a redox group). A flow through detector placed downstream records the desired physical parameter, such as UV-visible absorbance, fluorescence absorbance or electrochemical characteristics.

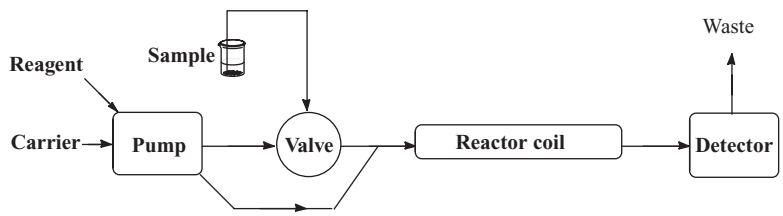


Figure 4.6.5 *Schematic diagram of basic flow injection set-up. Filters and degassing units can be placed online and an additional reagent and reactor coil can be used*

A standard FIA system usually consists of a high quality multichannel peristaltic pump, an autosampler, an injection valve, a coiled reactor and a detector such as a photometric flow cell (Figure 4.6.5). Additional components may include a flow through heater to increase the speed of chemical reactions, columns for sample reduction, degassing units and filters.

The typical FIA flow rate is 1 ml/min, typical sample volume consumption is 100 μl per sample, and typical sampling frequency is two samples per minute. With regard to precision, FIA determinations are usually comparable with other techniques, such as chromatographic methods, typically having reproducibilities (expressed as %RSD) of a few percent.

A summary of FIA methods used in the assay of alcoholic beverage components is given in Table 4.6.1. In essence, FIA methods are superautomated versions of manual or semi-automatic methods for specific analytes (such as ammonia, ethanol, glucose, glycerol, metal ions, sulfite, tartaric acid and urea) or families of analytes (such as nitrogen compounds, polyphenols and reducing sugars). Detection usually requires reaction of analyte(s) with a reagent that confers properties to the analyte(s) suitable for a specific detector, such as UV-visible, fluorometric, or electrochemical properties. Furthermore, FIA has been instrumental in the development of miniaturized electrochemical sensor and biosensor devices used for field work and routine quality control. Some FIA methods require more than one reagent, whereas a few involve direct detection, such as in the assay of metal ions in wine (Lapa *et al.*, 1996; Segura *et al.*, 1999; Wangkarn and Pergantis, 1999; Chuachad and Tyson, 2005; Elçi *et al.*, 2009) and beer (Fernandes and Rangel, 1997; Segura *et al.*, 1999; Ampana *et al.*, 2002), determination of total soluble solids (density) of wines by refractive index measurement (Mataix and Luque de Castro, 2001a), the assay of ethanol in wine by density measurement (González-Rodríguez *et al.*, 2003) and the assay of total phenolic content by the 280 nm absorbance method (Section 4.4.3) (Mataix and Luque de Castro, 2001b).

Lapa *et al.* (1996) used flow injection zone sampling technique to determine Ca, K, Mg and Na in wines by flame photometry and flame atomic absorption spectroscopy, whereas the same elements in beer were determined in beer by Fernandes and Rangel (1997) using flame atomic emission and atomic absorption spectroscopy. Computer operated rotatory valves allowed a wide range of reproducible dilutions and also permitted the most appropriate dilution level according to the analyte concentration for each sample during the same analytical run (Lapa *et al.*, 1996). Thus, identical dilutions for samples with different concentrations of analytes were avoided.

Flow injection has been combined with hydride generation atomic absorption spectroscopy (see Section 4.4.4) for the direct determination of arsenic in beers and wines, without sample pretreatment (Segura *et al.*, 1999), and combination of FI with inductively coupled plasma mass spectrometry (ICPMS – see Section 4.4.5) was used to determine ultratrace levels of arsenic in wine (Wangkarn and Pergantis, 1999).

More recently, cadmium in wine, and lead in wine and rum were determined by FI using an electrothermal AAS detector with chemical vapor generation from a BH_4 anion exchanger (with in atomizer trapping) and flame quartz atomizer AAS detector with hydride generation, respectively (Chuachad and Tyson, 2005; Elçi *et al.*, 2009). Lead hydride (PbH_4) was generated using potassium ferricyanide as oxidant and sodium tetrahydroborate ($NaBH_4$) as reductant Elçi *et al.*, 2009). For lead in wines (diluted prior to analysis) and rum (assayed neat), the detection limit was 0.16 μg/l, recoveries were in the range 95–110% and reproducibility (as %RSD) ranged from 6.1% (wine) to 12.0% (rum).

In the determination of total soluble solids (Mataix and Luque de Castro, 2001b), the sample/carrier concentration gradient produced on injection created a refractive index gradient, which was detected by a UV-visible spectrophotometric detector. The magnitude of this gradient was measured by allowing a nonabsorbed light beam to pass through the detector volume. Using a simple manifold, the total phenolic index (measured by absorbance at 280 nm – see Section 4.4.3) could be determined sequentially to the total soluble solids assay.

Table 4.6.1 *Examples of the use of flow injection analysis in the determination of alcoholic beverage components*

Analyte(s)	Beverage	Detector/sensor	Comments/references
Density	Wine	Refractive index	Mataix and Luque de Castro (2001a); total soluble solids.
Metal ions And other elements	Wine	AAS/flame photometry	Lapa *et al.* (1996); Ca, Mg, Na, K.
	Beer	FAES/FAAS or spectrophotometric	Fernandes *et al.* (1997); Fernandes *et al.* (2000); Ampan *et al.* (2002).
	Wine	Electrothermal-AAS	Chuachad and Tyson (2005); Cd.
	Wine/rum	HG-AAS	Elçi *et al.* (2009); Pb.
Ethanol	Wine	Density detector	González-Rodríguez *et al.* (2003).
	Wine	UV-visible	Segundo and Rangel (2002). Enzymatic.
	Wine	UV-visible	Borges *et al.* (2006).
Glucose	Wine	Electrochemical	Schachl *et al.* (2002). Biosensor.
	Wine	Electrochemical	Ulasova *et al.* (2003); residual glucose.
Reducing and other sugars	Wine	UV-visible	Oliveira *et al.* (2001).
	Wine	Electrochemical	Esti *et al.* (2003). Amperometric biosensor.
	Beer	ESI-MS	Mauri *et al.* (2002).
Glycerol	Wine	UV-visible	Segundo and Rangel (2002). Enzymatic.
Polyphenols	Wine	UV-visible	Mataix and Luque de Castro (2001b).
	Red wine	UV-visible	González-Rodríguez *et al.* (2002a). TPI and TAI
	Wine	Chemiluminescence	Costin *et al.* (2003).
	Wine	UV-visible	Schoonen and Sales (2002).
Ascorbate Sulphite and other preservatives	Beer	UV-visible	Luque-Peréz *et al.* (2000).
	Wine	UV-visible	Segundo *et al.* (2000).
	Wine	Electrochemical	Alipázaga *et al.* (2002).
	Wine	Electrochemical	Corbo and Bertotti (2002).
	Wine	UV-visible	Tzanavaras *et al.* (2009); total sulphite.
	Wine	UV-visible	Rivero-Molina *et al.* (1999); sorbic acid.
Nitrogen compounds	Wine	UV-visible	González-Rodríguez *et al.* (2002b); ammonia, involving pervaporation.
	Must and wine	UV-visible	González-Rodríguez *et al.* (2002c); readily assimable nitrogen.
	Wine	Chemiluminescence	Costin *et al.* (2004); Proline
	Wine	CE/ESI-MS	Santos *et al.* (2004); biogenic amines.
Tartaric acid	Wine	UV-visible	Silva *et al.* (2002).
Multiple	Wine	Electrochemical	Esti *et al.* (2003). Fermentation monitor.

González-Rodríguez *et al.* (2003) used pervaporation for separation of ethanol from the sample and a vibrating tube type detector (Section 4.6.3). Pervaporation is a nonchromatographic membrane separation technique that selectively separates volatile components of liquid mixture by partial vaporization through a nonporous polymeric membrane (Section 2.13.3); it has been used in many FI methods for the determination of more volatile components, such as ammonia, SO_2 and ethanol. Good correlation was achieved between

the FIA method and a reference distillation method, the latter being much more labor intensive and somewhat less precise.

The majority of flow injection (FI) techniques use at least one reagent to convert the analyte to an easily detectable product. In many cases, the reagent type, reaction conditions and method of determination are similar to those used in nonautomated reference methods, but FI has inspired a large number of modified or new methods, particularly with regard to the use of electrochemical sensors (Section 4.5.3).

UV-visible detection of derivatized or oxido reduced analytes is frequently used in FIA. Total phenolic content/index (TPC/TPI) of red and white wines has been determined by FI versions of the Folin–Ciocalteu method (Section 4.4.3) (Celeste *et al.*, 1992; Mataix and Luque de Castro, 2001b). Alternatively, TPC of wines has been determined using the colored complex formed between phenols and 4-aminoantipyrine in the presence of oxidizing agents, such as ferricyanide, peroxodisulfate or periodate (Schoonen and Sales, 2002). Interferences from sugars, tartaric acid and others suffered by the Folin–Ciocalteu method, were not observed for this method, which was precise (RSD $< 3.9\%$) and accurate (relative error, by the Folin–Ciocalteu method $<5.1\%$).

Total anthocyanin index was simultaneously determined with the total phenolic index of red wines using an FI version of the SO_2 bleach method (absorbance at 520 nm) and 'I_{280}' method (absorbance at 280 nm) (Section 4.4.3), respectively (González-Rodríguez *et al.*, 2002a). The valve in the injection mode allowed monitoring absorbances at 520 and 280 nm. After this, a switching valve supplied potassium metabisulfite to the stream and a new sample aliquot was injected. The absorbance at 520 nm was now lower because of bleaching of free anthocyanins. The difference of absorbance between the two measurements at 520 nm gave the free anthocyanin index value.

The determination of ethanol, glycerol and sugars in alcoholic beverages are of fundamental importance, particularly in the case of ethanol and sugars, since legislation imposes certain limitations on the content of these two components, depending on beverage type, area of production and country. Also the extent of tax or duty is dependant on the ethanol content, usually expressed as a percentage (v:v).

Ethanol in beverages can be determined by flow injection analysis with electrochemical detectors, provided that interferences such as carboxylic acids are removed beforehand. Such a method, using an oxidized nickel wire was used to analyze the sample eluent of from a strong anion exchanger (Chen *et al.*, 1997). The optimal conditions for the detection of ethanol were an applied potential of $+0.60$ V (versus Ag/AgC1) in a carrier of 100 mM sodium hydroxide solution. The recoveries of ethanol from wines spiked with standards ranged from 101 to 103 % with an LoD of 10^{-5} M.

Ethanol has been determined in Portuguese wines and fortified wines by combining FI with an immobilized alcohol dehydrogenase reactor and NAD^+ cofactor. The ethanol content was calculated from the absorbance of NADH produced by the enzyme reaction, but ethanol was first separated from the sample medium by gas diffusion, thus maximizing dilution and minimizing matrix intereference (Rangel and Toth, 1999). More recently, ethanol, glycerol and reducing sugars have all been determined in wine using a flow injection version of the appropriate enzymatic essays (Segundo and Rangel, 2002). Immobilized ethanol and glycerol dehydrogenase enzymes, in the presence of NAD^+, were used as the reagents, with determination of NADH at 340 nm after oxidation of the analytes. Relative deviations between the FI method and reference methods were $<3.7\%$ for ethanol and $<3.4\%$ for glycerol, but as always the FIA, the analysis rate was far higher at 45 determinations per hour.

An FI method that uses neither immobilized enzymes nor chromogenic reagents has been developed for the assay of ethanol in wine (Borges *et al.*, 2006). This procedure is based on a falling drop system that is supplied by employing a flow injection manifold. The detection system comprised an infrared LED and a phototransistor. The sample drop grew between these two devices, thus causing a decrease in the intensity of the beam of radiation coming from the LED. The size of the drop was related to the ethanol content of the sample and hence this could be related to the intensity of radiation reaching the phototransistor.

Reducing sugars in wines were determined by oxidation of glucose and fructose with potassium ferricyanide in alkaline solution, using microwaves to increase dispersion and reduce hydrodynamic pressure, by observing the reduction of the absorbance at 420 nm due to the reduction of ferricyanide (Oliveira *et al.*, 2001). Good precision (%RSD = 1.9), good accuracy (% recovery in the range 99.2–104.6) and good agreement with a reference method, with a sampling rate of 54 per hour, were obtained with this method.

Certain oligosaccharides in beer (α-glucans or dextrins) (Section 2.6.2) make important contributions to organoleptic character and hence their analysis is of considerable interest. Oligosaccharides in beer have been determined by a flow injection procedure linked to an electrospray ionization-mass spectrometer (Mauri *et al.*, 2002). The method allowed characterization and quantification of six maltooligosacchirides (up to maltoheptaose) from 1 μl injections of 1000-fold diluted beer samples, thus eliminating matrix effects.

Urea has been shown to be a major precursor of the potentially carcinogenic ethyl carbamate (EC) in alcoholic drinks and hence its assay is of importance. UV-visible detection has also been used in the determination of nitrogen compounds, tartaric acid and sulfites in alcoholic beverages by FIA. A flow injection–pervaporation method for monitoring urea and ammonia in must and wine has been developed (González-Rodríguez *et al.*, 2002b). The method was based on separation of the ammonia from the sample matrix by pervaporation using a PTFE membrane followed by reaction with salicylate, hypochlorite and nitroprusside to form a diazonium salt with maximum absorption at 647 nm. Urea was converted to ammonia by the enzyme urease prior to pervaporation and analysis. The method had double the sampling rate of a reference method, with adequate reproducibility (1.34 versus 0.21 mg/l) and limit of detection (0.90 versus 0.15 mg/l) for urea determination. Readily assimilable nitrogen (RAN) due to ammonia, ammonium and amines in wines was determined by a very different FI method (González-Rodríguez *et al.*, 2002c). Here, the reaction of analyte amine functions with formaldehyde reagent caused a change in medium pH, which was monitored by the corresponding change in absorbance of bromothymol blue indicator (at 616 nm) present in the reagent stream. The detection limit was 10 mg/l and the RAN content of 120 samples of must and wines was found to be 25–35 mg/l.

A multisyringe FI system for the determination of sulfur dioxide in wines, using UV-visible detection, was developed by Segundo *et al.* (2000). The method is based on the *p*-rosaniline method: reaction of SO_2 with formaldehyde and *p*-rosaniline. The manifold included a gas diffusion unit with a hydrophobic membrane (0.22 μm pore size) in order to prevent the color interference of red wines with the spectrophotometric measurement. The method was successfully applied to the determination of free and total SO_2 in wines. A sampling rate of 25–30 samples per hour was possible with good repeatability for 10 consecutive injections of wine samples (%RSD <3.2). Reaction of SO_2 (after its diffusion through a teflon membrane into an alkaline stream at pH 8) with malachite green and detection of the product at 620 nm has also been used to determine the sulfite content of white wine (Melo *et al.*, 2003).

Tartaric acid is generally the most abundant acid of grape must and wine and as it makes an important contribution to wine quality, it needs frequent determination. A flow injection method using a cellulose acetate dialysis unit was developed to analyze tartaric acid in wine by reaction with vanadate in acetate buffer at pH 5 (Silva and Álvares-Ribeira, 2002). Tartrate was included in the reagent stream to reduce background noise, and the UV-visible detector was set at 490 nm. The method was compared with the modified Rebelein method (Section 4.4.3), which uses metavanadate rather than vanadate. Agreement between the two methods was good, with the FI method giving tartaric acid concentrations in 17 Portuguese table wines in the range 0.37–3.19 g/l and in 13 Port wines in the range 0.72–1.89 g/l. The factorial designs and simplex chemometric techniques were used to optimize the operating parameters of the method.

Electrochemical sensors of a wide variety of types have been extensively used in FIA for more easily oxidizable components of alcoholic beverages, such as glucose, reducing sugars and sulfites. Ulasova *et al.* (2003) used a Prussian blue type biosensor (Section 4.5.3) for the determination of residual glucose in wines

and Schachl *et al.* (2002) used a carbon paste electrode modified with glucose oxidase as the biocomponent and manganese dioxide as the mediator working at 0.48 V versus Ag/AgCl reference electrode. The sensor could be operated for 12 h without signal loss under FI conditions: flow rate 0.2 ml/min of 0.2 M phosphate buffer (pH = 7.5) at 21 °C. Both methods were used to assay the glucose content of white wines.

Antioxidant and antibacterial agents, such as sulfite and sorbic acid respectively (see Chapter 2.5), are very useful, but highly regulated additives in the alcoholic drinks industry and hence need regular assessment. FIA involving a variety of electrochemical detection methods have been widely applied to the assay of sulfites. Most of these methods include a membrane system for diffusion of SO_2 from the sample and use either potentiometric (Araujo *et al.*, 1998) or amperometric detection (Azevedo *et al.*, 1999; Cardwell and Christophersen, 2000). Araujo *et al.*, (1998) used a homogeneous crystalline tubular electrode as the potentiometric detector for SO_2 in wine, whereas Azevedo *et al.* (1999) used a glassy carbon electrode modified with electrostatically assembled films of tetraruthenated porphyrin working at +0.90 V (versus Ag/AgCl (saturated KCl)). In contrast, a platinum electrode, set at +0.90 V (versus Ag/AgCl), was used by Cardwell and Christophersen (2000) to determine SO_2 in wine, while ascorbic acid (sometimes used as an additive in conjunction with sulfite) was assayed simultaneously at a glass carbon electrode, working at +0.42 V (versus Ag/AgCl).

A sensor consisting of a copper electrode working at 0.60 V (versus Ag/AgCl (saturated NaCl) has been developed to determine sulfite in wine in an alkaline (1 M NaOH) medium (Corbo and Bertotti, 2002). The flow injection manifold included a PTFE membrane to extract sulfite from the sample as SO_2. Changing the pH of the donor stream allowed determination of ethanol (which is electroactive and permeates the membrane) only, then ethanol plus sulfite were assayed and the sulfite concentration was determined by difference. Agreement between the FI method and a recommended method for sulfite assay was excellent.

Another indirect FI method for the determination of sulfite in wine is based on the amperometric measurement (E = 0.1 V) of Cu(III) chemically generated by the sulfite induced oxidation of Cu(II)/tetraglycine by dissolved oxygen (Alipazaga *et al.* 2002). SO_2, displaced by a stream of argon through the acidified sample, was collected in water and injected into the acceptor stream. The detection limit of the method was 1 μM and the precision (as %RSD) for the determination (10 μM) was 1.4% (n = 20). The results compared favorably with those from a standard iodometric method.

Sequential injection analysis (SIA) is a recent development of FIA. The apparatus usually consists of a single channel high precision bidirectional pump, a holding coil, a multiposition valve and a flow through detector. The system is initially filled with a carrier stream into which a zone of sample and a zone of reagent(s) are sequentially injected into a holding coil. These zones soon become overlapped and flow reversals and flow acceleration promote mixing and hence reaction between sample and reagent. The multiposition valve is then switched to the detector position, and the flow direction is reversed, pushing the sample/reagent zones through the flow cell. SIA only uses a small fraction of sample and reagent(s) compared to FIA, and therefore produces less waste. Typical reagent consumption is only around 20–30 μl per assay. This often makes SIA more suitable for remote site and online process monitoring. On the down side, SIA methods generally have lower sampling frequencies than FIA methods. Segundo and Rangel (2002) partially resolved this problem by incorporating an additional pump into their SI system for the analysis of ethanol and glycerol in wine, thus enhancing the flow rate and reducing analysis time by 30%.

All the above examples involve the determination of one or two analytes (sequentially or simultaneously), using a single detector or sensor. An array of electrochemical biosensors has been used to monitor wine fermentation in industrial-scale wineries by a flow injection method (Esti *et al.*, 2003). The electrodes were platinum based, coated with enzymes for the determination of ethanol, fructose, glucose and glycerol. Before analysis, the biosensors were validated on musts and wines using spectrophotometric methods.

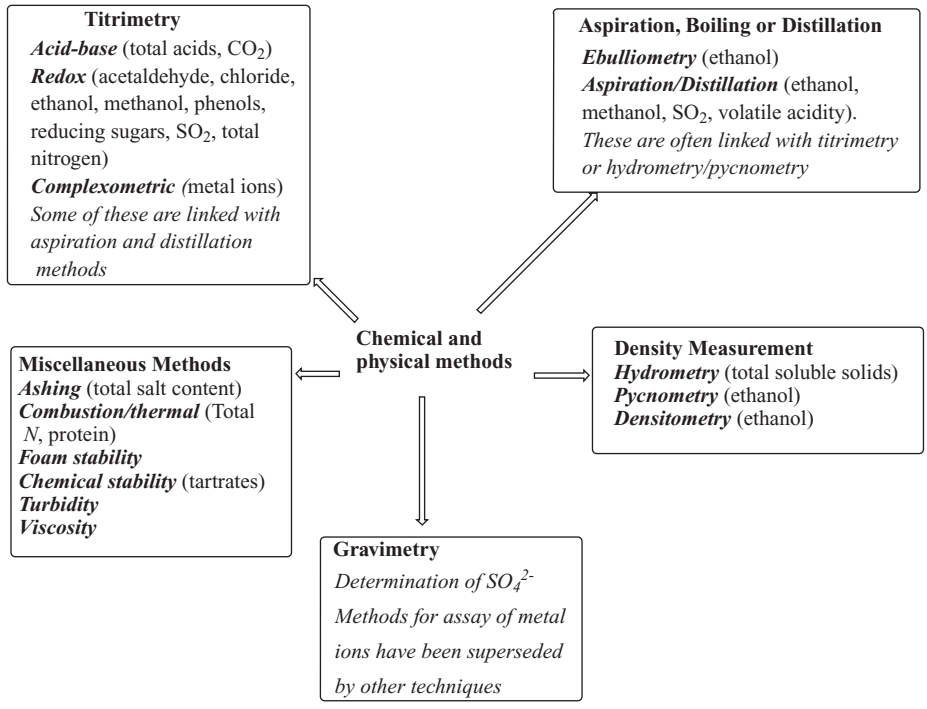


Figure 4.6.6 *Summary of chemical and physical methods*

4.6.3 Chemical and Physical Methods

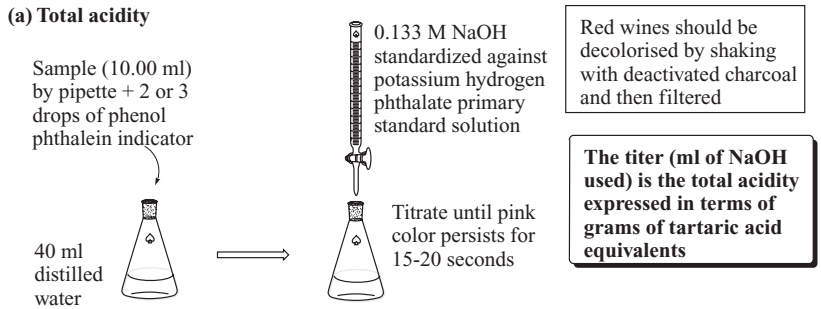
For the purposes of this section, chemical methods are defined as those that involve the use of chemical reactions that are monitored by eye and do not involve the use of spectrometers, chromatographic or electrochemical equipment. Likewise, physical methods are defined here as those that involve the measurement of physical parameters, without the use of such instruments. Many of these methods are still in routine use and some are standard reference procedures, although many others have been replaced by instrumental and/or automated methods. This account is not intended to be exhaustive, rather it is meant to be a summary of the more important or more widely used chemical and physical methods, as outlined in Figure 4.6.6.

Volumetric and Gravimetric Methods

Titrations and gravimetric methods were once much used in the determination of a range of components in alcoholic drinks. Some titrimetric methods are still used today, but gravimetric procedures have been replaced by methods that are quicker, more reliable, more sensitive and more robust.

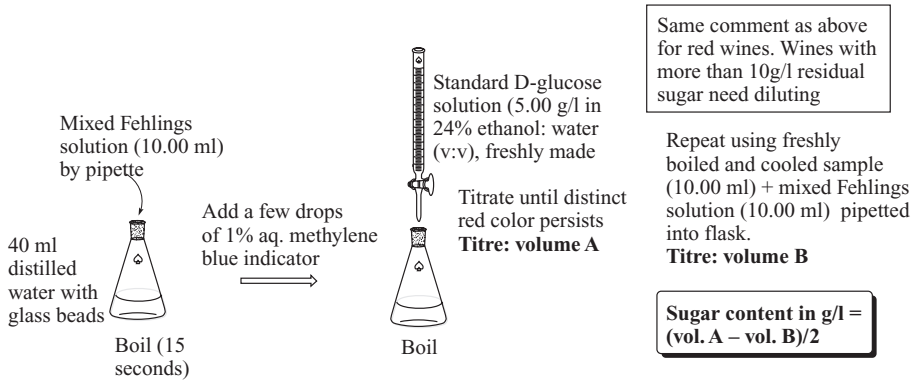
Acid–base titrations using chromogenic indicators are still in use for the determination of total acidity, but even here, in larger wineries or breweries, automated pH titrations (Section 4.5.1) or automated flow injection spectrophotometric techniques (Gaiao *et al.*, 1999) are often preferred. For the smaller winery, the procedure described by Jackson and Schuster (1981) is convenient (Figure 4.6.7(a)).

The majority of titrimetric procedures still used in alcoholic beverage analysis are of the redox type. Residual reducing sugars in drinks can be assayed using Fehlings solution. Although there are many versions



(b) Sugar levels below 2.2 ^{o}Brix (1 ^{o}Baume): Lane and Eynon method

Fehlings solution A [$CuSO_4.7H_2O$ (69.2 g/l)]; Fehlings solution B [sodium potassium tartrate (346 g/l) and NaOH (100g/l)]. Mix 1:1 immediately before use.



(c) Total and free sulfur dioxide content: Ripper method

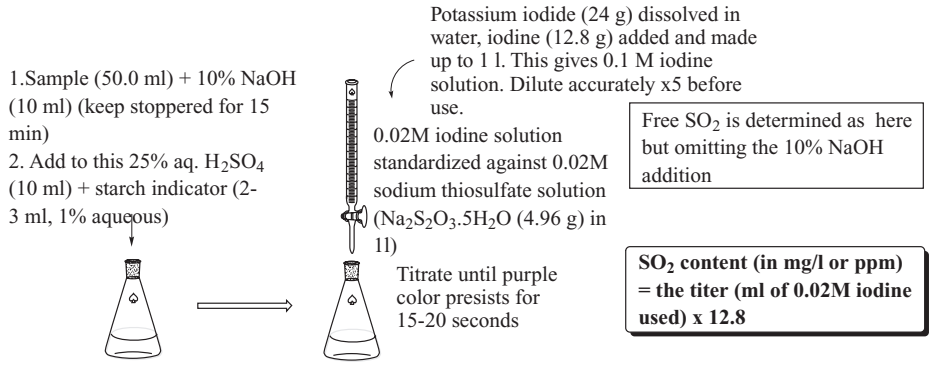


Figure 4.6.7 *Some important convenient titration procedures, suitable for a small winery*

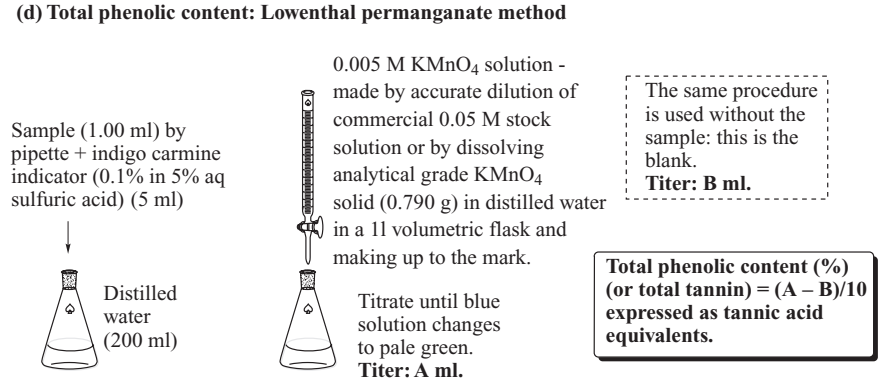


Figure 4.6.7 *(Continued)*

based on this solution (Amerine and Ough, 1980a), they all involve the reduction of blue Cu(II) to red-brown Cu_2O and the Lane and Eynon method is probably the best known (Jackson and Schuster, 1981). For a small winery or brewery, a summary of the Lane and Eynon procedure is given in Figure 4.6.7(b).

The diastatic power of malt (its ability to hydrolyze starch to sugars) can be measured by use of titrations involving Fehlings solution or a modification of it made by Soxhlet (JECFA, 1992). It is often expressed in units of degrees Lintner, defined as follows (JECFA, 1992): 'a malt has a diastatic power of 100 °Lintner if 0.1 ml of a clear 5% infusion of malt, acting on 100 ml of a 2% starch solution at 20 °C for 1 hour, produces sufficient reducing sugars to reduce completely 5 ml of Fehling's solution.' European brewers often use Windisch–Kolbach units (°WK) rather than °Lintner units (°L), the two being related by Equation 4.6.3. See also Section 4.4.3 for colorimetric methods for the determination of overall diastatic power and the activity of individual amylolytic enzymes.

$$^{\circ}\mathrm{L} = \frac{^{\circ}\mathrm{WK} + 16}{3.5} \tag{4.6.3}$$

In the JECFA procedure, which refers to standard procedures of the American Society of Brewing Chemists (ASBC), the Soxhlet solution is standardized using a solution of pure sucrose of accurately known concentration. A filtered malt infusion (prepared in 0.5% sodium chloride solution), after dilution with sodium chloride solution, is allowed to saccharify a buffered starch solution for 30 min, after which time sodium hydroxide solution is added to stop the diastasis. A blank is prepared in the same way, but adding the sodium hydroxide to the diluted malt infusion before the adding the starch solution. The saccharified starch solution (in the burette) is titrated against the gently boiling soxhlet solution, adding methylene blue late in the titration to determine the end point (blue to lavender). An identical procedure is performed on the blank. The diastatic power determined by this method is not strictly the Lintner value, because of the 0.5% NaCl solution, rather than water, used for extraction of diastase.

The official methods of the Institute and Brewing and Distilling and the American Society of Brewing Chemists use a procedure that gives results in Lintner units, whereas the European Brewery Convention (EBC) has a somewhat different procedure that gives results expressed as Windisch–Kolbach units, as mentioned above. Yet other procedures exist (particularly involving other malted cereals, such as sorghum), which involve other units – see EtokAkpan (2004) for a comparison of the IoB/ASBC, EBC methods for malted

barley and the South African Bureau of Standards (SABS) method for malted sorghum (this gives diastatic activity as sorghum diastatic units, SDU).

The grain bill of a beer mash (Section 2.6.2) should have a diastatic power of at least 40 °Lintner to ensure efficient conversion of starch to sugars in the mash; most pale barley malts have a diastatic power up to 160 °Lintner.

Total and free sulfur dioxide, mostly arising from the use of some form of sulfite as a preservative (Section 2.5.2), can be determined by a number of iodometric procedures (Amerine and Ough, 1980b), of which the Ripper method is probably the best known (Jackson and Schuster, 1981). See Figure 4.6.7(c) for a summary of this method, suitable for a small brewery or winery. The Ripper method suffers from interferences due to reactions between iodine with aldehydes, phenols and sugars and the iodine solution needs to be freshly prepared. More reproducible results are obtained using aspiration (aeration) methods, discussed later (Figure 4.6.10), or by an iodate titrimetric method (Amerine and Ough, 1980b), but for routine analysis, particularly of dry white wines, the Ripper method is satisfactory.

Ethanol, after a suitable distillation procedure (Figure 4.6.9 – see later), can be determined by oxidation with excess acidified standard dichromate, the unreacted dichromate being titrated against iodide, which produces iodine that is then titrated with standard thiosulfate solution (Amerine and Ough, 1980c). Acetaldehyde can be assayed using a similar distillation apparatus (Figure 4.6.9), but distilling the sample (containing less than 30 mg of acetaldehyde) from saturated sodium borate solution (pH $\sim$ 8) into a solution of potassium metabisulfite, with which it forms the bisulfite addition compound (Amerine and Ough, 1980c). The distillate is then acidified and titrated against 0.1 M iodine, using starch indicator. This removes excess bisulfite. Sodium borate solution is then added and the liberated bisulfite is titrated against 0.02 M iodine solution.

The chloride ion content of alcoholic drinks or water is of interest because of legal restrictions in the EU ($<$152 mg/l) on chloride levels in wine and because of beneficial influences of low to moderate levels in brewing water. Chloride is nowadays assayed mostly by ion chromatography (Section 4.3.3), capillary electrophoresis (Section 4.6.1) or by electrochemical methods (Section 4.5.1), but can be determined volumetrically by precipitation of silver chloride in the presence of ferric nitrate solution and titration of the excess silver nitrate with potassium thiocyanate solution to a persistent brick-red endpoint, in the presence of diethyl ether (Amerine and Ough, 1980d). Highly colored samples (e.g. red wines) should be passed through a strong anion exchange resin before titration (as the color masks the endpoint), eluting with 1 M nitric acid. Residual color can be removed acidifying the eluent with 20% aqueous nitric acid and a few drops of saturated $KMnO_4$ solution.

Calcium is an important constituent in wine because of the problem of precipitation of calcium tartrate and oxalate; these salts are slow to precipitate and usually form in the bottled product. Calcium is also important in the brewing water of certain beers (Section 2.6.2), giving beneficial pH effects in the mashing step, which is also of benefit in subsequent brewing steps. Calcium in beverages is derived from the soil (via the raw materials) and water, and from various beverage production processes (e.g. addition of $CaSO_4$, $CaCO_3$; treatment with calcium bentonite; storage in concrete tanks etc). Nowadays, it is mostly determined by atomic spectroscopic techniques (Section 4.4.4), but it can be assayed by ion chromatography (Section 4.3.3), electrochemical methods (Section 4.5.1) and colorimetric methods (Section 4.4.3). Volumetrically, calcium may be determined by passing the beverage through a strongly basic anion exchange column (to remove interferences), evaporating the eluent to dryness, ashing and titrating the ash (dissolved in dilute acid) with EDTA solution, in the presence of ethanolamine (to prevent interference from Al^{3+}, Fe^{2+}/Fe^{3+} and Mn^{2+}) and with calcon indicator (color change: red-blue) (Amerine and Ough, 1980d).

Sulfate is present in most nondistilled alcoholic drinks and may be legally added (as gypsum, $CaSO_4 \cdot 2H_2O$) to must for the production of flor Sherry and similar wines (Sections 2.10.2, 2.10.3) or to the mash in the brewing of certain beer types (a process known as 'Burtonization' – see Section 2.6.2). Today, sulfate is generally analyzed by ion chromatography, capillary electrophoresis or potentiometric methods, but it may

be determined in desulfited drinks by a gravimetric procedure involving its precipitation with barium chloride solution (Amerine and Ough, 1980d).

Total nitrogen is of importance to winemakers and brewers, since ammonium salts and amino acids are required for effective yeast function and certain proteins contribute to the formation of hazes, whereas others are important in foam formation. Total nitrogen content is determined by the Kjeldahl or Dumas methods (see also the last paragraphs of Section 4.6.3), the former of which involves conversion of all N in the sample to NH_4^+, by 'digestion' of the sample in concentrated sulfuric acid in the presence of selenium oxychloride or copper sulfate catalyst and with added sulfate salts to increase the boiling point. After vigorous heating, the clear solution (initially charring occurs) is cooled and concentrated sodium hydroxide solution is added until alkaline. This converts ammonium salts to ammonia, which in the original method is distilled directly into a 4% boric acid solution, where the ammonia is converted to ammonium borate. In a titration reaction, ammonium borate behaves like free ammonia and is determined by titration against standard hydrochloric acid. Alternatively, the ammonia can be distilled into excess standard hydrochloric acid solution and the unreacted HCl is determined by titration with standard alkali, such as NaOH. A blank is taken through exactly the same procedure, with pure water instead of the sample.

Some Kjeldahl instruments involve determination of NH_4^+ in the digestion mixture (without distillation) by use of Nessler or Biuret color development reactions, with manual or automatic colorimetric monitoring (Section 4.4.3). Also, instead of the distillation step followed by titration, the ammonium salts formed in the digestion step can be determined by use of an ammonia specific ion electrode (Section 4.5.1). In these cases, a set of standards is analyzed under identical conditions and a standard curve is used for the determination of ammonium or ammonia in the sample. An account of a manual Kjeldahl determination is given in Amerine and Ough (1980e), but nowadays, automated versions of the procedure are generally used.

Ammonia content (as NH_4^+) of alcoholic beverages can be determined by volumetric methods (Amerine and Ough, 1980e), even though ammonia is mostly analyzed by ion chromatography, capillary electrophoresis, colorimetric or ion selective electrode methods these days. Ammonia may also be assessed by use of a weak cation exchange/distillation process, followed by acid–base titration (Amerine and Ough, 1980e) (Figure 4.6.8).

Total α-amino nitrogen can be estimated using column chromatography to remove ammonia, followed by evaporation of the eluate to dryness, drying the residue and performing a formol titration on it, to a pH 9.0 endpoint (Amerine and Ough, 1980e).

The Kjeldahl or similar methods, such as the Dumas method, are still used to assay total N (see last paragraphs of this section), whereas amino acids are determined by chromatographic (Sections 4.3.2 and 4.3.3) or colorimetric (Section 4.4.3) methods, and total proteins may be estimated by a number of colorimetric methods including one that uses the biuret color reaction – see Section 4.4.3, or by NIR spectroscopy (Section 4.4.2). In the latter mode of analysis, data for construction of a database of optimized calibration models usually comes from Kjeldahl or Dumas determinations.

Distillation, Aspiration (Aeration) and Ebulliometric Methods

Certain volatile constituents of alcoholic beverages may be analyzed by methods that involve distillation, aspiration or ebulliometric (boiling) procedures. Classic amongst these are the determination of ethanol and volatile acidity by distillation, sulfur dioxide by aspiration and ethanol by ebulliometry. Although these methods are still in use, they have been largely replaced by less labor intensive and more rapid instrumental methods (e.g. GC for ethanol, HPLC or CZE for acetic acid).

Ethanol may be assayed by use of distillation equipment similar to that shown in Figure 4.6.9. The specific gravity of the distillate can be measured using a Brix specific gravity hydrometer or a pycnometer, adjusting for temperatures other than 20 °C by using the correction table (Table 2) in Appendix 2. Distillation removes

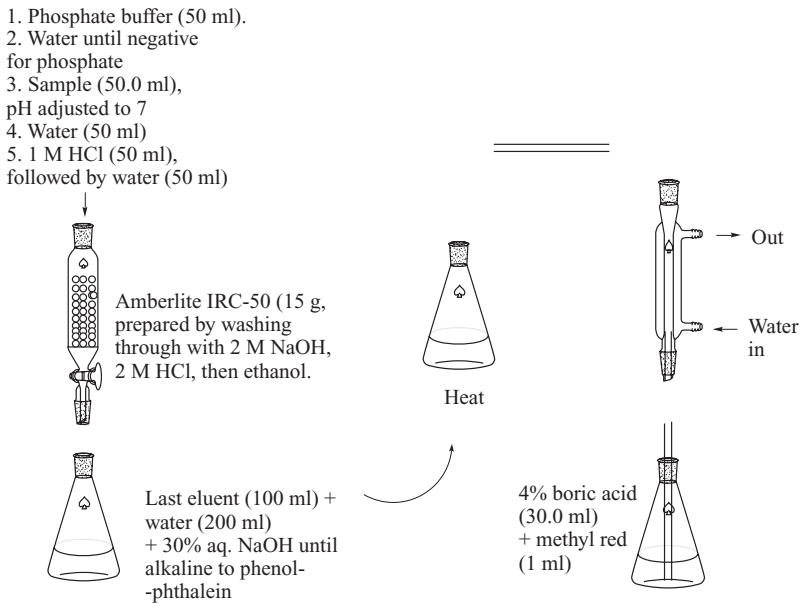


Figure 4.6.8 *Determination of ammonia content of beverages by titration*

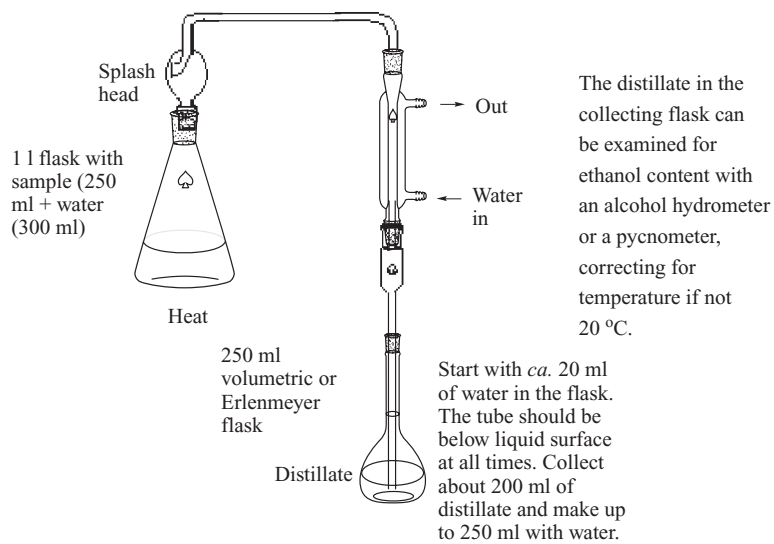


Figure 4.6.9 *Assay of ethanol content by distillation*

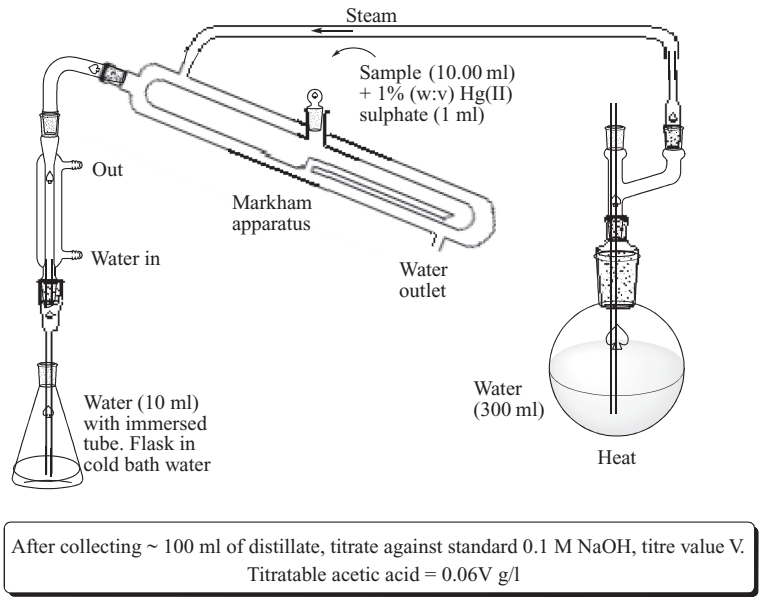


Figure 4.6.10 *Determination of volatile acidity using Markham apparatus*

interferences from dissolved constituents, such as salts and sugars. A rather more elaborate distillation set up, using a Markham apparatus, is required for the determination of volatile acidity (Jackson and Schuster, 1981) (Figure 4.6.10). Volatile acids (mainly acetic acid) are driven off as the steam bubbles through the sample in the Markham apparatus. They are collected in the receiver flask, where after addition of phenolphthalein indicator, they are titrated with standard 0.1 M sodium hydroxide solution.

The aspiration method (Figure 4.6.11) (Jackson and Schuster, 1981) is still used to determine free and bound sulfur dioxide in beverages, as it is more accurate than the standard Ripper method, which can be used

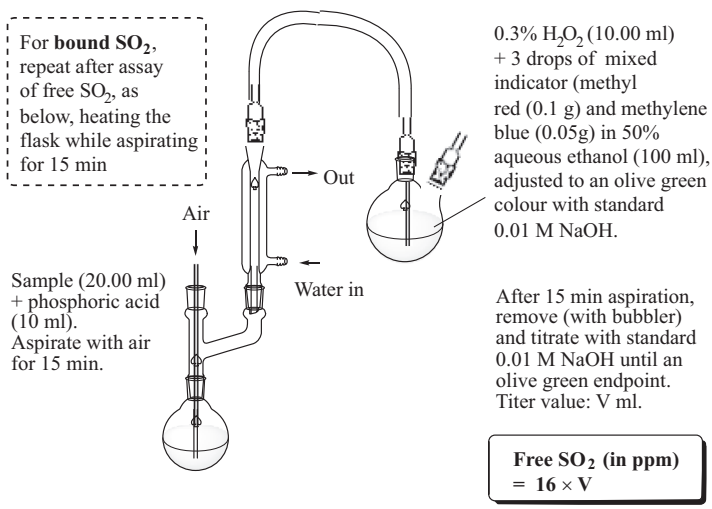


Figure 4.6.11 *Apparatus and outline of aspiration method for the determination of free and bound SO_2*

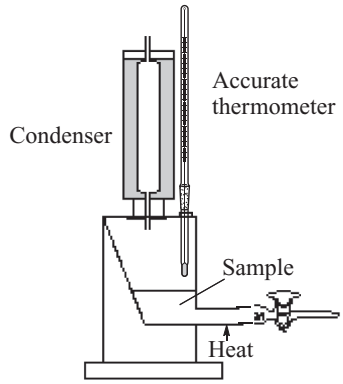


Figure 4.6.12 *Schematic representation of a typical ebulliometer*

for routine checks throughout the production process. Before bottling, or as a final check, it is better to use the aspiration method. Regular assay of known sulfur dioxide standards should be used to check the method accuracy.

Ebulliometry is based on the elevation of the boiling point of a liquid caused by the presence of solutes. It is used primarily for the determination of the ethanol content; mainly degassed beers, ciders and wines, but samples with high sugar content and spirits should be diluted before determination, in which case the result is multiplied by the dilution factor. The instrument used is known as an ebulliometer or ebullioscope, of which there are many commercial versions, those of Braun and Dujardin-Salleron probably being the best known (see Figure 4.6.12 for a general example). The boiling point of pure water is first determined and the instrument calculator is set at 0.00. The condenser and thermometer are removed, the ebulliometer chamber emptied and then rinsed well with the sample. Sample (50 ml) is added to the chamber, the thermometer is reinserted, the condenser (filled with fresh cold water) is screwed down and the steady boiling point of the sample is determined. The ethanol content (as % ABV) is read on the calculator.

Ebulliometer readings can be corrected for the presence of other solutes, such as sugars (say, in sweet wine) by a number of refinements, two of which are shown in Equations 4.6.4 and 4.6.5, the latter being specifically suggested for the Malligand instrument.

$$\text{Ethanol \% (v:v)} = E\% - 0.05S\% \tag{4.6.4}$$

$$\text{Ethanol \% (v:v)} = E\% \left(\frac{100 = 0.62S\%}{100} \right) \tag{4.6.5}$$

where $E\%$ is percentage ethanol (v:v) determined by ebulliometer and $S\%$ is percentage sugar (w:w).

Density and Viscosity Measurements

Density measurements are important in the determination of soluble solids in fruit must and beer wort, as well as in the determination of ethanol content. Additionally, progress of fermentation or distillation can be judged by density measurements. The time honored instrument for measuring relative density or specific gravity (the density of a liquid compared with the density of pure water; 1.000 g/ml at 20.0 °C) is the hydrometer. It comes in many shapes and sizes with different calibrations (sometimes with two or more calibrations): specific gravity (e.g. 1.097), °Oechsle (97); °Brix/Balling or Plato (22.8); °Baumé (12.7); % sugar (22.8); or

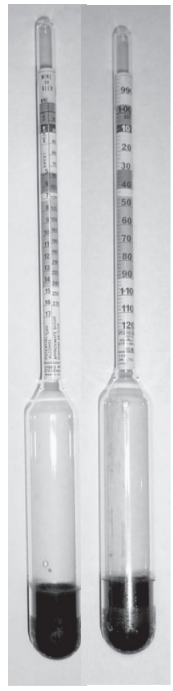


Figure 4.6.13 *Hydrometers and a pycnometer. These hydrometers are calibrated in specific gravity, potential alcohol (% ABV) and sugar equivalent (g/l). Other hydrometers are calibrated in °Baumé, °Brix or °Plato*

(potential) % alcohol (13.4) (see Appendix 2, Table 1 for interconversions). Hydrometer readings are subject to error by the presence of suspended solids and must be corrected for temperatures other than 17.5 °C or 20.0 °C, depending on the manufacturer and type of hydrometer (see Appendix 2, Table 2). Figure 4.6.13 shows some hydrometers and a pycnometer.

The pycnometer is considered by many to be more accurate than the hydrometer in the measurement of sugar or ethanol content: the pycnometer is weighed empty on an analytical balance (to four decimal places), filled with water, reweighed, then filled with sample (after thorough rinsing) and reweighed. The specific gravity (with temperature correction) is calculated according to Equation 4.6.6.

$$\text{Specific gravity} = \frac{\text{Mass of pycnometer with sample} - \text{Mass of empty pycnometer}}{\text{Mass of pycnometer with water} - \text{Mass of empty pycnometer}} \tag{4.6.6}$$

Density detectors (densitometers) are well suited to the in line monitoring of ethanol content, say of an alcoholic fermentation or a distillation. Two major kinds are the vibrating tube and ultrasound velocity types. In the case of the vibrating tube density detector a vibrating (usually) U shaped tube causes mechanical resonant vibrations in a liquid contained in the tube. The square of the resonance frequency is inversely proportional to the sum of the mass of tube and tube contents (Equation 4.6.7). Since both the tube mass and the tube inner volume are known values, the vibrating tube method is able to measure the density of an unknown fluid in a single measurement. Previously, the instrument will have been calibrated with two fluids, usually water and air, which give low and high tones for high and low density, respectively. The U tube is kept oscillating continuously at the characteristic frequency, which depends on the density of the sample.

The oscillation period is measured and converted into density by the equation of the mass-spring-model equation (4.6.7)

$$f = \frac{1}{2\pi}\sqrt{\frac{k}{(m + \rho V)}} \tag{4.6.7}$$

where f is frequency of resonant vibration, k is the spring force constant, m is the mass of the tube and contents, ρ is the density of the liquid and V is the volume of the liquid.

This method has been used in conjunction with flow injection to measure the ethanol content of wine (González-Rodríguez *et al.*, 2003).

An ultrasonic transmitter/receiver type density detector is based on the principle that the sound velocity depends on the density of the medium. The time an acoustic echo takes to travel a fixed path length in the detector element is used to determine sound velocity. The detector is calibrated using a series of ethanol-water standards over a range of temperatures; if a wide range of ethanol contents need to be measured, the detector must be calibrated separately for high ethanol content (say 95–40%) and low ethanol content (say 25–0%). The percentage ethanol (v:v) is calculated by a computer from a complex polynomial of velocity and temperature, using least squares or similar methods. An ultrasound detector has been used to monitor the ethanol content of batch (pot still) distillation run off (Forrest and Taylor, 2004). The detector element was installed just before the spirit safe, along with a temperature sensor. The precision of the instrument was $\pm 0.05\%$ (v:v), or better and its accuracy was sufficient to enable diversion of spirit at the foreshots and feints cut off points – very important in batch distillations (Section 3.2.4). The instrument, linked up to automated systems, has been used in several Scotch malt whisky distilleries.

Viscosity (symbol η, units millipascals per second) measurement is especially important in grain whisky distilleries that use cooked wheat prior to mashing with malted barley, since this can produce high viscosity starches (Section 3.2.3). High viscosity can cause problems during cooking, filtration and evaporation stages, leading to longer process times and higher production costs. There are many kinds of viscometers (instruments for the measurement of viscosity), such as the Ostwald and Ubbelohde U tube capillary, falling sphere, falling piston, bubble, vibrational and rotational viscometer types (including some that measure kinematic viscosity – viscosity (η)/density (ρ)).

Ostwald type viscometers consist of a U tube, where on one arm there is a vertical section of precise narrow bore (the capillary). Above this is a bulb, and there is another bulb lower down on the other arm. In use, liquid is drawn into the upper bulb by suction, then allowed to flow down through the capillary into the lower bulb. Two marks (one above and one below the upper bulb) correspond to a known volume and the flow of sample between these marks is timed, the time being proportional to the sample's kinematic viscosity. Most commercial instruments are provided with a conversion factor, or can be calibrated by a fluid of known density and viscocity. In pilot scale studies associated with grain whisky production, U tube type viscometers have found application in the determination of the viscosity of in grain residue of alcoholic strength distillations, after adjustment of volume with water, centrifugation and filtration (Agu *et al.*, 2006).

The Newport Scientific Rapid Visco Analyzer™ is a cooking and stirring viscometer that has been optimized for testing the viscous properties of starch, grain, flour and foods. It has in built temperature control facilities and thus can be used to measure 'pasting curves' of cereal starches – variation of viscosity with a particular temperature ramping programme. It has found application in the determination of suitable wheat samples for grain whisky production (Broadhead *et al.*, 2004) and in the determination of performance of various barley types in relation to alcohol production (Agu *et al.*, 2008). This instrument can also be used to investigate the extent of modification (Section 2.6.2) of malted barley samples; poorly modified malt gelatinizes at a lower temperature than well modified malt and gives a higher viscosity mash (Broadhead *et al.*, 2004).

Foam is a very important property of most beers (Section 2.6.9), some ciders and sparkling wines. Foam stability is defined as the ability of the foam, once formed, to resist deterioration; it is considered as a function of the bulk liquid viscosity and surface viscosity, as well as the repulsion between electrical double layers of molecules and the Marangoni effect (the ability to repair broken film or bubble structures). Various aspects of foam stability can be measured (Section 2.6.9), the most commonly determined parameters being collapse rate (drainage or retention) and foam adhesion (cling or lacing) (Hughes and Baxter, 2001). One of the most widely used beer foam assessors is the Nibem Foam Stability Tester, which measures the foam collapse time over a distance of 30 mm. After filling the glass with highly reproducible foam made using special sampler and head devices, the glass is placed in the instrument and the foam collapse time is measured by decrease in conductivity.

Similarly, the Nibem Cling Meter calculates the glass area covered by foam by driving a conductivity scanning head down into a rotating beer glass. From the proportion of the area covered by beer foam and the total scanned area, the cling value is calculated and expressed in percentage foam coverage.

Bikerman's method is sometimes used to measure cider foam parameters (Blanco-Gomis *et al.*, 2007). The equipment consists of a glass column (60 cm $\times$ 1 cm) with a sintered glass disk (pore size 10 μm) at the bottom. Degassed cider (10 ml) at 20 °C is placed in the column and carbon dioxide is forced through the bottom at 30 ml/min. Maximum height and stable height can be recorded while the gas is flowing continuously, and foam collapse time can be determined once the flow of CO_2 is stopped.

Light Scattering and Refraction Methods

The extent of haze can be estimated using light scattering methods. Shallow light scattering instruments are better for hazes formed by larger particles, whereas wider angle instruments, measuring scattered light at 90° to the incident beam, are better for assessing hazes produced by smaller particles (say, 0.1–0.5 μm) (Bamforth, 2002).

In typical nephelometric turbidimeters, such as those of the Hach series, light from a tungsten filament lamp is passed through a sample cell containing the sample, where a 90° slit allows scattered light to reach the photomultiplier detector. The intensity measurement is compared with that of a turbidity standard, which has a predesignated turbidity expressed in nephelometric turbidity units (NTU). Common standards are formazin (a copolymer of hydrazine and hexamethylenetetramine) and polystyrene-divinylbenzene copolymer beads (AEPA-1).

With beers, there is a tendency for temporary or chill hazes, generally composed of smaller particles, to mature into permanent hazes (Section 2.6.9), composed of bigger covalently linked particles, upon storage. The haze stability of beers designed for a long shelf life is important and consequently is often assessed by light scattering devices, particularly in conjunction with accelerated ageing programmes. Here, the effect of a 24 h period of cold (0 °C) storage and 24 h of warm (37 °C) storage (reckoned to be equivalent to several weeks of normal storage) is used to predict the likelihood of permanent haze formation.

In winemaking, the 90° angle turbidometer (called a nephelometer) is the most widely used type, giving a result that describes the wine clarity in terms of nephelometric turbidity units (NTU). By observation, wines can be described as brilliant, clear, cloudy, dull or turbid. Brilliant corresponds to NTU values of $<$1.1, $<$1.4 and $<$2.0 for white, rosé and red wine, respectively, whereas turbid is characterized by NTU values $>$4.4, $>$5.8 and $>$8.0 for white, rosé and red wines, respectively (Ribéreau-Gayon *et al.*, 2000).

In brewing, light absorption can be used to assess the quality of barley for malting, particularly with regard to water take up by the starchy endosperm during steeping (Section 2.6.2). Shining light through pearled barley can distinguish between mealy and glassy endosperms, the former of which absorb water more easily during steeping. Pearled barley with glassy endosperms appears bright in the beam of light, whilst those with mealy endosperms appear dark, as a result of reflection and absorption, respectively.

The refractive index of sugar solutions and of ethanol-water solutions varies according to the concentration of sugar or alcohol. Consequently, refractive index measurement, using an Abbé or similar refractometer, can be used to assay sugar content of wine and cider musts or beer worts or the ethanol content of drinks, using a sodium lamp. Modern instruments have temperature control devices (correction must be made for temperatures other than 20.0 °C) and scales for both refractive index and sugar content, calculated as glucose or sucrose. The instrument may be calibrated with a proprietary standard, or by use of a series of standard solutions of sucrose in water or ethanol-water solutions, and refractive index can be related to concentration by use of tables in such reference texts as the *CRC Handbook of Chemistry and Physics*.

Thermal and Combustion Methods

The Kjeldahl method for the determination of total nitrogen content of foodstuffs is the time honored method, nowadays automated, but in recent years has been challenged by the more rapid and environmentally safer Dumas method. The Kjeldahl procedure involves heating (~370–410 °C) the accurately weighed sample in the presence of concentrated sulfuric acid with dissolved sulfate salts – usually K_2SO_4 and $CuSO_4{\cdot}5H_2O$, or a combination of Na_2SO_4, $CuSO_4{\cdot}5H_2O$ and $FeSO_4{\cdot}7H_2O$. Under these vigorous conditions, all the nitrogen in the sample is converted to ammonium sulfate. There are many commercial versions of varying sizes (macro and micro) and degree of automation (see, for example the Buchi AutoKjeldahl and Foss Tecator Kjeltec series), that differ in detail regarding the digestion, dilution, distillation and titration steps. Most Kjeldahl instruments use block digesters, with a fume removing device, such as a water or mechanical aspirator. The block digesters are operated by a controller that allows automatic timed temperature ramping and multiple temperature programmes. Distillation can be carried out (often automatically) by classical distillation or by more rapid steam distillation (3–8 min), in which case the procedure is often known as 'rapid Kjeldahl.' Determination of ammonia is carried out by titration (again often automated), as described earlier in Section 4.6.3 – see also Section 4.4.3.

The Dumas method (also automated, see for example www.gerhardt.de) is now often used to determine the protein content of powdered cereal grains and malted cereals, via determination of the total nitrogen content. An accurately weighed sample is combusted (at ~1000 °C) in excess oxygen in the presence of a catalyst, so that carbon dioxide, water vapor and oxides of nitrogen are formed from the carbon, hydrogen and nitrogen content of the sample. The gases are passed through a reduction furnace where oxides of nitrogen are converted to N_2 in the presence of copper. They are than passed over a Nafion® membrane, where most of the water is removed. The remaining water, along with the CO_2 is removed in self-regenerating adsorption traps. The elemental nitrogen is measured in a thermal conductivity detector, after calibration with a standard. EDTA (containing 9.56% N) is often used as a calibration standard. The results are reported as %N, which are then converted to percentage protein by use of a 6.25 conversion factor. The protein content is then usually expressed on a dry weight basis by taking into account the amount of moisture in the sample, which can be determined by drying a quantity of the flour at 105 °C for three hours. See Section 4.4.3 for colorimetric methods for the determination of protein and other forms of nitrogen in alcoholic beverages and their raw materials.

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4.7
Sensory Analysis

Some readers may find certain expressions far-fetched. Cynics scorn the use of descriptions such as 'old straw, 'linoleum' or 'cheese rind' yet these are not fanciful: the physical and chemical compounds that produce the smell of linoleum, for example, can be present in wine.

—Michael Broadbent, 1991

4.7.1 Introduction

The best ingredients, combined with the most careful production methods and the most rigorous in line quality assurance may well give a consistently top notch alcoholic beverage, but whatever the history of the product, the final judgement regarding its quality, or at least its acceptability, will come from the drinker. Therefore, all alcoholic drinks manufacturers will include in their production process a program of sensory analysis, often at different stages along the production line. Sensory analysis, sometimes called organoleptic analysis, involves the assessment of the beverage by what is often loosely called 'tasting', but other than in its simplest form, sensory analysis is much more than this. It is an attempt to assess and describe the character of a drink in universally understandable terms using the natural senses of vision (for color, clarity), smell (for aroma/flavor), taste and touch (for texture and temperature).

All the natural senses, apart from sound, play an important part in sensory analysis. However, in this section, use of the senses of smell and taste will be emphasized, because they play a key role in assessing and describing the character of a drink. Description of color and reference to color scales (particularly for beers and wines) has been outlined in Sections 4.4.4, 2.6.2 and 2.6.13. Likewise, reference to clarity assessment is made in Section 4.6.3. The sense of touch is more or less limited to the impact of 'mouthfeel' (which includes astringency) and temperature of the drink in the upper digestive tract, along with the impact of its viscosity, relating to dissolved substances such as glycerol and sugars, and its homogeneity, relating to the presence or absence of suspended solids or bubbles of gas. Measurement of viscosity is outlined in Section 4.6.3.

The senses of smell and taste are sometimes known as the chemical senses, because of their reliance on the interaction between chemicals (aroma, flavor and taste chemicals) and the relevant sensory organs (chemoreception). The chemical sensory organs are especially well developed in most animals, where chemical signals play a most important role in their daily lives, including communication. In primates, humans and birds of

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

Table 4.7.1 *Examples of organizations and institutes that supply sensory analysis guidelines and information*

Organisation	Country/website	Organisation	Country/website
AENOR (Asociación Español de Normalisación y Certificación)*	Spain/www.aenor.es/	DIN (Deutsches Institut für Normung)	Germany/www.din.de
AFNOR (Association française de normalisation)*	France/www.afnor.org	KATS (Korean Agency for Technology and Standards)*	South Korea/www.kats.go.kr
ANSI (American National Standards Institute)*	USA/www.ansi.org	SA (Standards Australia)*	Australia/www.standards.org.au/
ASTM (American Standards for Testing and Materials)	USA/astm.org	SABS (South African Bureau of Standards)*	South Africa/www.sabs.co.za/
BSI (British Standards Institution)*	UK/www.bsigroup.com		

*Member bodies of International Organization for Standards (ISO), Geneva, Switzerland (www.iso.org). Many of their publications carry ISO identities.
Source: Many of these sites have available for purchase: information, guidance for training and assessing panelists, laboratory design, methods of sensory analysis and other sensory related topics.

prey, sight and sound is more highly developed than smell and taste, but nevertheless, the latter senses are still of considerable importance.

Sensory reference literature regarding food aromas and flavors includes Burdock (2001), Civille and Lyon (1996), Ashurst (1999) and *Flavours and Fragrances* (2002). Also, for recommendations regarding experimental conditions, details of types of sensory tests and general information, see Table 4.7.1. Additionally, the reader is directed to a website on the science and art of flavor construction (with definitions) (Fischetti, 2002), an account on flavor chemistry (Cole and Noble, 2003) and a recent review on wine chemistry and flavor (Ebeler and Thorngate, 2009).

4.7.2 Olfaction and Taste

The olfactory system is located in the nasal cavity (Figure 4.7.1), where conchae (or turbinates) form a number of cavities and the superior conchae and olfactory clefts are lined with olfactory epithelium (or mucosa). The olfactory epithelium consists of receptor cells lodged between supporting (sustentacular) cells and basal cells, as well as olfactory (Bowman's) glands, which (along with the supporting cells) supply the mucous secretion that coats the epithelium. Receptor cells are developed from basal cells in about a month and perform their receptor function for a week or so before dying, breaking up and being replaced by new receptor cells.

The receptor cells may be thought of as specialized, primitive type bipolar neurons (Netter, 1991). Evidence suggests that there are many kinds of receptor cells, but that the signals from one type are led to a common area of the olfactory bulb. The receptor cells are elongated, with their nuclei situated at their upper ends and each possesses 10–15 olfactory hairs or cilia (of up to 0.3 mm in length) at their lower ends, which project from the epithelium surface into the covering mucus (Figure 4.7.2). The upper end of a receptor cell narrows and gives rise to an unmyelinated axon (0.2–0.3 μm in diameter). Large numbers of these axons are bundled together under the protection of a Schwann cell sheath, eventually passing through the cribiform

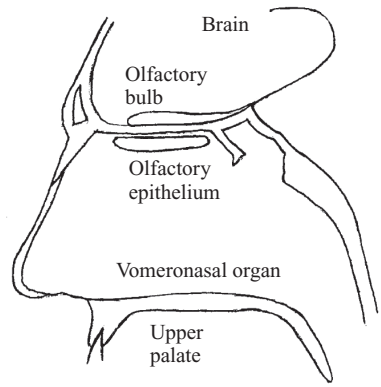

Figure 4.7.1 *Nasal cavity and location of olfactory system*

plate of ethmoid bone to the olfactory bulb, a flattened oval mass near the lateral margin of the cribiform plate (Figure 4.7.1). It is estimated that about 100 million olfactory axons enter the olfactory bulb.

The cilia have a fine structure made up of microtubules, which are essentially cylindrical lipid bilayer cell membranes. The membranes possess embedded receptor proteins, whose undulating structures (mostly α-helices) pass through the membrane at seven points, but with one terminal either side of the membrane, the inner terminus being linked to a G protein. It is thought that the odor binding sites are situated on three

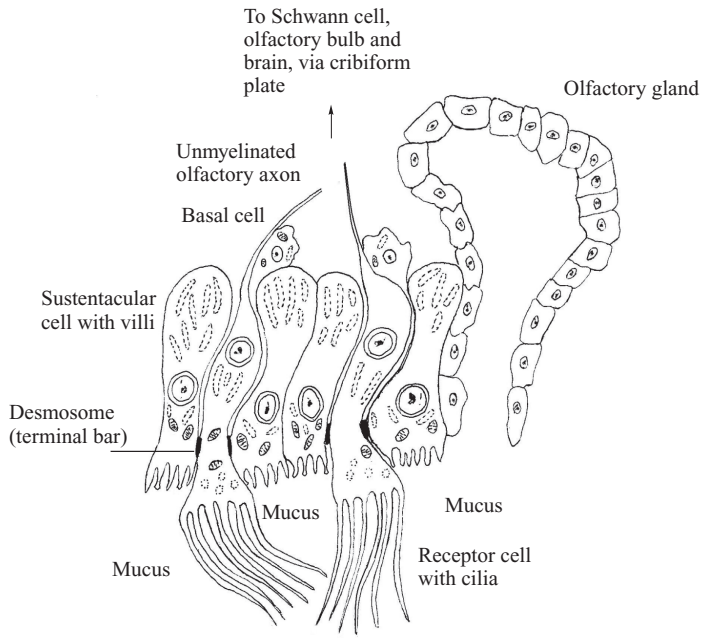

Figure 4.7.2 *Section of a small region of the olfactory epithelium*

neighboring protruding α-helical structures. The receptor proteins expressed by different receptor cells are similar except for the regions of hypervariability associated with the binding sites of the three α-helices. Also located in the membrane are numerous ion channels and the entire olfactory epithelium is covered in viscous mucus, which is has the important job of acting as a vehicle for odorant molecules, ions and a large number of secreted proteins.

For detailed discussions of the anatomy and functioning of the olfactory system, the reader is referred to Shepherd (1979), Netter (1991) and Matheis (1999).

Many theories of olfaction have been put forward over the years, most of them attempting to explain the interaction between the odorant molecules and receptors in the olfactory epithelium, the notion of receptors being universally accepted a long time ago. These theories include radiation, thermodynamic activity and membrane penetration as the mechanisms of olfaction, but nowadays the theories that are most highly regarded are those based on either recognition and/or vibration (Sell, 1997). Recognition theories strive to explain the olfaction mechanism in terms of some 'matching' mechanism that links the odorant to the receptor, rather like the 'lock and key' mechanism of enzyme action. The best known modern recognition theory is that of Amoore (1970), which regards primary odors as contributors to the broad spectrum of perceived odors, rather in the way that all colors are formed from the primary colors blue, red and yellow. He postulated that molecular shape or electrical charge was the key recognition factor between odorant molecule and receptor protein. Other recognition theories sought to explain odorant–receptor interaction in terms of intermolecular bonding, steric bulk and frontier orbital topography associated with the odorant molecule.

Vibration theories attempt to explain odorant molecule-receptor protein interactions in terms of a reaction (ultimately giving rise to an electrical signal) in the receptor caused by the sensing of specific vibration(s) in the odorant molecule. Turin (1996) proposed that electron tunneling provides the electric signal that results in the perception of a certain odor in the brain. The mechanism involves the receptor protein having two prosthetic groups, NADPH (as electron donor) and Zn^{2+} (as electron acceptor), but when there is no odor molecule specific to this particular protein present, electron transfer between the two sites is unable to occur, because the potential energy barrier is too high. When an odor molecule is bound between the two sites, an electron is able to tunnel through the energy barrier by using the odorant molecule's orbitals, thereby causing characteristic excited vibrational modes and creating a drop in electrical potential.

The many theories have tried to explain the mechanism of olfaction on the basis of empirical structure–activity relationships (SARs). Either some feature of structure or activity that is seemingly important in the observed SARs is postulated as being responsible for biological activity, or a biological mechanism is first postulated and then is linked to odorant structural features that would be in accord with the mechanism.

All these theories are deficient in that they are unable to account for all the known facts regarding olfaction and a great deal still needs to be learned. The problem is that there are many stages between the genesis of odor at the site of interaction and the perception of odor in the brain.

A modern picture of olfaction genesis is outlined in Figure 4.7.3. It involves the participation of odorant binding proteins (OBPs), of which there are many types. These probably have varying interactions with different odor molecules and receptor proteins, and their job is probably to chaperon the odor molecules to the relevant receptor protein where an interaction complex is formed, thus starting the process of events leading to odor perception. Formation of the interaction complex is thought to cause a change in conformation of the receptor protein, possibly by the formation of specific intermolecular attractions (such as hydrogen bonding) or by electron tunneling.

The change in conformation triggers a protein associated with the receptor protein (called a G protein) to activate the enzymes adenylyl cyclase or phospholipase C, which catalyze the formation of the second cell messengers cyclic adenosine monophosphate (cAMP) and inositol triphosphate (IP_3), respectively. These

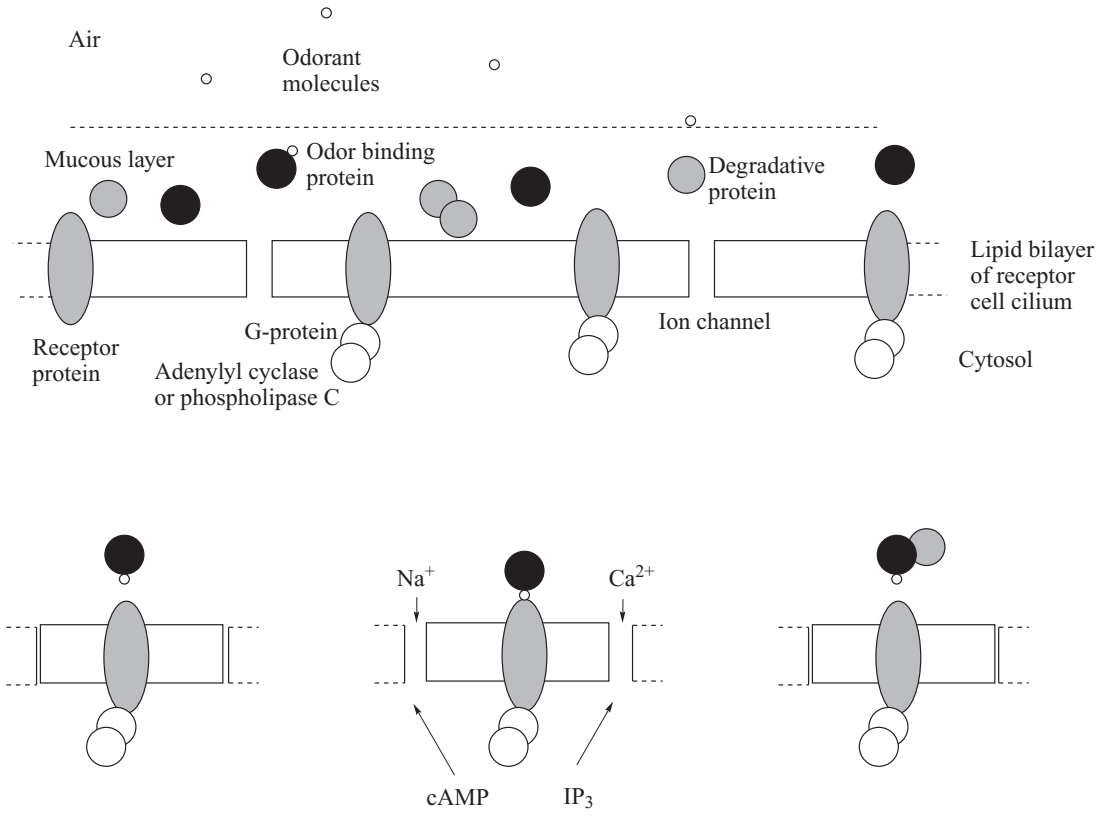

Figure 4.7.3 *Outline mechanism of initiation of olfactory response*

cause cAMP and IP_3 gated ion channels to open, allowing sodium and calcium ions to enter the cell resulting in a change in cell electrical potential (the cell becomes less negatively charged). If the stimulus persists, the potential change is propagated along the length of the neuron and is transmitted directly to the brain, where it is converted to an odor sensation.

There is a negative feedback mechanism associated with olfactory signaling, in that increase in calcium ion concentration within the cell decreases the sensitivity of the ion channels to cAMP, so that an action potential is maintained only by a strong odor stimulus.

The second messengers also trigger a process that deactivates the receptor protein by phosphorylation, which presumably dissociates the interaction complex, leaving the OBP and its odor molecule to be demolished by degradative proteins that are known to be present in the layer of mucous. Thus, signal generation ceases after removal of the odor source.

Most people can detect a very wide range of odors, associated with a large number of molecules. This is because most odorants are recognized by more than one type of receptor and most receptors can receive more than one odorant. This cross reactivity means that the few hundred types of receptors in humans work in concert to detect a wide spectrum of odors.

The odor of an alcoholic beverage, such as the bouquet of a wine or the hop aroma of a beer, is a very pleasurable experience. It depends on the persistent evaporation of aroma compounds from the surface of the beverage. Likewise, the drinking of an alcoholic beverage such as wine is accompanied by the perception

of a wide range of aromas and flavors, which changes as the drink is swallowed, suggesting that odorants are released from the drink at different rates and to different extents as a result of their different mass transfer characteristics between air and aqueous ethanol solution. Evaporation of volatile substances at the air–liquid interface has the effect of slightly warming the headspace above the liquid and slightly cooling the bulk liquid at the surface. In order to maintain a steady headspace concentration of volatile substances there must be continual movement of these substances in the bulk liquid to the interface. The Marangoni current is a major mechanism for doing this, the driving force being surface tension differences at the surface, which may in turn be caused by temperature or concentration changes. In water-ethanol solutions, ethanol preferentially accumulates at the interface, lowering the surface tension and thus increasing Marangoni convection, compared with purely aqueous solutions (Tsachaki *et al.*, 2008).

Dynamic headspace headspace studies on model wine solutions, designed to simulate the conditions when wine is sniffed, have indicated that the presence of ethanol (at 12% ABV) maintained the headspace concentrations of a number of odorants close to equilibrium values during gas phase dilution over 10 minutes (Tsachaki *et al.*, 2009). This was explained by an increase in mass transfer (additively to partition coefficient variation) due to the presence of ethanol, and this was supported by the observation of convection of odorants above model wine solutions (as opposed to aqueous solutions) using thermal imaging.

Real wine samples have been found not to maintain headspace concentration of odorants to the same extent. Results of dynamic headspace dilution experiments on model wine solutions spiked with proteins suggest that this is probably due to the presence of small quantitities of certain proteins (e.g. bovine serum albumin, β-casein and β-lactoglobulin) in real wine samples ((Tsachaki *et al.*, 2009). It appears that the proteins interfere with ethanol at the air–liquid interface. More mature wines will contain fewer proteins than young wines, because over the years the proteins will precipitate with tannins and tartrate salts as a deposit. This could partly explain why many wines gradually improve their quality and intensity of bouquet after spending many years in bottle and also why a very fine wine of great age will often release and maintain an intense perfume for a limited period of time before 'collapsing.'

GC-O and sensory analysis has shown that the nonvolatile matrix of wines exerts a marked effect on the perception of odor (Sáenz-Navajas *et al.*, 2010). For example, by adding red wine nonvolatile fractions to a white wine caused the suppression of white, yellow, citrus and tropical fruit notes, so that black, red and dried fruit notes became dominant. In particular, red wine nonvolatile matrices appeared to retain strongly the ester 3-mercaptohexyl acetate (an important odorant of Sauvignon Blanc wines), thus significantly reducing its odor impact. Also, Sáenz-Navajas *et al.* (2010) have shown that the wine nonvolatile matrix influences the release of aroma molecules, so that the headspaces above white wines are richer in fruity esters and volatile acids than those above red wines.

Another phenomenon that has interested aroma scientists is the observation that perception of certain aromas and flavors during the drinking of alcoholic beverages is sometimes time delayed, relying on the hydrolysis of flavorless conjugates to form aroma/flavor compounds after swallowing. For example, *S*-cysteine conjugates were found to be precursors of flavorful thiols, released from wines of Sauvignon Blanc some 20 seconds or so after swallowing (Tominaga *et al.*, 1998a; 1998b). Recently, Starkenmann *et al.* (2008) showed that similar cysteine *S*-conjugates in bell peppers are converted to odorous thiols by anaerobic bacteria (*Fusobacterium nucleatum*) in saliva, the mouth thus acting as a reactor producing odorants, but with the saliva moderating overall olfactory responses by trapping a proportion of the free thiols.

The odors and flavors of an alcoholic drink are notoriously difficult to describe in a universally acceptable way – using descriptions that are recognized unambiguously by all. Hence it is possible to devise nonequivalent, but broadly similar, flavor wheels or profiles for a particular drink, such as Scotch malt whisky (see Section 3.2.6, Figure 3.2.13; see also this chapter, Figure 4.7.7). However, these profiles, with their specific descriptors, work well in their own contexts, where tasters (panellists) have been trained with typical examples

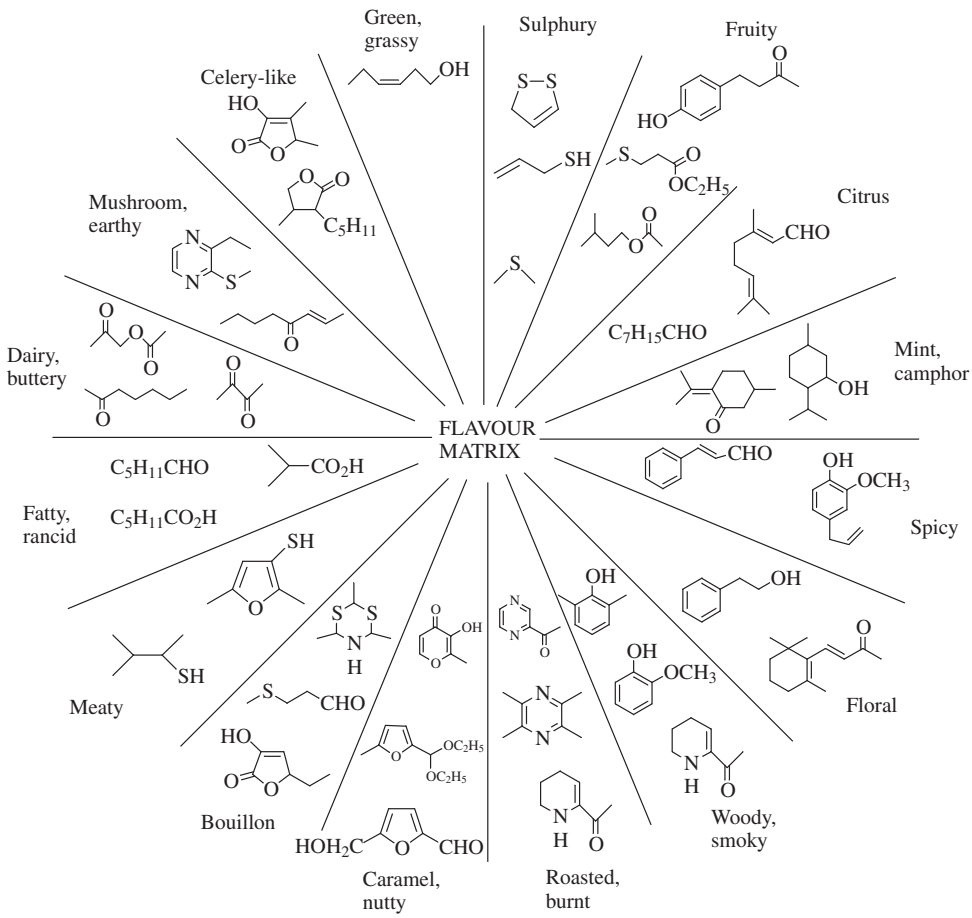

Figure 4.7.4 *Flavor matrix or wheel used for foodstuffs in general, illustrating a few representative flavor molecules*

of the flavor descriptors, sometimes with pure compounds that are associated with the flavors (Section 4.7.4). It is possible to construct general flavor wheels or matrices that account for the major flavor attributes of foodstuffs in general. Two such wheels are shown in Figures 4.7.4 and 4.7.5, where it can be seen that, while they are not equivalent, there is a broad similarity between the two. Figure 4.7.4 also includes some typical flavor molecules associated with each descriptor, whereas Figure 4.7.5 includes taste and mouthfeel as well as aroma/flavor and also illustrates how primary descriptors can be subdivided.

Although there is no universal agreement on a set of primary aromas and flavors, it has long been agreed that taste can be described by just four sensations; bitter, salty, sour and sweet. The sensations of taste originate from interaction between taste buds and taste molecules or ions. Taste buds are located all over the mouth, including the epiglottis, the top third of the esophagus, the larynx, the soft palate and the uvula (at the back of the mouth). However, the biggest concentration of taste buds is on the upper surface of the palatine section (front two thirds) of the tongue. Here, the taste buds are located in close packed short projections known as papillae, of which there are four types depending on their anatomy; filiform, foliate, fungiform and vallate.

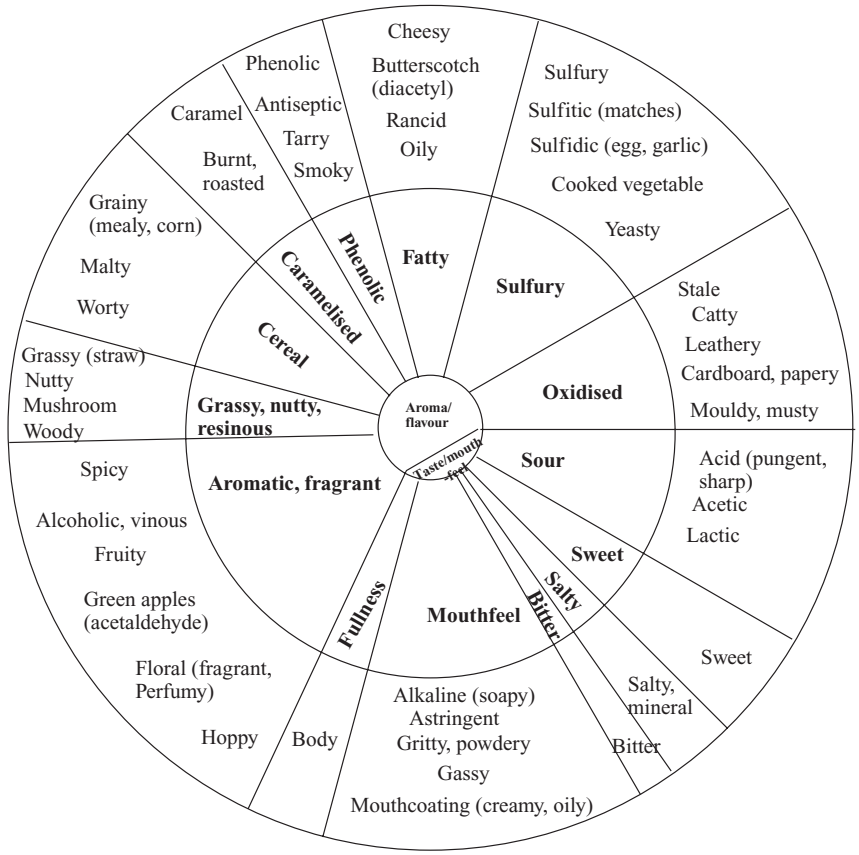

Figure 4.7.5 *Flavor, taste and mouthfeel wheel, showing subdivisions of major descriptors*

Figure 4.7.6 shows a simplified cross section of the central part of the tongue's upper surface, where it can it can be seen that the taste buds are embedded in the highly undulating epithelium, with apical pores on the outer surface and sensory nerve fibers leading off into the connective tissue. The nerve fibers are bundled into a succession of cranial nerves, which enter the brain via the brain stem and thence to the hypothalamus, the thalamus and sensory cortex areas of the brain.

The tongue is covered in mucous secreted by glands in the moat surrounding the vallate papillae and the salivary glands provide saliva with its various hydrolytic enzymes (such as amylases) and degradative enzymes (such as lysozymes).

The filiform or conical papillae are most numerous, but lacking taste buds, they are involved in trapping of taste substances. The flat topped fungiform papillae have taste buds giving both sweet and sour responses, whereas the the taste buds of the vallate and foliate papillae are associated with bitter (and sour) and salt (and sour) responses, respectively. The distribution of the different types of papillae on the tongue's surface reflects the dominant taste sensations in different parts of the tongue. Thus salt and sour taste sensations are strongest at the sides of the tongue, whereas sweet sensation is most pronounced at the tip and bitter taste sensation is prevalent toward the back of the tongue.

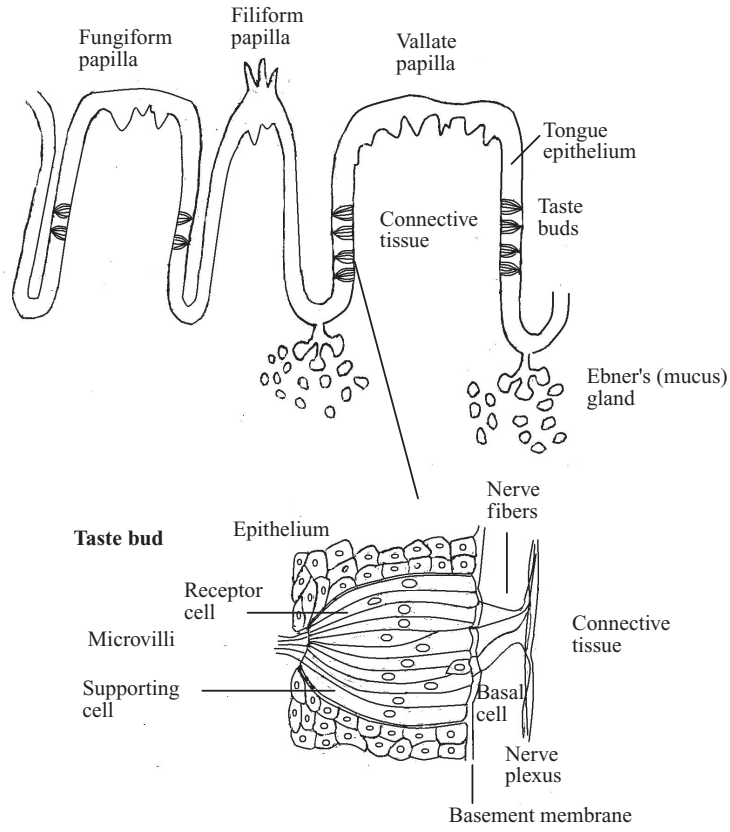

Figure 4.7.6 *Section through tongue papillae and section through taste bud*

The perception of astringency and bitterness are common in the consumption of many alcoholic drinks, such as beer, bitters, certain liqueurs, vermouth and wine (especially red wine). Bitterness is perceived by the taste receptors on the tongue, whereas astringency is regarded as a tactile (feeling or touch) sensation, characterized by a feeling of dryness and puckering of the mouth cavity. Interestingly, astringency is defined in a chemical way, as the ability to precipitate proteins, a property that probably gives rise to the characteristic mouthfeel. Both sensations are time dependent, taking time to establish maximum intensity and then gradually receding.

In wine, the major cause of bitterness and astringency is the presence of phenolic substances. Preleg *et al.* (1999) found that the flavan-3-ol monomers were more persistently and more intensely bitter than flavan-3-ol dimers and trimers formed from flavan-3-ols and (+)-dihydroquercetin. Astringency, on the other hand, was higher for the oligomers, but of the same duration as the monomers. Moreover, the bitterness and astringency depended on the type of linkage in the flavan-3-ol dimers. Thus, catechin-(4→6)-catechin (cat-(4→6)-cat) was more bitter than either cat-(4→8)-cat or cat-(4→8)-epicat, and cat-(4→8)-cat was more astringent than either cat-(4→6)-cat or cat-(4→8)-epicat.

The feeling of astringency in the mouth is thought to be a result of the formation of insoluble saliva protein (e.g. α-amylase)–polyphenol complexes – these, particularly if tannins are involved, reduce oral lubrication

and give rise to sensations of constriction, dryness and roughness (Haslam, 1981). Soares *et al.* (2009), using nephelometry (Section 4.6.3), dynamic light scattering and fluorescence quenching, have demonstrated how polysaccharides in tannic foods reduce astringency by forming either soluble protein/tannin/polysaccharide ternary complexes or soluble tannin/polysaccharide complexes, the polysaccharide competing successfully against saliva proteins. In the experiments, the tannins were procyanidins, the polysaccharides were cycoldextrin, gum Arabic and pectin, whereas the protein was porcine pancreas α-amylase, which is reckoned to be similar to the human enzyme.

In the case of wine and vermouth, the residence time in the mouth was found to have a strong influence on the perception of maximum astringency and on the rate of its decrease, which obeyed an exponential law (Valentová *et al.*, 2002). Furthermore, solutions of tannic acid and catechin were more astringent in water than in wine or orange juice, and added sugar decreased the astringency of red wine, illustrating the existence of masking effects. In the case of sugars, it is thought that their presence in alcoholic beverages increases saliva flow, so that either bitter or astringent substances are washed out of taste buds or sugar molecules interfere with the formation of complexes between salivary proteins and astringent components (Valentová *et al.*, 2002).

4.7.3 Odor Theshold, Odor Activity and Low Impact Odorants

Because of their profound effect upon the chemical senses, aroma compounds play a definitive role in the quality and character of alcoholic beverages. In the case of most drinks, aroma and flavor are influenced by the presence of hundreds of volatile and semivolatile compounds, which are normally determined by methods involving gas chromatography (Section 4.3.2). In total, however, aroma compounds normally comprise only a small fraction of the components present in the drink. For example, the concentration of total aroma compounds of wine is *ca.* 0.8–1.2 g/l; about 1% of the ethanol concentration (Rapp, 1998). Of these, higher alcohols ('fusel oils') make up about 50%, with the levels of the remaining odorants ranging from 10^{-4}–10^{-10} g/l (Rapp and Mandery, 1986).

The human olfactory system exhibits a rather sensitive and variable reaction to aroma compounds in alcoholic beverages. A measure of the contribution (or more crudely the 'strength') of a particular odorant is its odor threshold value (OTV, sometimes known as threshold odor concentration (TOC), or olfactory perception threshold), defined as the concentration of odorant at which the sensory difference from a control sample can be detected by 50% of panellists in a sensory test. Determination of OTV is thus an example sensory analysis, which requires testing to be carried out under conditions laid down in standard procedures provided by various statutory bodies and institutions related to the food and alcoholic beverage industries (Section 4.7.4).

As an example, consider the determination of the OTV of methyl-2-methyl-3-furyl disulfide (MMFDS) in whisky (Watts *et al.*, 2004). This was determined in 20% (v:v) ethanol following a common procedure used for sensory analysis of Scotch whiskies (Lee *et al.*, 2000). The MMFDS samples were prepared in a range of concentrations (100, 50, 10, 5 and 1 ng/l; i.e. 0.1–0.001 ppb) in 30 ml of 20% (v:v) ethanol in 130 ml clear nosing glasses. These samples were nosed by a sensory panel consisting of 13 members, who were required to say whether they could identify an aroma in each of the samples and whether they could describe that aroma. The test results were interpreted using specialist software (Section 4.7.3). The number of panellists detecting and recognizing (successfully describing) the aroma of MMFDS at each concentration was calculated. A probit regression was carried out on the data to determine detection and recognition thresholds. These were estimated at probit 0.5, the point at which 50% of the panel could detect or recognize the aroma. The recognition threshold and odor threshold (detection threshold) for MMFDS were found to be 18 ng/l and 15 ng/l respectively.

Although several aspects in the above procedure are specific to Scotch whisky (e.g. dilution of sample to 20% ABV, size of sample and glass, nosing only – no tasting; see Section 4.7.4) it can be regarded as being typical, and most importantly, the target odorant is sampled in the matrix most commonly used for sensory analysis of Scotch. It is important to determine OTVs of odorants dissolved in a relevant matrix or solution.

An alternative method of determining OTVs is illustrated for 1,3-dimethoxybenzene (*m*-DMB), a volatile component of Port wines (Rogerson *et al.*, 2002). Here, the method used was that of triangular tests according to forced choice ascending concentration series method of limits (ASTMS, 1991). A panel of seven tasters were given eight three-alternative forced choice (3-AFC) tests, which consisted of two blanks and an odd sample with added *m*-DMB to make up the triangle. The concentration of *m*-DMB in the eight odd samples differed by a factor of two, from 1.95 μg/l up to 250 μg/l. The tests were repeated until a correct answer (yes or no, with correct odor descriptors – hazelnut, medicinal, sweet) resulted for at least three in a row for each panellist.

The panel threshold was calculated as the geometric mean of the best estimate threshold for each panellist. OTVs of *m*-DMB were determined in two model wine systems (12% and 20% ABV; tartaric acid, 5.0 g/l, pH 3.5 to resemble Douro wine and Port, respectively): they were 21 μg/l and 47 μg/l, respectively.

Similar tests were also used to determine OTVs for abhexon (5-ethyl-3-hydroxy-4-methylfuran-2(5H)-one – a spicy flavor component of Sauternes) (Bailly *et al.*, 2009) and (*R/S*)-3-sulfanylhexan-1-ol, a flavor component of bell peppers and wines of Sauvignon Blanc (Starkenmann *et al.*, 2008). For abhexon OTV determination, the blanks were water:ethanol (88:12, v:v), and abhexon was spiked in this medium at 2.5–12.5 μg/l, whereas for (*R/S*)-3-sulfanylhexan-1-ol the blanks were water and the odorant was spiked in water at 4–500 ng/l.

OTVs vary widely, typically from 0.1 mg/l (10^{-4} g/l or ppm levels) to 0.1 ng/l (10^{-13} g/l or ppt levels) (Rapp, 1998), with many in the 1–0.01 μg/l (10^{-9}–10^{-11} g/l or ppb) level. It is widely held that if a component is present at concentrations much lower than its OTV, it will provide only a minor or background contribution to the overall aroma (but see later paragraphs of this section).

Odor activity values (OAVs) were constructed in order to give an estimate of the extent of contribution of an odorant, depending on its concentration and OTV (Equation 4.7.1) (Salo *et al.*, 1972).

$$\text{OAV} = \frac{\text{Concentration}}{\text{OTV}} \tag{4.7.1}$$

The OAV of an odorant is thus a dimensionless unit that indicates the likely extent of contribution of a particular odor compound to the overall smell of the drink; the larger the value the greater the contribution. On this basis, it has been proposed that only components with OAVs greater than 1 are likely to make a significant contribution (Guth, 1997)

Another way of measuring odor potency or impact (which can be compared with OAV), is gas chromatography olfactometry aroma extract dilution analysis (GC-O AEDA). For a review of the subject, the reader is referred to Delahunty *et al.* (2006) and Plutowska and Wardencki (2008) for general applications of GC-O. For more discussion on GC-O, see Sections 4.7.4 and 4.3.2. In GCO-AEDA, firstly, characteristic odor components of an alcoholic beverage are extracted (usually by liquid–liquid extraction) and determined by GC-O and identified (usually) by GC-MS. Then, GC-O is carried out on successively diluted samples of the extract until the odor can no longer be perceived for certain of the components, which are noted each time. Eventually, dilution will be so great that no odors are perceived by GC-O. The maximum dilution for each component is known as the flavor dilution (FD) factor and is a measure of the odor potency: the larger the FD, the greater the odor impact of that component. GC-O AEDA is thus an incremental GC-O technique. Another such method is charm analysis, which constructs an aromagram – a chromatogram with peak areas proportional to the odor activity (Acree *et al.*, 1984).

A more recent GC-O method involves sniffing of the eluent of a single GC experiment by 6–10 panellists simultaneously. The method is known as detection frequency analysis (DFA) and relies on the relative number of panellists detecting odors at particular retention times during a GC run being related to the relative importance of each aroma component (Falcão *et al.*, 2008). The nasal impact factor is defined by Equation 4.7.2.

$$\text{NIF} = \frac{N_t}{n} 100 \tag{4.7.2}$$

where N_t is the number of panellists recognising an odor at retention time t and n is the total number of panellists.

Such a technique was used to analyze Brazilian Cabernet Sauvignon wines, with the panellists recognizing aromas using 14 impact aroma descriptors. Furaneol was associated with a jam or caramel aroma and 2-methoxy-3-isobutylpyrazine (MIBP) was associated with vegetal or bell pepper aroma (Falcão *et al.*, 2008),

Recently, Poisson and Schieberle (2008a) used AEDA to determine 45 odor active components in Bourbon whisky with FD factors in the range 32–4096 (E)-β-damascenone and γ-nonalactone had the highest FD values (4096 and 2048 respectively), with and *cis* and *trans* whisky lactones also having high values (1024 and 512, respectively). The aroma compounds were extracted from diluted Bourbon whisky using diethyl ether and separated from nonvolatile components by solvent assisted flavor evaporation (SAFE) (Engel *et al.*, 1999). They were then separated into fractions containing components of increasing polarity by column chromatography. For AEDA, fractions were stepwise diluted with diethyl ether (1:1, 1:2, 1:4, 1:8 . . . , 1:256, . . . , 1:4096). Thirty one of the 45 most odor active components in Bourbon whisky were quantified by stable isotope dilution analysis (Poisson and Schieberle, 2008b) and their OAVs were calculated using this data and OTVs determined in water: ethanol (6:4 by volume). Twenty-six of these had OAVs greater than unity and when recombined they mimicked the overall aroma of the whisky.

As previously mentioned, GC-O and AEDA are the most frequently used methods to determine the most active odorants in an alcoholic or other beverage. Omission tests and reconstitution tests are often applied in conjunction with GC-O and AEDA data to judge the importance of one component or a group of components in a reconstituted mixture (see the example above). It is here that there is often a discrepancy between the overall odor of a reconstituted mixture of high impact odorants and that of the original beverage, even though the levels of major odorants are identical in both mixtures. This defect is sometimes known as 'reconstitutional discrepancy' and is believed to arise because the omission of low impact odorants in the reconstituted mixture (Bult *et al.*, 2001).

Low impact odorants (those with low OAVs) have been traditionally considered to be unimportant in the global aroma perception of a foodstuff or alcoholic beverage. However, there is a growing opinion that the presence of aroma components at sub- or peri-threshold levels may either modify olfactory perception of high impact odorants or act synergistically to influence the overall aroma profile (see for example Escudero *et al.*, 2004 and 2007; Ryan *et al.*, 2008; Sánchez-Palomo *et al.*, 2007; Caven-Quantrill and Buglass, 2008 and references therein). It should be noted that low impact odorants make up the big majority of aroma compounds in alcoholic and other beverages.

It seems that predictive mathematical models that reduce odor interactions to a set of simple rules are unreliable and at best can give a crude representation of a real odor (Ryan *et al.*, 2008). Instead, it appears that a combination of odor molecules interact with the olfactory system in a more complex way than in the proposals of simple addition/proportional odor representation and vector predictive models. For example, Atanasova *et al.* (2004; 2005) studied the qualitative interaction of binary mixtures of wine odorants with woody notes and fruity notes and found that a woody olfactory sensation dominated the odor of mixtures

where the perceived intensities of each unmixed compound were equal. Also, quantitative results at supra threshold levels indicated that perceptual interactions were not symmetrical and depended on concentrations.

There are many examples where inclusion of minor odorants (according to GC-O results), in a reconstitution mixture, at concentrations well below their OTVs, results in an overall aroma that is much closer to the real thing. It appears as if the minor components act in synergy to modify the olfactory perception of the major odorants. For example, when dearomatized wine was spiked with 2-methoxypyrazine (pepper notes) at levels found in wines, it was found that the odor perception was merely an indefinite earthy note (Escudero *et al.*, 2007). However, if 1-hexanol and (*Z*)-3-hexenol were also added (both at levels well below their OTVs), a peppery aroma was more easily discernable.

The importance of materially minor aroma components in overall odor perception can be illustrated in a different way – by the correlation between analytical and sensory data. The sulfury characters of 58 new make Scotch malt whiskies were determined by a highly trained and experienced panel of assessors and the scores were correlated with the levels of 36 known organosulfur congeners (obtained from headspace GC experiments) using partial least squares regression (PLSR) (Jack and Fotheringham, 2004). The correlation was 0.67. On the other hand, when the levels of another 20 (unidentified) sulfur components in the analytical data were included in the correlation analysis, correlation was improved to 0.79, indicating that these unknown components made a significant contribution to the global sulfury aroma perception of new make malt whiskies.

Because of repeated observations of the modification of high impact odorant perception by sub- and peri-threshold odorants, it has been suggested that the traditional GC-O AEDA and similar methods need to be extended to include neurological analysis (Ryan *et al.*, 2008). In particular, electroencephalography (EEG) and functional magnetic resonance imaging (fMRI) together could be linked with GCO/sensory analysis for a better understanding of odor perception.

4.7.4 Sensory Tests

Sensory evaluation is a valuable tool for both quality control and research in all areas of the alcoholic beverage industry. Standards and conditions for the various kinds of sensory analyses are in place in all countries, laid down by organizations or institutes dedicated to overseeing standardization and uniformity in many areas of commerce and industry. Many of these institutes are member bodies of the International Standards Organization (ISO), based in Switzerland (Table 4.7.1). Furthermore, individual institutes associated with the drinks industry have their own guidelines for sensory evaluation (e.g. The Institute of Brewing and Distilling). These standards are designed to maximize the quality and consistency of data generated from sensory tests and they refer to experimental procedure and conditions that are deemed to be necessary to achieve such data. They deal with all aspects of experimental set up from the type of room, including its sampling booths, temperature and lighting, to how the sample is presented, including volume, type of container and so on. Some of the standards also deal with the education and training of panellists.

However, for the sensory analysis of spirits, there are several aspects of sample handling and presentation that are not mentioned in the standards, as these are often nonspecific and apply to foodstuffs or alcoholic beverages in general. Consider the case of Scotch whisky sensory evaluation. Firstly, flavor sensory analysis is based entirely on aroma (known as 'nosing'), because of the detrimental effects of tasting or ingestion on the judgement and health of blenders or taste panellists, since they may need to judge a large number of samples during the course of a normal working day. Furthermore, Scotch whisky samples are diluted to 20% ABV prior to analysis for reasons of comfort (less pungency on the nose), and since they are not ingested, the same sample can be shared between a number of panellists. Hence, modifications of or additions to the general standard procedure need to be implemented in order to minimize variation in sample condition due to

Table 4.7.2 *Types of sensory analysis procedures and their uses*

Type of test	Examples	Applications
Descriptive	Free choice profiling method Flavor profiling method Quantitative descriptive analysis Trueness to type method	All these tests require a panel of experts or trained tasters. The aim is to describe the flavor of one or more samples using a specific vocabulary of sensory terms.
Difference	Duo-trio test Triangular test	Experts or trained panelists determine whether two or more samples are different or if they are identical.
Flavor scaling	Ranking test Estimation of magnitude test	Technical tasters determine intensities of one or more flavor descriptors in several samples.
Preference	Paired comparison tests	Consumer and trained tasters assess drink on the presence or absence of a flavor descriptor.
	Preference mapping tests	Tasters find relationships between different drinks.

Scotch whisky procedure mentioned above (Jack, 2003). The major guidelines suggested for Scotch whisky sensory evaluation included the following:

- Presentation of sample (30 ml) (20% ABV) in tulip glasses (130 ml) covered with watch glasses that are kept in place between evaluations
- Samples are all well equilibrated to room temperature, which is comfortable (e.g. 21 °C) and constant
- The length of time between sample preparation and evaluation should be minimized, as should the number of panellists, within practical limits
- In tests where samples are being compared, if a sample is spilled, all the samples are replaced.

In this way, sensory samples are presented to the panellists in optimum condition, allowing the achievement of high quality sensory data.

The many different kinds of sensory tests are summarized in Table 4.7.2, where it will be seen that the majority of them are conducted by either expert tasters or a panel of tasters who have undergone an extensive program of training in sensory assessment beforehand. Sensory panellists need regular training and assessment.

Descriptive tests and difference tests are probably the most frequently used sensory tests. Descriptive tests aim to provide an overall account of the aroma/flavor of an alcoholic drink using a vocabulary of flavor terms that has been agreed for that drink, whereas difference tests, on the other hand, aim to determine whether two or more samples are either identical or different.

Of paramount importance in descriptive tests is aroma/flavor terminology, the language of sensory analysis, which requires consistency in use and a common point of reference for meaningful communication (Simpson *et al.*, 2004). Ideally, a limited vocabulary of flavor descriptors should be available for a particular alcoholic beverage, and they should be and universally used by sensory analysis panels when assessing that kind of beverage. In practice, however, this has only been partially achieved. The most desirable attributes of a flavor vocabulary are (Simpson *et al.*, 2004):

- One-to-one correlation between flavor and descriptor
- A limited number of objective descriptors that are consistent with the beverage or sensory test
- Lack of subjective terms.

Vocabularies vary enormously in size from beverage to beverage, from 122 terms for beer (Meilgaard *et al.*, 1982) to 16 or so for certain spirits (Phelan *et al.*, 2004; Simpson *et al.*, 2004). As an example, in developing a vocabulary for gin, the experienced sensory panel was first given a number of samples of gin, gin concentrates and botanicals to assess and generate descriptors (Phelan *et al.*, 2004). The collection of terms was summarized and rendered down to a vocabulary of 16 descriptors: aniseed, buttery, citrus, floral, fruity, herbal, juniper, oily, pungent, soapy, solventy, sour, spicy, stale, sulfury and sweet (see Figure 4.7.7). The panel was then trained to understand and recognize each of the descriptors by use of training aids (including botanical distillates and raw botanicals) and practice sessions.

There are two basic approaches to developing a vocabulary, firstly by allowing the generation of personalized descriptors (free choice profiling) by trainee panellists and secondly by the imposition of descriptors on the trainee panellists in advance of the sensory assessment. The second approach is generally reckoned to be

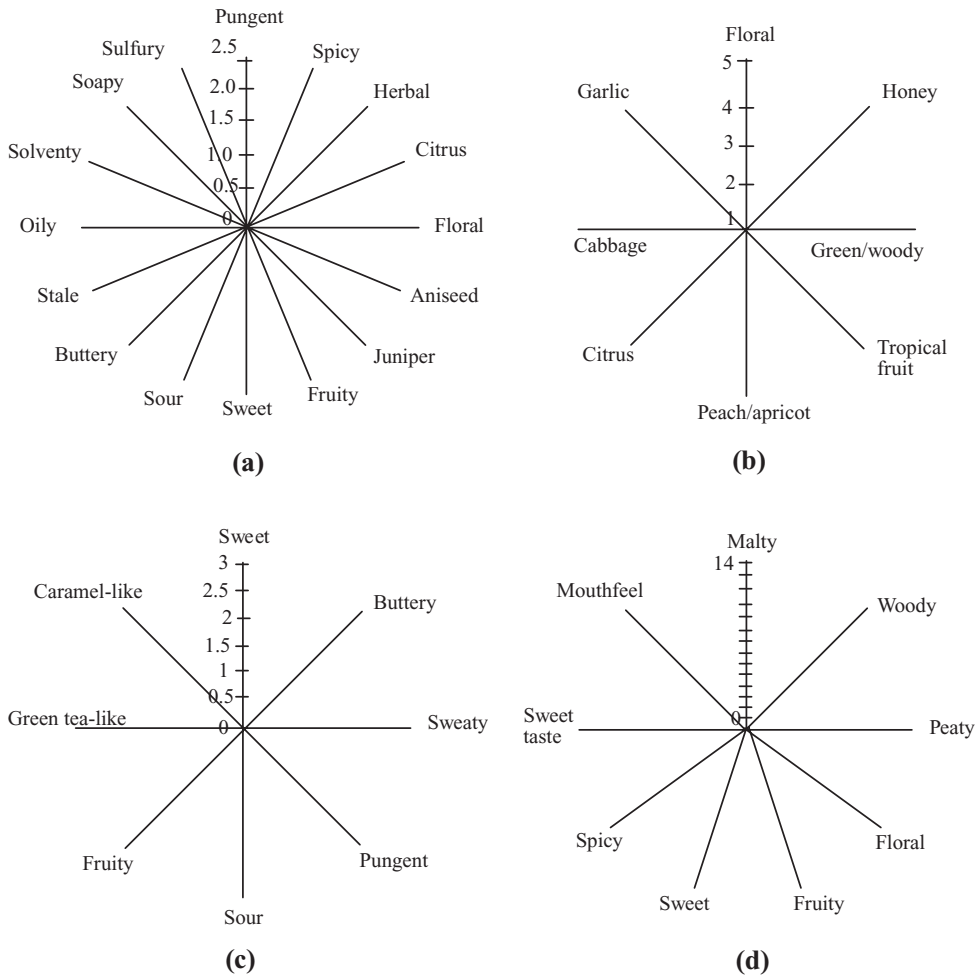

Figure 4.7.7 *Flavor profile templates for (a) gin and gin botanical distillates, (b) lychee, (c) strawberry juice and (d) Indian whisky. Scale used is shown only on one axis. Phelan* et al.*, 2004; Mahattanatawee* et al.*, 2007; Schieberle and Hofmann, 1997; Maitlin and Stephen, 2004*

rather more satisfactory, but sometimes a combination or hybridization of the two approaches is used. The choice of descriptors can be derived from authentic samples that have distinctive flavor attributes, such as botanical distillates for gin descriptors (see above), or peated malt whiskies for peat smoke descriptors, or Earl Grey tea for the bergamot descriptor used for wine made from Touriga Nacional and Port wine, for example. Alternatively, chemical reference standards can be used for training of panellists, where diluted pure flavor chemicals are added to model wine or neutral spirit, as has been proposed for whisky flavor training (Lee *et al.*, 2000).

Chemical flavor standards need to be of high purity food grade quality, but also need to be consistent from batch to batch. Most require very high dilution before they can be used as flavor standards and their solutions are not necessarily stable, often requiring fresh preparation. To overcome this problem, a series of microencapsulated flavor reference compounds are available for beer sensory analysis training and more recently for distilled spirits assessment (Simpson *et al.*, 2004). The flavor compound is released from a free flowing powder contained in a small capsule when added to a beer or spirits base. Each capsule contains a precise weight of flavor compound and the content of the capsule, unlike many diluted flavor references, is stable for years at room temperature. Spirits flavor standards include 2,3-butanedione (diacetyl, buttery), *o*-cresol (medicinal), dimethyl trisulfide (sulfury, onion), ethyl hexanoate (apple), ethyl maltol (sweet), eugenol (spicy), guaiacol (smoky), hexanal (grassy), isoamyl acetate (banana), isovaleric acid (cheesy), 2-phenylethanol (floral), vanillin (vanilla) and whisky lactone (coconut).

Sensory profiling is probably the most widely used kind of quantitative descriptive test (Stone *et al.*, 1974). Here, the already trained panellists are required to assess the samples, under standard sensory analysis conditions, according to the set of training descriptors. This is done using a scale for scoring each descriptor – the higher the score for a particular descriptor, the greater the presence of that descriptor in the global aroma or flavor. Mean scores are calculated for each descriptor and for each sample. The results can be visualized in a number of ways, but spider web diagrams are probably the most widely used representations of flavor profiles these days. Here, the mean scores for the flavor attributes are plotted on lines emanating from a central point at regular angles and the scores are then joined by straight lines, so the finished plot looks rather like a portion of a spider's web. Examples of spider web plots for gin (and gin botanicals), Indian whisky, lychee and strawberry juice can be seen in Figure 4.7.7. Examples of flavor profile templates used for sensory profiling of different kinds of Scotch malt whiskies can also be seen in 3.2.6 (Figure 3.2.13).

Special data collection software (such as those of Compusense) and standard spreadsheet, graphical and statistical software (such as Microsoft Excel or Unistat software) can be used to process the results in other ways than constructing spider web diagrams. Multiple comparison tests can be used to identify differences in the assessment of duplicate samples, thus ensuring that the panel is using the vocabulary reproducibly. Principal component analysis (PCA) can be used to classify groups of samples according to their sensory profile and also to determine the major descriptors that discriminate amongst samples. Analysis of variance (ANOVA) is frequently used to determine individual differences between panellists.

Descriptive sensory tests have been used to create flavor profiles for a wide range of alcoholic drinks and their precursors, only a few of which are quoted as examples here: gin (and gin botanicals) (Phelan *et al.*, 2004 – see Figure 4.7.7), lychees (Mahattanatawee *et al.*, 2007 – see Figure 4.7.7), strawberry juice (Schieberle and Hofmann, 1997 – see Figure 4.7.7), whiskies (Jack, 2003; Maitlin and Stephen, 2004 – see Figure 4.7.7; Poisson and Schieberle, 2008b) and wine (Carlucci and Monteleone, 2001; Genovese *et al.*, 2007; Guedes de Pinho *et al.*, 2007). Depending on the requirements of the test (e.g. for wine, aroma only or aroma, flavor, taste and mouthfeel), the samples and reference standards will be sniffed, or sniffed and tasted (sometimes swallowed). Likewise, whether the samples and standards will be sniffed, or sniffed and tasted depends on the nature of the sample and the accepted protocol for sensory analysis in that part of the alcoholic beverage industry. Beer, cider and wine, for example, can be sniffed and ingested, whereas Scotch whisky is sniffed ('nosed') only.

The design of a descriptive sensory test depends upon the specific goals of the analysis, but the major steps that are needed are summarized below:

- Selection of panellists and choice of descriptors to be used
- Training of panellists with samples and reference materials
- Testing, maybe in several sessions
- Receiving and processing the results by statistical methods
- Assessment and recommended adjustments for future tests.

All sensory sessions, and probably the basic experimental design, will be conducted according to protocols and guidelines laid down by the various standards or similar institutes (e.g. ISO or one of its member bodies, such as AFNOR).

Panellists will normally be experienced sensory assessors or they may have been chosen from preliminary aptitude tests. Choice of descriptors can be made from a consideration of sensory literature (e.g. see Mahattanatawee *et al.*, 2007), but is more likely to be made by preliminary sensory sessions with the panellists generating an initial set of descriptors, followed by deciding the most appropriate terms, usually 8–16 descriptors (e.g. Carlucci and Monteleone, 2001; Phelan *et al.*, 2004; Guedes de Pinho *et al.*, 2007). The descriptors can be entirely aroma/flavor related, as in the sensory analysis of lychee fruit (e.g. Mahattanatawee *et al.*, 2007) or can include taste and mouthfeel as in the assessment of young red wines (Carlucci and Monteleone, 2001).

Further panellist training sessions are then conducted in order to improve recognition and discrimination, using reference standards at various concentrations, as well as samples to be tested later. For example, 2-phenylethanol, cherry jam, aqueous quinine hydrochloride and aqueous tannic acid were used as reference materials for rose odor, cherry odor, bitter taste and astringent mouthfeel descriptors, respectively, generated by the panel for the description of young Aglianico wine (Carlucci and Monteleone, 2001). Similarly, bergamot oil or Earl Grey tea were used as reference standards for the bergamot odor descriptor of Touriga Nacional wines (Guedes de Pinho *et al.*, 2007).

Testing is usually carried out on a strictly limited number of samples per session, with the panellists testing the reference standards then assessing the intensity of each of the descriptor contributions in the samples. A 100 mm unstructured scale with anchor points at 'hardly perceptible' and 'intense' (or similar phrases) is often used for scoring, but point category scales are also used. The testing is usually performed in duplicate or triplicate.

Other sensory analysis tests that are often performed on alcoholic beverages include triangular tests (which can be used to establish OTV) and rank order tests. Triangular tests are designed to determine differences or similarities between samples. Trained panellists are subjected to three samples; a reference containing a component relating to a particular descriptor and two unknowns, one with and one without the same descriptor. In a study of the role of methional in the aroma of oxidized white wines, the samples were oxidized wine (reference) and ethanolic extracts or HPLC fractions of nonoxidized and oxidized wine of the same type (Escudero *et al.*, 2000). Panellists sniffed the reference first and then the first unknown and assessed the similarity of aroma using a 100 mm unstructured scale, anchored with 'identical to reference sample' on the left and 'different from reference sample' on the right. This was repeated for the second unkown sample. The results were treated in two ways: by the total and correct answers and by univariate analysis of variance of scale responses, in mm from the left anchor.

Rank order or preference tests are often used to determine acceptability of a certain product (e.g. a new product compared with current products of the same company) or to determine panellist preferences (e.g. amongst a new product and similar products of different companies). In these ways, new melon spirits and melon liqueurs, made in different ways (e.g. unmacerated spirit, spirit macerated with melon seeds and

placenta, etc.) were assessed for preference. Likewise, the most favored melon liqueur was assessed against well-known commercial liqueurs for acceptability (Hernández-Gómez, *et al.*, 2009).

The quality of an alcoholic beverage is partly a subjective matter and is difficult to either quantify or generalize, and just as difficult to predict from the beverage's chemical composition, despite many studies on the chemical origins of certain perceptions of quality, such as fruitiness, oak character and others. Recently, sensory analysis has been used, alongside GC-O to assess the quality of 25 premium Spanish red wines (Ferreira *et al.*, 2009). Panellists were given the coded wines to smell and taste, and to sort them into five categories, ranging from 5 (exceptional) to 1 (defective or rejectable). The quality index of each wine was obtained by averaging all the individual scores. The results were matched with partial least squares analysis of three analytical variables: defective odorants (mainly simple phenols), negative odorants and fruity-sweet odorants. It was found that quality, as decided by the panellists, was primarily related to the presence of defective or negative odorants, rather than to desirable fruity-sweet odorants.

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Part 5
Nutritional and Health Aspects

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Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

5.1
General Introduction

Evidence from prehistory indicates that alcohol may have been actively produced for human use in China and the Middle East as far back as perhaps 9000 BCE to 8000 BCE, but there are more certain archeological indications of wine production starting around 5000 BCE and beer from around 4000 BCE (Wolf *et al.*, 2008) (Section 1.2.1). Over subsequent millennia, its social effects and medicinal properties have continued to be highly valued with increasing and substantial quantities of alcoholic beverages being consumed as relatively safe and sterile sources of important nutrients, especially as communities began to grow. Bacterial contamination was essentially preventable during production of alcoholic beverages by the use of boiling, the presence of yeast in high concentrations and the relatively low pH, and the ethanol (alcohol) content per se also helped to prevent spoilage and preserve some of the nutritional value. From these early origins, the progression of knowledge and the craft of producing alcoholic beverages have resulted in their spread across the globe (Section 1.2.1). Essentially, alcoholic beverages with relatively low alcohol content are produced by the fermentation of grains, such as beers (Chapter 2.6), and fruits, such as ciders (Chapter 2.8) and wines (Chapter 2.9). Distillation of these primary products of fermentation yields beverages of relatively high alcohol content, such as spirits and liqueurs (liqueurs are often referred to as cordials, depending on location), enabling the more practical transport and storage of alcohol (Part 3). Generally, the fermentation and maturation processes are relatively short for beers and ciders to reach their optimum state for consumption, compared with wines and the distillation and aging of spirits (Parts 2 and 3).

The numbers and variations of alcoholic beverages based on these generic styles have increased substantially because of the many additional ingredients that may be used, frequently when concocting, for example, the numerous cocktails created from spirits or wines mixed with fruit juices. Therefore, manufacture of the vast numbers of multifarious alcoholic beverages is now possible by the use of a multitude of ingredients and many different processes, from small 'home brew' production through to the industrial scale of multinational concerns with their capacity for distribution throughout the world. In view of this, it is not surprising that there is substantial variation in the nutrient content of the vast array of alcoholic beverages currently available. Although these differences may be especially apparent between the main generic styles of alcoholic beverages, the typical nutrient content of which can be easily compared through general reference sources, such as Table 2.13 in *McCance and Widdowson's, The Composition of Foods* (Food Standards Agency, 2002), individual products within these styles also show considerable variation in nutritional value. Furthermore, as so many different alcoholic beverages are readily available from a substantial variety of sources, extremely variable quantities are also consumed according to individual preferences and patterns of drinking, with

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

significant implications for nutritional status and concomitant health consequences (see the general texts of Morgan and Ritson, 2003; Watson and Preedy, 2003). Unfortunately, because of the vast number and variety of styles, it is not possible in this chapter to research and cover the entire range of nutrients present in all of the conceivable alcoholic beverages. However, the content and potential nutritional value of the generic styles of alcoholic beverage, with specific examples of individual products wherever appropriate, will be considered, along with the evidence based metabolic sequelae, and possible health and social consequences of their consumption.

Although nutritional and health aspects are in reality inter-related, in this book, nutritional topics are emphasized in Chapters 5.2–5.5, whereas health topics are focused in Chapters 5.6–5.11. In view of this, Chapter 5.5 acts as a 'bridge' between the two, leading into Chapter 5.6, where it will be seen that many of the detrimental effects of extraneous alcohol consumption are, at least in part, a result of nutritional changes. Chapters 5.7 and 5.8 deal with health related topics concerning the carbohydrate content and antioxidant content (respectively) of alcoholic drinks. The later chapters, Chapters 5.9–5.11, focus on health aspects related to the toxic effects of minor beverage components, namely additives, residues and trace substances.

5.1.1 'Alcohol' in Perspective

In the context of alcoholic beverages the term 'alcohol' is usually applied when referring to ethanol (also known as ethyl alcohol), which is a primary alcohol with the formula CH_3-CH_2-OH, and, essentially, this generic use will be continued here. However, the definition of 'alcohols' includes a large number of related molecules with similar chemical and structural properties, many of which are toxic and some of which may occasionally appear as components of the diet. In very simple terms, alcohols are characterized by a hydroxyl group bound to a carbon atom of an alkyl group (alkyls take the form C_nH_{2n+1}; e.g. methyl CH_3 and ethyl C_2H_5), and they differ from each other by the number and position of carbon atoms present in the molecule. Alcohol (ethanol) is relatively toxic and so excessive exposure, especially through the very high levels that may be ingested on some occasions, is not to be recommended. However, alcohol may be considered to have some nutritional value that can be utilized by the human body because it provides energy, at 29 kJ/g alcohol, from its breakdown to the metabolic end products, carbon dioxide (CO_2) and water (H_2O); see 'Alcohol Catabolism' (Section 5.5.1). For general information, 1 kJ is equivalent to 0.239 kcal (i.e. 1 kcal = 4.184 kJ) so that 29 kJ/g alcohol is equivalent to 7 kcal/g alcohol, which is the alternative value seemingly most often quoted for the energy value of alcohol.

Certain alcohols, such as methanol, 1-propanol, 2-propanol and higher (secondary and tertiary) alcohols, which may be intentionally or accidentally ingested on occasions, are far more toxic and cannot be considered acceptable constituents of the diet, whereas others, such as sugar alcohols, are more readily accepted as dietary components. Glycerol, for example, is a sugar alcohol that may be a fairly major product of fermentation, or may be added to alcoholic beverages for character, but without the intoxicating properties of ethanol. Indeed, glycerol itself and certain other sugar alcohols are widely utilized as food additives, especially on a commercial basis by the food industry, because they do not cause intoxication, and significant levels of these alcohols may be present in many of the readily available and more highly processed foods found on supermarket shelves. Other alcohols that are produced during fermentation processes are known as fusel alcohols or fusel oils and are considered important to alcoholic beverages because certain of these more complex alcohols may add desirable character to some (e.g. see Section 3.7.2), but may impair the character of others if present at high levels (e.g. see Section 2.9.1). Levels of 1- and 2-propanols, butanol, furfural and amyl alcohol, for example, may be increased during fermentation under certain modifiable conditions (including higher temperature, lower pH and less than optimum yeast activity), and may be selectively included, or not, in distilled beverages as particular distillation processes are differentially applied according

to the resultant character desired (Chapters 2.2 and 3.1). Collectively, organic acids, esters and higher alcohols that may be distilled along with ethanol are known as congeners and the degree of difference amongst these many and varied substances may influence the amounts likely to be consumed of any particular beverage. Although detoxification of the relatively low amounts of methanol, and possibly other alcohols that may be ingested under usual circumstances of social drinking, provides small amounts of energy and carbon units that are disposed of through the usual metabolic pathways, such alcohols are not considered to be acceptable as nutrients because of the relatively high toxicity of these compounds and of their metabolites. For example, formaldehyde is an extremely toxic product of methanol catabolism (Section 5.5.2) that can lead to blindness and even death when methanol is ingested in sufficiently large quantities.

Relatively simple standardization of the alcohol content in different types of alcoholic beverage has been implemented in order to try to promote greater understanding of the implications of the large range of alcohol levels available from the different beverages, especially for health and legal reasons. Alcohol offered for sale in the UK (above 1.2%) must have its alcohol content clearly stated and displayed on the product itself or at the point of sale, usually as the amount of alcohol by volume (ABV) as percentage (e.g. 14% by volume, for a red wine). The 'unit' of alcohol in the UK is equivalent to 10 ml or 7.89 g of pure alcohol (the density of ethanol is 0.789 g/ml), which is the exact amount found in a 25 ml measure of 40% ABV spirits, and approximately that in a 125 ml glass of about 8% ABV wine or a half pint of 'session' beer (Section 5.6.1) of about 3.6% ABV. The current UK Government guidelines for healthy adults recommend no more than 3 to 4 UK units of alcohol per day for men and 2 to 3 UK units for women, with lower levels in pregnancy (less than 1 to 2 units on only a couple of occasions per week at most); see the website of the British Nutrition Foundation (http://www.nutrition.org.uk) for general information on dietary guidelines. The UK unit is not to be confused with a nominal and very variable 'standard' amount of alcohol applied to alcoholic beverages around the world, which are generally higher than the UK unit (see Section 5.6.1). In the USA, the standard amount is equivalent to 14 g or 17.75 ml (1.2 tablespoons or 0.6 fluid ounces), and in many other countries this amount is even greater; relatively high standard amounts are applied in Hungary (21.5 ml, 17 g) and Japan (25 ml, 19.75 g), for example. For a comprehensive review on the topic of standardization of alcohol and other important aspects of alcohol consumption and health, see Foster and Marriott (2006).

It is important to note that most wines and very many beers are currently being manufactured at much higher alcohol content than those produced previously, which is a source of some confusion to consumers as they may seriously underestimate their actual alcohol intake. Certain commonly consumed red wines now contain perhaps 14% ABV and those beers and lagers generally considered to be 'regular' may be at least 5% ABV; therefore, a traditional or standard serving is often equivalent to upwards of 1.5 UK units. Also, many measures, especially spirits and wines, and whether or not consumed at home or in retail outlets, are now served in greater quantities than traditional standard measures. For example, a 'glass' of wine is now often served as a 175 ml or 250 ml measure rather than the 125 ml standard measure. Another complication for the consumer is that alcoholic beverages may be offered for sale in variable and nonstandard volumes in retail outlets. For example, in contrast to the regular pint (568 ml) and half pint (284 ml) measures in UK public houses, beers and lagers may be available in cans of 330 ml or in bottles of 500 ml, making difficult the ready estimation of the number of 'units' available for consumption. Therefore, despite the generally open displays of alcohol levels at retail outlets and on the products themselves, it seems that a majority of consumers probably lack a full appreciation of their total alcohol intake when consuming a majority of alcoholic beverages, putting themselves at risk of acute and chronic harm due to relatively excessive consumption of alcohol to which they may be unaware and insensitive. For informative purposes, another representation of the alcoholic strength of a spirit is the alcoholic 'proof,' a value that approaches double (1.75×) that of the ABV (%) in the UK – so a 40% ABV spirit is actually equivalent to 70 degrees proof spirit.

5.1.2 The Extent and General Implications of Alcoholic Beverage Consumption

Estimates of alcohol intake and the associated costs to health and social implications vary somewhat according to the particular country and region of interest concerned (Prime Minister's Strategy Unit, 2004, www.cabinetoffice.gov.uk; Association of Public Health Observatories, 2007). Very generally, in the 'Western World' average yearly alcohol consumption for each adult approximates 10 l of pure alcohol from all of the multifarious alcoholic beverages. The proportion of people consuming alcohol in different communities also varies considerably (Sieri *et al.*, 2002), with some societies banning alcohol consumption altogether. Individual responses to alcohol and, indeed, the likelihood of individual susceptibility to alcoholism are determined by both genetic and environmental factors, and the extent of their interaction (Gurr, 1996). Multiple genes have been implicated in the heritability of dependence (Gurr, 1996), and demographic and social factors are also important in determining alcohol intake and its impact on health (Klipstein–Grobusch *et al.*, 2002; Rehm *et al.*, 2003), as are patterns of alcohol consumption (e.g. Puddey *et al.*, 1999; Rouillier *et al.*, 2004; Duffey and Popkin, 2007; Smith and Shevlin, 2008).

Alcoholic drinks may be consumed in addition to normal dietary requirements or may effectively replace other foodstuffs due to a number of possible changes in appetite and certain behavioral factors. Firstly, additional satiety influences (positive and negative) may be imposed on normal appetite control, such as those produced physically by increased volumes ingested and, also, through hormonal feedback mechanisms in response to digestion and assimilation of certain nutrients. Secondly, modifications in feeding behavior, such as changes to otherwise regular healthy eating patterns imposed by more frequent and extended drinking sessions, including missed meals, snacking to replace traditional meals and inappropriate food selection, alter intake of these nutrients compared to the usual diet. Lastly, patterns of food and nutrient consumption are also influenced according to acute or chronic use and low to moderate or excessive consumption at any particular time span (Forsander, 1998; Yeomans *et al.*, 2003; Yeomans, 2004; Caton *et al.*, 2007; Kokavec, 2008; see Section 5.5.3).

Although nutritional and health information is known to influence consumer perceptions, these perceptions are apparently not the most compelling determinants of drinking behavior and choice (Wright *et al.*, 2008). In Britain, about 90% of the adult population consumes or has consumed alcohol to some extent with about a quarter of consumers regularly drinking above recommended guidelines (Prime Minister's Strategy Unit, 2004; www.cabinetoffice.gov.uk). Most consume alcohol 'sensibly' but for many others consumption is excessive leading to possible dependence and harm, especially if this occurs over the long term. Dependence, both physical and psychological, is a major public health concern (Morgan and Ritson, 2003), even in the young (McArdle, 2008), and the vast extent of short- and long-term health and social issues has led to calls for nationwide strategies to tackle alcohol misuse, albeit with some consensus disunity (Babor, 2008).

However, although certain of the many factors associated with consumption of alcoholic beverages have the potential for serious harm, especially when alcohol is ingested in large quantities, other factors appear to confer some health benefits, even if only marginal. For some aspects of health the relative risk of alcohol consumption appears to follow a 'j' shaped curve in which low to moderate levels of intake are associated with lower risk to health than in abstainers or very occasional drinkers (Section 5.6.1) (Gurr, 1996). The apparent cardiovascular benefits observed on an epidemiological basis have led to deliberations concerning possible underlying protective mechanisms (e.g. Agarwal, 2002; Puddey *et al.*, 2003), including the effects of alcohol on blood platelets (Ruf, 2004), for example. However, the risk to cardiovascular health rises considerably as alcohol intake increases beyond that considered moderate, being highest in excessive drinkers and alcoholics (Gurr, 1996). Therefore, the possible nutritional and health consequences for individuals are extremely complex and potentially very serious indeed for high consumers, with major implications for population health (Table 5.1.1), now and extending further into the millenium (Foster and Marriot, 2006; for summary, see British Nutrition Foundation website, http://www.nutrition.org.uk). These effects of alcohol consumption

Table 5.1.1 *Potential health consequences of imbibing alcoholic beverages**

Accidents and injury	Unintentional and immediate physical lesions and even death due to intoxication related accidents, with possible short- and long-term consequences depending on severity, with violence and crime related intentional injuries associated with excessive alcohol intake.
Addiction	Possible long-term psychological and physical dependence.
Amnesia	Loss of memory for variable periods due to excessive alcohol intake.
Cardiovascular complications	Although there appear to be some cardiovascular health benefits especially at low levels of alcohol intake (e.g. reviewed by Puddey *et al*., 1999; Agarwal, 2002; Suter, 2004) and blood coagulability appears to be reduced by alcohol per se due to effects on platelet aggregation along with possible polyphenolic component effects (Puddey *et al*., 2003; Ruf, 2004), greater alcohol intake and concomitant metabolic challenges predispose to increased arrhythmias, cardiomyopathy, vascular hypertension and coronary heart disease (Morgan and Ritson, 2003).
Drug/alcohol interactions (pharmaceuticals and drug abuse)	Alcohol may reduce the capacity of the body to detoxify certain pharmaceuticals and xenobiotics, e.g. through various detrimental interactions with cytochrome P450 during catabolism of alcohol through the microsomal ethanol oxidizing system (MEOS; Leiber, 2000; Section 5.5.1).
Gastrointestinal problems	Gastritis, esophagitis and pancreatitis may develop over the short term with long-term exacerbations such as Mallory–Weiss syndrome (severe esophageal bleeding) as a result of the direct effects of alcohol per se on the gut with malabsorption of important nutrients potentially a major consequence.
Hepatic disease	Fatty liver, hepatitis and cirrhosis may be long-term consequences of excessive alcohol consumption due in major part to the metabolic challenges encountered during alcohol catabolism.
Hypersensitivity, allergy and autoimmune responses	Certain constituents of alcoholic beverages, other than alcohol, have been associated with specific hypersensitivity, allergic or autoimmune reactions, such as biogenic amines and polyamines in wines and beers causing allergic reactions (Kalac and Krizek, 2003; Vally and Thompson, 2003; Ancín-Azpilicueta *et al*., 2008) or the antigens gluten (wheat) and hordein (barley) exacerbating existing *celiac disease* (Ellis *et al*., 1990; Dostálek *et al*., 2006).
Hypoglycaemia	Changes in the flux of intermediary metabolites through usual metabolic pathways caused by high levels of alcohol intake may result in seriously low blood glucose concentrations.
Malignancies	Excessive long-term alcohol intake is associated with a number of malignancies, although alcohol per se may not be the agent specifically responsible.
Malnutrition	Inadequate nutritional status is associated with alcohol abuse either through reduced dietary intake of other nutrients or by the direct effects on nutrient absorption and metabolism.
Methanol toxicity	Methanol is a by-product of fermentation, which may be concentrated significantly particularly during illegal distillation, and its ingestion in sufficient quantities may lead to blindness and even death.
Myopathy	Occurs in skeletal muscle with a wide spectrum of symptoms and may be acute or chronic, with *rhabdomyolysis* a possible secondary complication of alcohol related disorders or its direct toxicity, with increased sodium permeability and consequent accumulation of calcium and associated hypophosphataemia reducing cellular ATP underlying the condition; tetany may also occur in alcoholics due to low levels of calcium and magnesium.

(*Continued*)

Table 5.1.1 (Continued)

Nervous system exacerbations	In addition to possible brain damage from cerebrovascular events (e.g. hemorrhagic stroke), alcoholic dementia, cerebellar degeneration, Wernicke–Korsakoff syndrome, Marchiafava–Bignami syndrome, peripheral neuropathy and neuropraxia, amongst other conditions, may also present, especially in chronic excessive alcohol abuse.
Osteoporosis	Alcohol appears to affect bone turnover leading to reduced bone mineral content and the occurrence of fracture may be more prevalent, although there are potential protective effects of silicon in beer against aluminium, the ingestion of which has implications for bone health (and aluminium is also associated with degenerative disorders of the nervous system).
Pneumonia	May occur due to aspiration of stomach contents during acute intoxication.
Poisoning	Acute excess quantities of alcohol may result in poisoning, with death occurring in half of all individuals when blood alcohol levels reach about 0.45%.
Psychological disorders and social consequence	Many psychological disturbances may occur with excessive and/or long-term alcohol consumption and its dependence that may lead to implications for acceptable social interactions with alcohol frequently implicated in suicides.
Sexual exacerbations, infertility and foetal disorders	Reproductive capacity can be significantly reduced in both susceptible men and women, with reduced fertility and increased risk of spontaneous abortion, and potentially serious risk to fetal health with fetal alcohol syndrome presenting in cases of serious alcohol misuse especially in the peri-conception period and early pregnancy, up to about 10 weeks, but throughout pregnancy there is an increased risk.
Skin disorders	Alcohol mediated skin lesions occur and increased risk of infective diseases is associated with alcohol abuse.
Withdrawal	Withdrawal from alcohol dependence is associated with sometimes severe physical and psychological reactions.

*Presented in alphabetical order, not in order of severity, and for general details of these health consequences, see Gurr (1996), Morgan and Ritson (2003), Paton and Touquet (2005), Smart (2007) and Watson and Preedy (2003).

on health are also well presented in a number of generally informative publications aimed at different levels of interest, background or requirements, including those of Gurr (1996), Morgan and Ritson (2003), Paton and Touquet (2005), Smart (2007) and Watson and Preedy (2003).

Although it is the amount of alcohol consumed that is of primary interest for health generally, there is also a lack of appreciation of the full implications of alcohol beverage intake on nutritional and health status. A vast majority of consequences of alcohol ingestion are clearly detrimental, as presented in Table 5.1.1. However, alcoholic beverage consumption is often associated with some purported health benefits. The nature of any particular type of alcoholic beverage and the specific circumstances surrounding its usage determines the volume likely to be consumed and, therefore, the total amounts of alcohol and nutrients (and potential toxins) likely to be ingested. Specific nutrient intakes are, for the most part, quite small in relation to dietary requirements for maintaining optimum nutritional status and health, although quantities of certain nutrients consumed in particular alcoholic beverages may be significant. Therefore, the intake of nutrients from alcoholic beverages may vary considerably in terms of their overall contribution to the diet and, of course, to fulfilling nutrient requirements (see Section 5.1.4). For example, a serving of 1 unit of lager may provide about 2% of purported daily requirement for thiamine but about 15–20% of that for folate (Section 5.4.1). When assessing the nutrient impact of alcoholic beverages, it is important to recognize that serving sizes differ considerably and that the overall nutrient load (i.e. the total amount ingested) is also variable

within and between individuals depending on consumer habits on a day to day basis. Such variability may be extreme as some consumers, possibly considered moderate drinkers on the basis of a weekly average, may be exposed to relatively low levels of alcohol intake almost every day, whereas others who are drinking equivalent amounts overall may be exposed to far higher intake levels during more intermittent sessions of 'binge' drinking, both modes of which may have implications for dietary intake and health (Puddey *et al.*, 1999).

5.1.3 Ingredients, Processing and the Nutrient Content of Alcoholic Beverages

Raw ingredients used in the production of alcoholic beverages are many and varied, some of which may contribute to the nutritional value of the final product, whereas others may not. In general terms, cereal grains are used for beers (Chapter 2.6) and fruits for wines (Chapters 2.9 and 2.11) and ciders (Chapter 2.8). Particular ingredients may be preferred for producing distilled beverages of specific character; whisky (Chapters 3.2 and 3.3) from fermented malt or cereal grains and brandy (Chapters 3.6, 3.7 and 3.8) from fermented fruit juices, for example. Other ingredients may be used for slightly less usual or speciality beverages, such as some root vegetables (e.g. for some vodka; see Section 3.4.3), molasses/sugar cane (cachaça and rum; see Sections 3.5.2 and 3.5.3), honey (mead; see Section 2.11.5), even flower petals for some wines, amongst many others. The relative availability of ingredients largely determines the styles of alcoholic beverages produced in a particular locality, with rice being the predominant staple grain in the East, corn (maize) in the USA and South America (the Incas, for example, were known to use corn for producing alcohol) and barley in the UK and Europe (Foster and Marriott, 2006). Almost any fruit may be used to produce wines locally, although grapes are the most abundant fruit used on a worldwide scale, and many fruits may also be used in producing fruit beers, such as in the UK and Belgium (Section 2.6.13).

Many beverages (alcoholic and nonalcoholic) are produced and consumed locally, even at the household level, such as some fermented milk products in Russia and parts of Africa, and many types of beers (e.g. pombe, shakporo, tella, and others made from various grains such as sorghum: see Section 2.7.2), wines and spirits, in particular geographical areas in different regions of Africa, for example. A fine description of how the wide variety of fermented beverages (including alcoholic wines, beers and spirits) are produced in Zimbabwe, as an example of an African Nation, at various levels from small household production through to large-scale manufacture, is provided by Gadaga *et al.* (1999). In South America, for example, beers were produced with manioc and peanuts as well as corn (Wolf *et al.*, 2008) (Section 2.7.2). Many of these household and local products are very crude in nature. However, because of the limited amount of processing involved, such products are usually perceived as having better nutritional qualities than the often clearer mass produced beverages. Homemade beers and wines are still quite a popular pastime in some countries such as in Europe, including the UK, although distillation is only permitted under license in the UK. Overall, however, homemade beverages are unlikely to have a major impact on nutritional status and health of the population in the more developed countries.

Supplementary ingredients, for instance fruit and herbs, and also the infused flavors of liqueurs, such as from chocolate, may be added to modify the product as processing progresses, as in the production of Vermouth (Section 2.12.2), apérifs (Section 2.12.3) and liqueurs (Chapter 3.9). Other ingredients, the additional flavoring hops added later in beer production for bitterness, or orange juice mixed with wine for making Sangria, for example, may be blended with particular beverages during and after the final production stages to modify the taste of the base product. Additives may modify the presentation or preservative properties, or simply produce a different type of beverage altogether, such as creating cocktails from a variety of mixers. Certain ingredients may be added during beer production in particular, in order to improve taste (e.g. some salts), or to provide body (e.g. maltodextrin) and smoothness (e.g. licorice sticks). Some waste products of fermentation,

such as spent ingredients and precipitates, may be allowed to remain in the beverage in greater or lesser quantities for particular character or may be removed completely during processing to produce a slightly different style of a particular beverage or simply to improve its presentation. Essential ingredients used in the initial stages of production, such as yeast, preservatives (e.g. sulfites) and finings (e.g. isinglass, egg white, bentonite, etc.) (Sections 5.9.2 and 5.9.3) may be partially or completely removed before the final product is ready. However, residual amounts of particular ingredients present in the finished beverage may have significance for health status (e.g. Vally and Thompson, 2003). For example, antigens such as gluten (wheat) and hordein (barley) present in some alcoholic beverages, specifically beer, may exacerbate existing celiac disease in susceptible individuals (Section 5.11.3) (Ellis *et al.*, 1990; Dostálek *et al.*, 2006); celiac disease is an autoimmune condition affecting the integrity of the small intestine leading to protein losing enteropathy and poor absorption of essential nutrients. Other constituents of finished alcoholic beverages, which are derived from the raw ingredients or produced during fermentation, may also have implications for health because of their potential biological activity in the chemical form as ingested or because they produce metabolically activated derivatives. These include the potentially beneficial phenolic antioxidants (Chapter 5.8) and phytoestrogens (e.g. Soleas *et al.*, 1997; Bravo, 1998; Eastwood, 1999; Puddey *et al.*, 2003; Aron and Kennedy, 2008) (Section 5.4.3) and the potentially detrimental biogenic amines (e.g. Ancin-Azpilicueta *et al.*, 2008; Kalac and Krizek, 2003 (Sections 5.3.4 and 5.11.3).

Distillation processes selectively concentrate alcohol at the expense of the other constituents, many of which are then not detectable at all or are only present in such small amounts that they could not make a significant contribution to the nutritional value of the diet or, indeed, to the possible detriment of health.

Reflecting this variability in ingredients and production methods, the nutrient content and, therefore, nutritional value to consumers of alcoholic beverages is also extremely variable. See Table 2.13 in *McCance and Widdowson's, The Composition of Foods* (Food Standards Agency, 2002) for comparison of nutrient concentrations in the generic classes of alcoholic beverage. Importantly, the contribution to nutritional status that might be expected from the constituent nutrients of alcoholic beverages under normal conditions may be significantly affected by alcohol induced alterations in nutrient bioavailability and disturbances of the usual metabolism of nutrients in the body during periods when alcohol consumption is sufficiently high (Lieber, 2000). Of further importance is that such alcohol induced alterations in nutrient bioavailability and metabolic disturbances may also adversely affect the absorption and metabolism of those nutrients normally available from other dietary sources ingested at or around the same time as high levels of alcohol are consumed (Lieber, 2000). Alcohol itself may interfere with normal healthy digestion and absorption processes and its metabolites may disturb certain intermediary metabolic pathways and cycles, blocking some reactions and redirecting the usual nutrient and energy fluxes into other pathways (see Section 5.5.4). In susceptible individuals, alcohol consumption does not necessarily need to be excessive by commonly accepted standards to influence nutritional status. Therefore, alcohol and its metabolites, formed during the breakdown of alcohol through intermediary metabolic pathways, may be of major detriment to health, but especially in high consumers and alcoholics. For more detailed information, please see Watson and Preedy (2003).

Alcoholic beverages may also contain a number of possible toxic contaminants derived from the raw ingredients per se or that may have originated during the production processes. Some congeners, for example, have been implicated in the toxic effects of alcoholic beverage consumption, especially at times of excess, perhaps exacerbating symptoms of a hangover, the effects of which are essentially transient in most consumers, although it is usually the light to moderate drinkers who incur most of the associated human 'costs' (Wiese *et al.*, 2000). However, severely debilitating conditions can result if high levels of the fermentation by-product methanol are consumed, especially as it may cause blindness. Potential toxins may arise from soil contaminants such as pesticides or heavy metals, depending on factors such as locality. Also, to reiterate, certain potential immunochemicals are also found in some alcoholic beverages. Gluten derived from grains, wheat in particular, or hordein in barley, which are the cause of celiac disease in genetically susceptible

individuals, may be present in finished beers. Although wines may be clarified with gluten instead of the usual animal proteins, it does not appear to be present in measurable amounts in the final products (Cattaneo *et al.*, 2003), and such immunochemicals are essentially absent from spirits due to distillation processes. However, some potential carcinogens, such as furans, may be introduced into spirits and wines that are matured in charred wood casks (Goldberg *et al.*, 1999). A number of other chemical constituents likely to be found in wines, beers and spirits, such as biogenic amines, have allergenic properties with health implications, especially for asthma sufferers (Vally and Thompson, 2003).

In contrast to potential toxicity, many of the constituents present in some alcoholic beverages, such as vitamins, minerals and some phytochemicals (compounds derived from plant metabolism; see Section 5.4.3), appear to be capable of positively promoting health in moderate consumers (e.g. Puddey *et al.*, 2003; Suter, 2004). Indeed, the ability of some plant derived antioxidants to scavenge and neutralize the free radicals or reactive oxygen and nitrogen species is believed to be particularly beneficial to health and, as a plethora of these phytochemicals are present in many wines, beers, ciders and certain spirits, alcoholic beverages are also considered to confer such health benefits (Section 5.8.5). However, with only a relatively small evidence base in terms of fundamental mechanisms and actual health outcomes, much of this belief is speculative and should be viewed with some caution (Soleas *et al.*, 1997). As most of the evidence is based on epidemiological studies, prospective and intervention trials are required for confirmation of the fundamental bases of purported health benefits of alcoholic beverages. One suggested rationale with some scientific credence is that the damage caused by free radicals may be prevented because plant derived antioxidants present in certain alcoholic beverages quench the oxidation potential of these free radicals before they can oxidize key structural and functional components of tissues and cells. Free radicals are produced during metabolic processes, including those involved in alcohol catabolism, and are purported to be detrimental to health because the oxidation of proteins and DNA, for example, by these free radicals is heavily implicated in some cancers and in exacerbating cardiovascular disease progression. The anomaly created by the apparent health benefits of consuming red wine, in particular, which supposedly helps to reduce the cardiovascular risk associated with high fat intake, especially saturated fat, and other inappropriate dietary and lifestyle practices is known as the 'French paradox,' albeit somewhat controversial (de Lorgeril *et al.*, 2002; Goldfinger, 2003; Ferrières, 2004). Such purported health benefits of wines (German and Walzem, 2000) have been extended to include beers (Denke, 2000; Baxter and Hughes, 2001; Kondo, 2004), and many spirits are known to contain antioxidants albeit representing a completely different spectrum of phytochemical components (e.g. Goldberg *et al.*, 1999). The reader is directed to Chapter 5.8 for more details on free radicals, antioxidants and health.

However, some of the health benefits usually attributed to other nutrients present in alcoholic beverages may in fact be due to some of the actions of alcohol per se; i.e. consumption of alcoholic beverages in low or moderate amounts may have health benefits independent of their direct nutritive value in that there are possible health gains through the relaxation aspects of moderate social drinking, associated lifestyle and nutritional practices, and other possible metabolic effects. Perhaps such factors may partially explain the j shaped curve of relative health risks. However, it should be noted that more than moderate consumption of alcohol, which is too often considered to fall within the bounds of reasonable 'social drinking,' has potentially serious consequences for health and acceptable social interactions (Morgan and Ritson, 2003) even though it is not outwardly perceived as being excessive.

5.1.4 Nutritional Considerations

In order to appreciate the full implications of the various levels of alcoholic beverage consumption in perspective of nutrient intake and concomitant nutritional and health status, it is important to understand the basic principles integral to the science of nutrition and dietetics. The amount of any particular nutrient that is

required to maintain health, that is the nutritional requirement, is very variable and depends on an individual's age, sex, physical activity levels, capability for absorption and metabolic utilization, and current health and disease status. Certain nutrients, such as the vitamins and some amino acids and fatty acids, are regarded as 'essential' in the sense that they cannot be produced endogenously, at all or in sufficient quantities, to support crucial metabolic processes, the lack of which will result in increased morbidity and mortality.

Dietary recommendations for the different population groups are defined by a series of dietary reference values (DRVs) which describe critical aspects of nutrient intake: DRVs include: (a) the lower reference nutrient intake (LRNI) below which nutrient intake is considered generally inadequate for individual members of any particular group, (b) the estimated average requirement (EAR) which is applied particularly to energy requirements in a population group and (c) the reference nutrient intake (RNI) which is applicable to micronutrients and protein, and is the value assigned to a population group in order to ensure that specific nutrient intake is sufficient for a vast majority of that group. The conceptual 'safe intake' may also be used which is a designated range of values between a higher limit, above which an increased health risk (potential toxicity) is considered significant, and a lower limit, below which there is no apparent risk of deficiency for any members of a particular group. Recommendations in the USA (dietary reference intakes; DRIs) may occasionally appear to differ from those of the UK; sometimes they are substantially different and, as such, these differences will be indicated where appropriate, but mostly any discrepancies are slight. More detailed descriptions of these principles and how they apply to the various classes of nutrients can be found on the websites of the British Nutrition Foundation (http://www.nutrition.org.uk), the Food Standards Agency (http://www.food.gov.uk/healthiereating) and the National Agricultural Library (NAL) at the United States Department of Agriculture (USDA - http://www.nal.usda.gov/).

During normal habitual consumption of food and drink in the absence of alcohol, dietary metabolic energy is differentially provided by the major food groups, fats (also known as lipids), carbohydrates and proteins, which are collectively known as the macronutrients (see Chapter 5.3). Essentially, recommended energy requirements for a diet considered generally healthy and balanced consist of about 35% from fats (lipids provide energy at 37 kJ/g – or 9 kcal/g), 50% from carbohydrates (at 16 kJ/g – often seen quoted at 4 kcal/g but, more precisely, 3.75 kcal/g) and 15% from proteins (at 17 kJ/g – or 4 kcal/g). For example, the energy intake recommended for an individual requiring a nominal 8368 kJ (2000 kcal) daily is, therefore, about 2929 kJ (700 kcal) or 78 g from fat, 4184 kJ (1000 kcal) or 250 g from carbohydrate and 1255 kJ (300 kcal) or 75 g from protein. This guidance for a healthy balanced 8368 kJ/day (2000 kcal/day) nominal intake from the various macronutrients quoted here is for illustrative purposes and is a very approximate average population value based on an intake of 6870 kJ (1632 kcal) for women and 9720 kJ (2313 kcal) for men (see British Nutrition Foundation website; http://www.nutrition.org.uk). The above values would need to be adjusted proportionally for application to an average dietary intake for individuals requiring different levels of energy intake, more or less according to whether they are more active or more sedentary. Of course there is great variation in energy requirements for individuals within the population according to body size and composition, as well as substantial differences in physical activity levels. Further details regarding dietary energy requirements are available in the Department of Health's COMA Report (Department of Health, 1991) and from the website of the British Nutrition Foundation (http://www.nutrition.org.uk).

Alcoholic beverages may, therefore, contribute significantly to overall energy intake because complete breakdown of alcohol to CO_2 and H_2O produces 29 kJ/g (7 kcal/g), a value that clearly falls in between those of the major food groups (16 kJ/g for carbohydrate; 17 kJ/g for protein; 37 kJ/g for fat). A single UK unit (10 ml; 7.89 g) of pure alcohol alone is equivalent to about 229 kJ; this is about 2.7% of the daily energy intake for an individual requiring 8368 kJ (2000 kcal) daily. However, in addition to the alcohol content, many alcoholic beverages also provide substantial amounts of energy from other food energy sources present, particularly carbohydrates. For example, a typical pint of beer or lager with moderate levels of alcohol (perhaps 3.6% ABV) that contains 837 kJ (200 kcal) of energy, in terms of alcohol plus carbohydrate content,

could provide 10% of the daily energy requirement of an individual for whom the daily requirement is 8368 kJ (2000 kcal). Some types of alcoholic beverage, for example premixed alcopops, are formulated for specific markets with possible large amounts of simple sugars and various chemical flavorings and colorings added to alcohol in various concentrations. Such concoctions are considered to be sources of calories only (so called 'empty calories'), with no additional nutrient value of any kind. However, some premixed concoctions do contain ingredients, such as fruit juices, with some potentially significant nutritional value.

Therefore, at times when alcohol is consumed in sizeable amounts, both the total amount of metabolic energy ingested and the balance of energy from the usual primary dietary sources may be altered significantly. Clearly, alcohol consumption without attendant reductions in energy intake from carbohydrates, fats and proteins, or certain other possible increases in behavior or changes in physiology, may lead to positive energy balance with concomitant weight gain; the extent of which is essentially dependent on the additional energy intake. Consider the potential implications of extra ingested alcohol being almost equivalent to fat in terms of metabolic energy (29 kcal/g – 7 kcal/g – energy from alcohol and 37 kJ/g – 9 kcal/g – from fat), rather than to carbohydrate or protein at only 4 kcal/g. However, not all consumers of alcoholic beverages gain weight as expected because of possible concomitant reductions in absolute levels of intake of the usual dietary energy sources, or changes in the relative balance of these energy sources, to compensate for the additional energy intake from alcohol. Indeed, many alcoholics exhibit characteristic weight loss, although this is often through associated malnutrition of different origins. Possible increases in physical activity may help to offset the additional caloric intake, most especially in some moderate consumers. In addition, production of energy in the form of heat through thermogenesis or futile cycles and its dissipation into the environment may increase depending on factors such as the predominant metabolic pathways (e.g. the microsomal ethanol oxidizing system – MEOS – see Section 5.5.1) which may be activated according to the amount of alcohol consumed and so may also contribute to weight maintenance, even though overall increased energy intake is apparent. However, the considerable intra- and interindividual variability in alcohol consumption imposes difficulties when assessing the relative contribution of alcohol to energy intakes and concomitant body weight control overall (Section 5.3.2) (Prentice, 1995). Although very low levels of alcohol or complete abstinence are generally recommended for optimum health, with few implications for energy balance, high or even moderate levels of alcohol intake can have major health sequelae beyond those of energy balance alone, especially as individuals have different abilities to detoxify alcohol with regard to genetic influences on the production of the specific enzymes needed for alcohol detoxification (Crabb *et al.*, 2004) (Section 5.5.1).

There are further implications for recommended dietary intakes because of differential habits of consumption of the various different types of alcoholic beverages and concoctions created from them. Firstly, current dietary recommendations in general are that sources of carbohydrates should be selected in order to avoid simple sugars (no more than 25% of dietary energy intake should be from simple sugars) in favor of the more complex forms of carbohydrate with their greater purported health benefits. A number of alcoholic beverages, however, are high in simple sugars, a large proportion of which may be added during processing. These include alcopops, certain liqueurs produced with sugar based infusions and cocktails made with mixers rich in simple free sugars. In other alcoholic beverages, especially some of the less refined fermentation products such as certain native beers (Chapter 2.7), the balance of carbohydrates present substantially favors those that are more complex in nature and may even make small contributions to the recommended daily intake of dietary fiber (essentially, dietary fiber is indigestible carbohydrate material derived from plant sources – see Sections 5.3.3 and 5.7.4). Secondly, high levels of saturated fats and cholesterol, predominantly from animal sources, and fats consisting mainly of *trans*-fatty acids are not recommended as part of a healthy diet (essentially, *trans*-fatty acids are used in the manufacturing of products containing fats for less expensive manufacturing options and with longer shelf life). Although alcoholic beverages rarely do contain any lipids whatsoever (negligible amounts are found in wines and beers, derived mainly from plant cell wall material), in those that do, their fat content has usually been added during the final stages of production, and includes

cream liqueurs (Section 3.9.5), for example, which could conceivably contain 5–6 g fat per 40 ml serving. Thirdly, the small amounts of protein in most alcoholic beverages are usually insignificant in terms of their contribution to recommended dietary requirements, although some products that are not treated by precipitation or filtration may contain plant proteins and particular beverages may have protein added as part of the final stages of production, e.g. from eggs or milk sources.

A healthy balanced diet overall should also have sufficiently varied foodstuffs to contain the recommended dietary requirements for vitamins and essential minerals or trace elements (collectively defined as micronutrients, Chapter 5.4), essential amino acids, and essential fatty acids, as well as other key nutrients such as the antioxidant phytochemicals. Certain alcoholic beverages may contain some or all of these essential nutrients in amounts that may be of some, if limited, benefit to health. However, significant intakes of alcohol and associated changes in behavior may also create imbalances in otherwise healthy dietary intakes, even in moderate consumers but, particularly, in heavy drinkers and alcoholics in whom such dietary anomalies may exacerbate the major health risks associated with ingestion of alcohol per se. For more easily accessible information on healthy eating and good nutrition, in general, see the websites of the British Nutrition Foundation (http://www.nutrition.org.uk) and the Food Standards Agency (http://www.food.gov.uk/healthiereating).

5.1.5 Summary

In conclusion, alcoholic beverages in their many forms and levels of consumption may have variable and extremely complex implications for nutritional status and health overall as they may influence levels of intake of specific nutrients to a greater or lesser extent, with both short- and longer-term consequences, many of which are detrimental, whilst others may be beneficial. Alcoholic beverages may be consumed on top of the usual diet or may replace more appropriate foods creating potentially harmful imbalances in otherwise healthy levels of nutrient intake. In contrast, certain types of alcoholic beverages are considered to contain significant levels of valuable nutrients; red wine, for example, is purported to be a significant source of antioxidants and beer is a source of B vitamins as well as antioxidants (Section 5.4.3 and Chapter 5.8). However, despite making available relatively greater amounts of such important nutrients, excessively high levels of alcohol intake, from wine and beer, as well as from stronger alcoholic beverages, may: (a) increase the bodily requirements for certain important nutrients, (b) interfere with the normal processing of macronutrients and micronutrients and (c) influence appetite in such a way that otherwise important sources of key nutrients are excluded from the diet. Indeed, these higher levels of alcohol consumption may compromise nutritional status to such an extent that medical intervention is required. It is also important to be aware that, although the nutrient content of certain alcoholic beverages may be beneficial to nutritional status and health if taken in moderation, a substantial number are providers of 'empty calories' only, in that the alcohol and free sugars contained in such drinks may contribute significant levels of energy, alone, with little or no additional nutrient value.

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5.2

Factors Influencing the Nutrient Content of Alcoholic Beverages

The wide ranging nutrient content of the multifarious alcoholic beverages is governed by which of the numerous possible raw ingredients are used and in what combination, along with particular production techniques. Many of the original ingredients persist unchanged into the final product, some are lost, some are altered chemically and many others are produced during fermentation and ageing. Additional materials may be utilized for optimizing the fermentation process, zinc for example, or for preservative properties of the final product, such as the antioxidant glutathione (also present in the grape and produced by the action of yeast). Although the major types of alcoholic beverage are somewhat defined in character and, therefore, in alcohol and nutrient content, there is still much potential for variability regarding nutritional value between apparently similar individual products within each class. Fermentation processes (Chapters 2.2 and 2.3) modify the raw ingredients not only because yeast converts the fermentable sugars, mainly glucose and fructose, into alcohol, but also because yeast utilizes nutrients from these ingredients for its own growth and multiplication and, significantly, yeast produces certain key nutrients itself, most notably members of the B group of vitamins. Yeast may also be the major source of purines and pyrimidines in alcoholic beverages as these nucleotides are components of the nucleic acids, DNA and RNA, which are manufactured as the numbers of yeast cells increase during fermentation. Beers, in particular, have relatively high levels of purines, the catabolic products of which are associated with certain health issues, particularly gout, which exacerbates with increased levels of uric acid (Yamamoto *et al*., 2005) (Sections 5.3.4 and 5.5.5). Some of the more refined beverages, such as spirits that are produced through distillation of fermented products (Part 3), tend to have greater alcohol content and reduced nutritional value than the less refined products of simple fermentation, such as beers, ciders and wines. Importantly, because alcohol is concentrated by distillation, lower volumes of intake of spirits, liqueurs and fortified wines are needed to provide the same alcohol load as beverages with lower levels of alcohol.

Alcoholic beverages that are essentially finished may still be exposed to further dynamic chemical processes, resulting in some derivatives that may be detrimental to the taste and character of the alcoholic beverage or, indeed, other derivatives that are perceived to enhance its character, and it is often the balance of these processes that is critical (Parts 2 and 3). The effects of such 'ageing' of particular products are valued to such an extent that substantial resources are allocated to their manufacture and storage, the conditions of which may need to be very precise to reproduce the character and consistency required. However, the extent

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

to which these processes may be of tangible benefit or detriment to the nutritional value of such alcoholic beverages is not known with absolute certainty. The reactions occurring during ageing may produce, for example, derivatives of reducing sugars and amino acids, which have potential nutritional value, although the amounts involved are likely to be nutritionally insignificant because such compounds are not considered to be 'essential.' Essential nutrients are those that the body cannot produce endogenously and so their absence in sufficient quantities in the diet could lead to specific deficiency diseases. A number of derived compounds such as acetate and ethyl esters, furanic ethers, Maillard products and carbonyls contribute considerably to a wide variation of complex tastes and aromas, along with alterations in color (Section 2.6.2). These, and a multitude of other reactions, such as the oxidation of higher alcohols or lipids (derived from plant cell walls), hydrolysis of yeast derived esters and degradation of numerous compounds, may occur to a greater or lesser extent in many alcoholic beverages, depending on the length and conditions of ageing. For example, reactions such as degradation of hop bitter acids may occur in beers, especially in some speciality or seasonal beers that may be allowed to age for many months in casks before being tapped or, in some beers, perhaps undergoing secondary fermentation (e.g. Vanderhaegen *et al*., 2003; 2007) (Section 2.6.7). However, apart from increasing the alcohol content and decreasing the fermentable sugar content during secondary fermentation, the relatively significant changes in taste and character potentially created by reactions of this type are unlikely to be reflected in the overall nutritional value of the alcoholic beverage concerned. Such chemical reactions would only be of any real nutritional significance if the resultant products were essential nutrients. This is especially true if the contribution of the beverage to the classes of nutrients involved is relatively insignificant when compared to other dietary sources.

In contrast, certain changes that can occur during ageing may be nutritionally significant, which, again, can be either detrimental or beneficial, or perhaps both. Oxidation of many compounds can occur in alcoholic beverages stored in wooden casks, e.g. oak (see Section 2.9.5). Such casks allow some alcohol to escape and air, particularly oxygen, to be drawn in to replace it in a very gradual process, and so oxidation of some constituent compounds may occur. Once these beverages are bottled, oxidation reactions essentially cease or are very slow (see Section 2.6.12). Storage in wooden casks, especially charred casks, enables polyphenols, furans and other potentially beneficial compounds to be drawn out of the wood, which contributes to the character of the beverage, as well as being of possible nutritional value because many of these compounds have important bioactive properties. Some furans and other components may have antioxidant, anti-inflammatory and anticancer potential but, importantly, some may actually be carcinogenic (Goldberg *et al.*, 1999). Certain of the larger and more complex phenolic compounds, for example the tannins, may undergo precipitation, especially during long periods of storage. If any of these larger and higher capacity antioxidant bioactives are ingested they may cause precipitation of certain proteins affecting the bioavailability of ingested proteins and denaturing important digestive enzymes in the gastrointestinal tract to perhaps impair the digestion and assimilation of other key nutrients (Bravo, 1998).

Whatever the production method and style of the finished alcoholic beverage, an even greater range of styles may be created by the addition of mixers for cocktails. Fruit juices, milk and egg products are used to create cocktails that may add significant nutritional value to the basic beverage. For example, wines may have orange juice added to make Sangria and vodka may have tomato juice added to make a 'Bloody Mary.' Such mixers may contribute significant levels of vitamins, minerals and antioxidants that are otherwise absent from the foundation beverage itself or in concentrations that are too low to be of any nutritional value. In addition, the relative alcohol concentration is reduced according to the proportion of mixer added.

As actual production techniques of the multifarious alcoholic beverages are presented in the other Parts in this book, specific to each type and style of beverage, only appropriate details concerning processes that have particular influences on nutrient content are presented in the rest of this chapter.

5.2.1 Wines

Wine can be made from a multitude of different fruits and vegetables, but grapes are generally preferred especially for large-scale production and for which there is a long history (Soleas *et al.*, 1997). The variable nutrient content of wine types, and individual products within each type, is due to differences in the grape and other ingredients and manufacture within quite a broad spectrum of production processes (see Chapter 2.9). The harvesting of the grape is a process that can influence greatly the character, quality and nutritional content of the finished wine. Harvesting depends largely on the wine growing region. Some practices rely on the use of hand selection of the appropriate quality and pureness of grape, whereas other regions rely on mechanical harvesting machines that take all grapes on the vine (ripe, unripe and some possibly contaminated with mold and rot that can compromise the quality of the finished product) along with extraneous material, such as stems and leaves. Greater amounts of tannins, for example, may be available from the less selectively harvested sources of grape. In essence, alcohol in wines is produced by a period of primary fermentation of carbohydrates present in the juice, or must, of the fruit, which are predominantly fermentable simple sugars such as glucose and fructose, although additional extraneous glucose or sucrose (a dimer of fructose and glucose) may be added. Some sugars are allowed to remain for sweetness if desired in certain wines, but only very small amounts are present, if any, in finished dry wines and so the caloric value of dry wines is predominantly due to the alcohol content (29 kJ/g or 7 kcal/g). The sweeter wines, containing more free sugars than the dry wines, may contribute significantly to caloric intake (Section 5.3.3) due to significant levels of both sugars (16 kJ/g or 3.75 kcal/g) and alcohol (29 kJ/g or 7 kcal/g). The more lengthy secondary fermentation is the process whereby proteins are broken down and fine particles precipitate out to allow clear wine to be bottled and, therefore, negligible protein content usually remains in the finished product.

Certain key ingredients of grapes contribute to the nutrient value of the finished wine. Informative details of the typical nutrient content of grapes and other fruits may be found at the website of the National Agricultural Library (NAL) at the United States Department of Agriculture (USDA): http://www.nal.usda.gov/. Grapes may typically contain about 15 g/100 g of sugars (7 g glucose, 8 g fructose) that ferment into alcohol, usually the main source of the energy content of wines. Complex carbohydrates mainly found in the skins of grapes may be almost entirely removed during production, especially if just the juice is used, but some may remain in those wines that ferment the whole fruit. Many nutritionally valuable elements also remain in the final product (Soleas *et al.*, 1997), where some may be found in even higher concentrations than in the ingredients themselves. The concentration of potassium is relatively high in both grape juice and wine (typically 130 mg/100 ml and 190 mg/100 ml, respectively) compared to that of sodium (3 mg/100 ml and 4 mg/100 ml, respectively). However, some vitamins, such as vitamin C (about 10 mg/100 g grapes), may be largely destroyed during processing and storage, whereas others, the B vitamins, for example, are also produced by the action of yeast and may be present in significant quantities in the final product.

The typical nutrient content of wines generally may be compared to other alcoholic beverages in readily accessible sources such as such as Table 2.13 in the Food Standards Agency publication, *McCance and Widdowson's, The Composition of Foods* (Food Standards Agency, 2002) or at websites such as NutritionData, http://www.nutritiondata.com/. It is uncertain as to whether or not many of the micronutrients from wine can make a significant contribution to nutritional status and health, especially at higher levels of alcohol consumption because, although drinking wine potentially makes available greater amounts of such essential nutrients, relatively high levels of alcohol intake from wine, as for other alcoholic beverages, may increase nutrient requirements. This is because alcohol intake interferes with normal metabolic processes involving key nutrients (Leiber, 2000; Watson and Preedy, 2003) (Sections 5.5.4 and 5.5.5), and differentially affects appetite and behavior to alter intake of these nutrients from the usual diet, according to acute or chronic

use, and low to moderate or excessive consumption (Forsander, 1998; Yeomans *et al.*, 2003; Yeomans, 2004; Caton *et al.*, 2007; Kokavec, 2008) (Section 5.5.3).

Red wines (Section 2.9.2) are produced from the entire grape, with the juice providing the fermentable sugars from which the alcohol is derived; whereas the skins essentially impart the color, and the skins, seeds and stalks are the source of the phenolic compounds that are purported to have antioxidant properties and are, therefore, considered to be of tangible benefit to health. Phenolic compounds are the substances that produce the intensity of color in fruit and are found in especially large quantities in berry fruits (Hancock *et al.*, 2007), many of which are used in wine making. The more intense colors have, therefore, been implicated in the greater purported health benefits of red wines, which have about five times the level of these compounds compared to white wines (German and Walzem, 2000). Tannins, for example, add astringency to red wines but, with ageing, may form polymers that settle out. The alcohol content of most red wines is typically about 12–14% ABV.

White wines (Section 2.9.1), produced from the juice of the grape, tend to have slightly less alcohol content than red wine, but some may have more free carbohydrates as a result, although dry white wines and red wines are generally comparable in this respect. To reiterate, because whole grapes are not usually used in white wine production (or, if the whole grape is used, contact with the skin of the grape and its disruption is minimized) there are lower levels of phenolic compounds, perhaps only about one fifth on average of red wines (German and Walzem, 2000), and, therefore, the level of contribution of white wines to health overall is currently uncertain. In fermentation processes where the skin of the grape is allowed to remain, far greater levels of antioxidants may be observed in the finished wine (Fuhrman *et al.*, 2001).

Rosé wines (Section 2.9.2) are produced by three variations of the basic wine fermentation techniques in which: (a) the skins of the red grape may be left in contact with the juice for a short period only, (b) some of the red juice may be removed at an early stage and then fermented separately and (c) red wine may be blended with white wine. The nutrient content may be slightly modified accordingly, perhaps producing something that is between red and white wines in terms of alcohol content and also phenolic compounds, the levels of which bear a relationship to the intensity of color (Hancock *et al.*, 2007).

Traditional sparkling wines and champagnes (Section 2.9.3) are essentially produced from still wines with an additional fermentation process in the bottle that traps the carbon dioxide formed as alcohol is produced. In general, this process seems to have no real significance in terms of beneficial or detrimental changes in nutritional content and value compared to the original fermentation product.

Sweet or dessert wines may be produced by, essentially, one of four processes: the late harvesting of grapes to ensure greater residual sugar and sweetness, the addition of grape juice after fermentation, the freezing of grapes to concentrate the sugars and the deactivation of yeast before fermentation is complete. The nutrient content of sweet wines produced by these various processes may, therefore, be quite variable, with higher levels of free sugars potentially contributing significantly to their calorie content.

Fruit (and vegetable) wines (Chapter 2.11), of which grapes are a specific example, may be produced from almost all types of suitable fruit (and vegetables), with the nutrient content essentially depending on variations in the raw ingredients and production processes. For example, wines made with highly colored berries may have greater amounts of antioxidants than other fruit or vegetable wines; berries have been dubbed, rightly or wrongly, 'super fruits' and the color of the final product may be representative of the concentration of antioxidants present (Hancock *et al.*, 2007).

5.2.2 Beers

Beers are produced from cereals, most typically malted barley, although unmalted grains, corn and rice, are also used for brewing beers in many regions of the world. The complex carbohydrates contained in malted

barley, mainly starch and β-glucans, are broken down during mashing into simple fermentable sugars by the enzymes α-amylase and β-amylase that are produced during the initial process of malting (sprouting) of the grains (Section 2.6.2) (Hornsey, 1999). Proteins may be broken down into smaller peptides and amino acids by activated proteinases to help to make the beer clearer. However, some proteins are allowed to remain as these are necessary in order to hold the head of the beer. The relatively low levels of protein in beer do not seem to contribute greatly to the recommended levels of protein intake for optimum nutrition as the amount in one unit (half pint) might only be somewhere about 3% of the daily requirement for protein (Section 5.3.4.). At the stage when the separated wort is boiled with hops (Section 2.6.3), other ingredients such as herbs and fruits may be added for character and flavor, and these may also influence the nutrient content of the beer. Similarly to wines, the various ingredients and processes involved in beer production ensure a large variation in nutrient content, as well as alcohol levels, and these may also be modified by secondary fermentation phases. In general, beers these days are quite different from the crude, less processed, brews consumed earlier in history that may have contained more protein, vitamins and minerals, perhaps with a more cloudy appearance. However, certain local or specialized brews still retain such characteristics, sorghum beers produced in sub-Saharan Africa, for example (Section 2.7.2).

The source of water used in beer production is an extremely important factor as water is the largest component in the finished beer and is a determinant of the type of beer that is best brewed from a particular water supply (Section 2.6.2). Making an important contribution to flavor, the water supply is also the source of various elements and electrolytes that are essential for optimum nutrition, with significant amounts of minerals also present in other raw ingredients such as malt. Compared to soft water, some hard waters may contain very approximately 100 times the concentration of sulfate (typically perhaps 5 mg/l in soft water versus 500 mg/l in hard water), with far greater concentrations in hard than soft waters of calcium (10 mg/l versus 240 mg/l), magnesium (2 mg/l versus 50 mg/l), bicarbonate (15 mg/l versus 250 mg/l), nitrate (5 mg/l versus 240 mg/l) and chloride (5 mg/l versus 50 mg/l). As chloride may be added to supplies of drinking water, this may influence the character of beer produced in many regions. Water may be modified by the deliberate addition of salts in order to produce certain desirable characteristics that are intended to be reminiscent of beers produced from the characteristic water of other regions. Calcium sulfate is often added in a process known as 'Burtonization,' which is intended to mimic the water of the Burton-on-Trent area of the UK, a region in which the water is naturally high in sulfates (Section 2.6.2). Sulfates optimize production of the lighter beers, which have significantly greater concentrations of calcium and sulfates in the final product than darker beers. Calcium has potentially important affects on the nutritional content of beers because it precipitates phosphates, creating lower acidity and thus modifying enzyme activity, promoting protein and oxalate precipitation, and preventing uptake of certain silicates, tannins and polyphenols. If excessive bicarbonate is present, greater acidification may be achieved by the addition of sulfate, chloride, phosphate or lactate ions, all of which may affect electrolyte content of the final product. The concentrations of other elements and electrolytes vary considerably amongst the different finished beers, depending on raw ingredients and production processes. Most beers contain relatively greater amounts of potassium and lower amounts of sodium, with the purported health benefits associated with this balance favoring potassium, especially the supposed promotion of cardiovascular health through mechanisms that might help lower blood pressure, or at least not contribute to raised pressure. Any such health benefits may also depend on the relative amounts present of potassium, calcium and magnesium, as potassium appears to act effectively in synergism with these particular elements, as well as independently (Rylander, 2003). Other beneficial trace elements found in beers include magnesium, chromium, iron, silicon and copper and there are also very few potentially toxic trace elements usually present in the finished beer. However, alcohol consumption may influence the rate of loss of important nutrients through the kidneys (Rylander, 2003).

In addition to providing suitable amounts of fermentable carbohydrates, malt is a significant source of other, more complex, carbohydrates that have potential health benefits due to their relatively low rates

of digestion and assimilation in the body. Some of these complex molecules are only digestible by the bacteria of the large intestine that produce simple derivatives such as volatile fatty acids that can be absorbed and metabolized. Indeed, butyrate is an acknowledged source of metabolic energy for the cells of the gut wall (Cummings and Macfarlane, 1997). Some other complex carbohydrates found in beers are essentially indigestible contributors to the fiber content of the diet, passing unchanged from the body. In general, if processing is kept to a minimum and as the alcohol content of beers is increased, so the fiber content may also be raised (Section 5.7.4). A very strong beer approaching 11–12% ABV may contain up to about 6 g fiber/l, which could be about one third of the daily amount of fiber considered adequate for optimum health in the UK, which is currently 18 g/day (British Nutrition Foundation: http://www.nutrition.org.uk; Food Standards Agency:http://www.food.gov.uk/healthiereating).

The grains used for beer production contain many potentially valuable nutrients, including silicates that are under scrutiny at present regarding nutritional importance (Section 5.4.2), and the malting process produces a number of water soluble vitamins that contribute to the essential requirements of the human diet (British Nutrition Foundation: http://www.nutrition.org.uk; Food Standards Agency: http://www.food.gov.uk/healthiereating). The process of malting also influences antioxidant capacity (Lu *et al.*, 2007). The production of a number of beers, e.g. dark milds and stouts, is based on roasted barley which results in an altered composition of the roasted grain compared to the original grain and also leads to modified nutrient content of the final product, notably phenolic antioxidants and furans (Section 2.6.2).

Although hops are added during the production of beer in order to provide bitterness, aromas and preservative qualities, they contain a number of potentially significant functional nutrients that may be infused into the finished beer, but not all of the these particular phytochemicals are considered to be exclusively beneficial or desirable (Milligan *et al.*, 2000). Indeed, hops have been used in herbal remedies over the centuries, long before their use in beer production. Certain vitamins, such as vitamins A, thiamine (vitamin B1), riboflavin (B2), niacin (B3), cyanocobalamin (B12), folate and vitamin C, and elements, such as calcium, magnesium, manganese, potassium, selenium and zinc, are found in hops, but whether or not hops are nutritionally significant sources of these nutrients in beer is uncertain, as they may also be provided in considerable amounts by the other raw ingredients, especially malt, and by the action of yeast. In particular, the use of live yeast cells to produce alcohol also yields significant levels of vitamins, especially those members of the B group such as thiamine (although yeast also utilizes B1 for optimum fermentation), riboflavin, pyridoxine and folate (Sections 2.2.9 and 2.6.4). As the yeast culture grows and the cells divide, more DNA and RNA are formed, which contain the nucleotide purines and pyrimidines, of which the purines are associated with certain health problems such as gout (Yamamoto *et al.*, 2005). However, hops contain other potential health promoting constituents, including some phytoestrogens (plant derived compounds with estrogenic activity, some of which may not be entirely desirable; Milligan *et al.*, 2000) (Section 5.11.2), phenolics and α- and β-acids. Although similar compounds may be present in wines, a number of these potentially health promoting hop and grain derived phytochemicals are present only in beer, but whether or not they can make significant contribution to nutritional status is still somewhat speculative and uncertain. The phytochemicals in beer, derived from both the malt and hops, include a number of phenolic compounds, including monophenols (phenolic acids, alcohols and amines) at typical levels of 10–30 mg/l, monomeric polyphenols (flavanols and flavonols) at up to about 25 mg/l and condensed polyphenols (di- and polymeric catechin, proanthocyanidins and prodelphinidins) at about 20–140 mg/l (for more details see Section 5.4.3; the generally informative chapter by Baxter and Hughes, 2001, specifically page 109; Denke, 2000). Many of these are potentially beneficial bioactive compounds that have been shown to lower plasma lipids and raise plasma antioxidants (e.g. Gorinstein *et al.*, 2007).

Yeast requires certain key nutrients for growth and optimum fermentation potential, most of which are provided by the water, malt and hops. However, zinc is of particular importance to the brewing process as it is not generally present in sufficiently high quantities in the basic ingredients, especially malt, for the

optimum functioning of yeast (Vecseri-Hegyes *et al.*, 2005; 2006). Inadequate amounts of zinc may introduce complications, including unwanted side products of fermentation adversely affecting the character of the finished beer. Therefore, zinc is often added to overcome this limitation. However, much of this exogenous zinc may be taken up by the yeast and so does not appear in the final product because yeast is largely separated out as it may settle or be filtered.

Processing may also destroy or remove some vitamins, and some minerals and other nutrients may be removed by precipitation and so are not present in such large quantities in the final product. Vitamin C is highly susceptible to processing, with most being lost, but other members of the water soluble vitamins may be preserved at significant levels despite some losses.

5.2.3 Cider and Perry

Cider (from apples) and *perry* (pears) are produced by taking the juice from the pressed fruit and fermenting to near completion before racking into another vessel for a second fermentation. The second fermentation utilizes the sugars remaining from the first fermentation or may have supplementary sugars added from which additional alcohol is produced (Chapter 2.8). Apples and pears are relatively good sources of a number of important nutrients, including the B vitamins, elements and electrolytes, significant amounts of which may be found in the finished product, depending on the extent of processing. Traditional cider production is generally a relatively crude procedure involving little by way of simple separation of insoluble and spent ingredients by racking and filtering (Section 2.8.5). Therefore, the skins of the fruit are in contact with the fermentation medium for sufficient time to allow extraction of some of the antioxidants that are present in relatively large amounts in the skins, quercetin for example. As such, much of the original raw material may remain in the final product, depending on the character required with some finished ciders allowed to remain 'cloudy.' Therefore, significant amounts of the original nutritional value of the apple are retained along with production of other nutrients through fermentation. Other ciders are produced for appeal to the 'popular' market and further processing may be applied to increase clarity, for example, and, of course, such processes may alter nutritional content substantially. Therefore, although significant levels of certain water soluble vitamins and phenolic compounds may be present in many of these products, there is great variability as some of the more highly processed ciders have low concentrations of such compounds (e.g. Marks *et al.*, 2007). Alcohol levels in ciders vary considerably from about 3% ABV to about 8% ABV, typically, and possibly more in some specialist products.

5.2.4 Distilled Beverages

Distillation processes (Part 3) are intended to concentrate alcohol at the expense of water and to increase preservative and sterile properties, as well as to improve ease of storage and transport of the beverage. Distillation may be repeated one or more times depending on required alcoholic strength and desired character of the beverage. Distillation also removes some of the other nonvolatile components of the initial fermentation products such as some potential toxins (e.g. pesticide residues) and certain plant derived immunochemicals (e.g. gluten or hordein). Essentially, distillation is a three stage process in which the first part (the head or foreshot), containing compounds such as ethyl acetate and ethyl lactate, and the last part (the tail), containing compounds such as fusel oils, are mostly discarded, with small amounts of some of these compounds retained out of choice for particular character, taste and aroma. However, most of the potentially beneficial nutrients present in the fermentation product may also be lost during distillation, including carbohydrates, elements, electrolytes and vitamins. Distillation also causes the formation of certain aromatic compounds and the

breakdown of others that might change the character of the product, but these are unlikely to have any real nutritional significance because they are usually present in relatively low concentrations.

Following distillation, the ageing processes, which usually occur in wooden casks (e.g. oak, pine) and are intended to impart character to the beverage, may also add small amounts of potential nutrients and other compounds (Goldberg *et al.*, 1999; Madrera *et al.*, 2003), some of which may be carcinogenic. Interactions with the casks enable chemical modifications of the spirit to potentially produce any of about 300 or more compounds for improved taste and character; some of these are also purported to be functional trace nutrients, such as the vast array of phenolic compounds and furans, especially those produced in charred wooden casks (see for example Section 3.2.5). It is uncertain as to whether or not these particular nutrients, although having very strong specific antioxidant potential, are in sufficient quantities to be of any major nutritional value. Although the presence of methanol and some other by-products of the fermentation process may actively be discouraged to avoid undesired characteristics, certain levels of fusel oils are considered necessary for tastes and aromas that are characteristic of individual spirits and liqueurs, although they are not usually considered to be acceptable components of the diet. As any sugars remain in the still as alcohol is concentrated in the distilled beverage, the metabolic energy content of the final product of distillation predominantly depends on its alcohol content (29 kJ/g – 7 kcal/g) producing 229 kJ (55 kcal) per regular serving of 25 ml (10 ml or 7.89 g pure alcohol), unless sugars are infused at any stage in order to produce liqueurs, some of the sweeter versions of which may contain very high levels (Chapter 3.9). The final alcohol content of the distilled beverage may be denoted as alcoholic 'proof' which is a figure that is almost double (1.75×) that of the value of the ABV (%).

Spirits are produced by distillation of the fermentation products of grains, fruits and vegetables and, typically, they contain at least 35% (ABV) alcohol and have negligible sugar content. The first distillation may result in a product of about 25–30% ABV (Section 3.1.2). A second distillation stage is normally applied to purify the beverage by removal of toxic contaminants such as urethane (ethyl carbamate or EC – see Section 5.11.5) and many of the fusel oils (higher alcohols), the latter of which may be selectively distilled and allowed to remain, depending on the character required for the final product. Water may be added back to the product, which at this stage may be about 60–70% alcohol (ABV) (Section 3.1.2) in order to achieve a spirit of 40% ABV. The character and potential nutritional value of spirits is, therefore, reliant on the initial ingredients, such as the type of malt used for whisky, the type of still used, the source of water and the type of wood used for the barrel in which spirits may be aged. Spirits may be used as a base for formulating numerous cocktails (Section 5.2.7). As there are such vast numbers and varieties of other miscellaneous spirits consumed worldwide, and also because their nutritional value per se is fairly limited, only a few of the spirits more commonly consumed in the UK are considered further here.

Varieties of *whisky* (Scotch) (Chapter 3.2) and *whiskey* (Irish) (Section 3.3.2) and, indeed, those from other countries such as Bourbon from the USA (Section 3.3.4), may be produced from a number of grains, notably barley or malted barley, maize (corn), rye or wheat, and once distilled, the storage in oak casks for a minimum of three years allows for ageing and the natural infusion of small amounts of the potentially nutritionally functional phenolics and furans, although some such compounds may have toxic or carcinogenic potential (Goldberg *et al.*, 1999).

Brandy is the distilled product of grape fermentation (Chapter 3.6), with other fruit, and pomace (grape pulp), brandies usually being prefixed by the specific name of the fruit, e.g. cherry brandy (Chapter 3.8). The extent of ageing in wood determines the special character of brandy that distinguishes it from some clear fruit brandies which lack such ageing processes from which further key nutrients may be obtained. Certain 'old' brandies are aged for up to 10 years or more, which may significantly modify the character of the final product, as well as the micronutrient content, i.e. by allowing the further gradual uptake of polyphenols from the casks into the brandy.

Distilled from fermented molasses and the juice of sugar cane, *rum* (Section 3.5.2) is initially clear and then is infused with color and character by ageing in wooden casks and from which it may also gain small amounts of potentially functional nutrients and possibly some toxins. There are many variations in the production of rum in terms of the level of alcohol and the ageing processes that may result in differential nutrition values according to the specific product.

Vodka (Section 3.4.3) is a distilled beverage produced from any of a large number of fermented raw materials, but predominantly grains, potatoes and sugar beet are the generally accepted standard ingredients. The extended distillation and filtering processes with no period of ageing ensure that vodka is fairly pure mix of alcohol and water with only very small amounts present of impurities, with possible flavorings added during production, thereby limiting its nutritional value to the energy provided by its alcohol content alone. Flavorings or mixers added later may have some nutritional value – fruit juices, for example, may provide significant levels of water soluble vitamins, elements and electrolytes. Vodka produced illegally ('bathtub' or black market production) often contains higher levels of toxic compounds such as methanol that can lead to cirrhosis and blindness if ingested in sufficient quantities, and death can result in extreme circumstances (Section 5.5.2).

There are a number of types of *gin* (Section 3.4.2) that are available, depending on ingredients and processing. Juniper berries, the usual flavoring of gin, may be added both before and after distillation of the white grain spirit fermented base. Other fruits and herbs may also be used in addition to juniper berries or certain ingredients may be used instead; the addition of sloe berries, for example. The relatively high alcohol content in gin provides a significant amount of energy, otherwise any nutritional value is usually quite limited, but with some possible variation as nutrients essentially originate from the added fruit or herbs only.

Tequila (Section 3.5.5) is distilled from fermented blue agave and is aged in wooden casks for varying amounts of time to produce many different types. Taken neat in Mexico, it may be accompanied by sangrita, a mix of fruit juices and hot chilies or sauces to give possible added nutritive value. Elsewhere, Tequila may be taken with salt and lime, or other accompaniments, mainly for modifying effects and flavor, but which may also provide certain additional nutrients, but these are probably only of limited significance.

Liqueurs (or cordials) are distilled alcoholic beverages that have additional flavorings and also typically have relatively high sugar contents (Chapter 3.9). The potentially high levels of simple sugars along with the high levels of alcohol in liqueurs indicates that the majority are significant sources of empty calories, essentially, unless they are mixed with other beverages or ingredients that have some nutritional value, such as fruit juices or cream. For example, Irish Cream is a liqueur produced from whiskey, cream, coffee and other ingredients; a single serving of 40 ml Irish Cream provides about 544 kJ (130 kcal) metabolic energy from 5–6 g of alcohol (146–176 kJ or 35–42 kcal), 8 g of carbohydrates (134 kJ or 32 kcal), 5–6 g of fat (188–226 kJ or 45–54 kcal) and 1 g of protein (16 kJ or 4 kcal).

5.2.5 Fortified wines

Fortified wines (Chapter 2.10) are wines that have spirits added to increase the alcohol content above that made possible by yeast fermentation, because the activity of yeast is significantly reduced by high alcohol concentrations; at such levels the yeast cells themselves may actually be killed. Examples of fortified wines include *Sherry*, *Port, Madeira* and *vermouth*. *Sherries* (Section 2.10.2) are wines fortified with brandy after completion of fermentation and so are usually dry unless sweet flavorings are added to make the fairly popular sweet Sherry style. *Ports* (Section 2.10.7) are wines fortified with brandy during fermentation in

order to stop the process and leave some sugars unfermented, making Port wines relatively sweet. *Madeira* (Section 2.10.5) wines are fortified, but are also exposed to higher temperatures during storage to influence the character of the beverage, and may be exposed to the oxidizing actions of air to alter their character, but with little effect nutritionally. *Vermouths* (Section 2.12.2) are imparted with the flavors of aromatic herbs and spices and contain varying amounts of sugars according to sweetness required, creating extra dry, bianco and sweet red (colored with caramel), with an alcohol content of about 16%. Therefore, the nutritional value of fortified wines is variable, being clearly dependent on many factors. As an example, a 50 ml serving of sweet Sherry might contain about 285 kJ (68 kcal) metabolic energy, provided mainly by about 7–8 g (205–234 kJ or 49–56 kcal) alcohol and 3.5 g (59 kJ or 14 kcal) carbohydrate, with a very small contribution from protein.

5.2.6 Alcopops

Alcopops (Section 1.3.9) are a relatively new fashionable way of targetting alcoholic beverages at specific markets, especially the younger age groups of consumers. Usually of between about 4% and 7% ABV and typically sold in measures of about one third of a litre (330 ml in Europe and 12 fluid ounces in the USA), alcopops have slightly different origins in Europe and the USA. In Europe, alcopops are mostly produced from a spirit base, such as vodka or white rum, to which may be added simple sugars, flavorings and colorings. In the USA, alcopops are essentially flavored malt alcoholic beverages that have been concocted from a fermented base product which has had all of its fermented malt character artificially removed, followed by the addition of spirits to provide the increased alcohol content, along with simple sugars, flavors and colors. Other alcopops are produced from various wines, also with sugars, flavorings and colors added in various amounts. A slightly more recent trend is for the addition of stimulants, such as caffeine, which are usually of negligible nutritional value. In essence, alcopops have little, if any, nutrient value beyond their 'empty calorie' content derived from alcohol and simple sugars. However, although some of the mixers used to formulate alcopops for flavor and color also have negligible nutritive value, if any, others, such as fruit juices, potentially containing significant levels of vitamins and other trace nutrients, or milk products with some protein, lipids and trace nutrients, may make some small contribution towards nutritional requirements.

5.2.7 Cocktails

Cocktails are multifarious alcoholic beverages made usually from any of a large number of distilled beverages in combination with one or more flavored mixers. Accordingly, the nutritional content of cocktails depends on the amount and nature of both the distilled beverage base and the mixer. Obviously, although the alcohol concentration may be less than the constituent spirit or liqueur if the mixer has lower or zero alcohol concentration, consumption of the whole drink provides the same total amount of alcohol as the 'shot' of spirit (or standard amount added). Liqueurs may also provide a relatively greater amount of simple sugars. Alcohol and simple sugars provide energy, but nothing else of nutritional value. However, many mixers used in cocktails may make a significant nutritional contribution to the cocktail; others may not. Fruit juices, such as the popular orange juice mixer, may provide some important vitamins (especially vitamin C), elements and electrolytes; see *McCance and Widdowson's, The Composition of Foods* (Food Standards Agency, 2002); the website of the National Agricultural Library (NAL) at the United States Department of Agriculture (USDA): http://www.nal.usda.gov/. However, it should be recognized that fruit squashes (unless supplemented) may

provide fruit flavorings only. Cream may be added to create certain cocktails, which are then the source of some lipids and fat soluble vitamins, but these probably have little real nutritional impact. Protein may be added in the form of eggs or milk, for example (Section 3.9.5).

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5.3

Macronutrient Content of Alcoholic Beverages

From the previous section, it can be seen that the various production methods involved may modify the nutrient content of any particular alcoholic beverage to be substantially different from the constituents of the original ingredients. Therefore, although nutrients contained in the ingredients may be known with some accuracy, chemical analysis of the final product is essential in order to ascertain the exact nutrient content of the various alcoholic beverages. Apart from alcohol concentration, which is required particularly for commercial and legal reasons, analyses of other constituents have been performed sporadically and often limited to specific nutrients according to the remit of the analyst involved, perhaps for commercial or research purposes. Therefore, although information regarding individual or groups of particular constituents in specific beverages may be obtainable from the literature, there is relatively little easily accessible reference information available for comparison of the entire nutrient content of alcoholic beverages, as determined by chemical analysis. A certain amount of relevant information has been collated by a relatively small number of authorities making accessible some summary information for the most commonly consumed styles of alcoholic beverage, for example in *McCance and Widdowson's The Composition of Foods* (Food Standards Agency, 2002), and other such sources include specialist websites (e.g. NutritionData, http://www.nutritiondata.com/). However, although generally applicable to other products within the same type, nutrient content may also vary considerably between alcoholic drinks that are otherwise very similar in style.

The potential nutrient intake or the nutrient load from alcoholic beverage consumption depends not only on the nutrient content, but also on the amounts likely to be consumed. Therefore, in order to avoid possible confusion, it is very important to recognize that nutritional information regarding individual products, styles or classes of alcoholic beverages may be presented as quantities present in different serving sizes (e.g. wine as 125 ml, 175 ml or 250 ml glass) and as concentrations (e.g. g/l or g/100g), or even as percentage of the daily recommended dietary intake per serving (e.g. 6%). Information on many nutrients is not consistently available. For example, values for individual free amino acids and free fatty acids are available for only a relatively few products, bearing in mind the vast numbers of beverages on the market, and often these simply represent typical values for generic classes of alcoholic beverages (see *McCance and Widdowson's The Composition of Foods* – Food Standards Agency, 2002; and NutritionData, http://www.nutritiondata.com/). Clearly, the vast range and complexity of alcoholic beverages precludes the presentation of the exact nutrient content of all possible styles and types. Table 5.3.1 provides typical values for water, alcohol and the macronutrient

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

Table 5.3.1 *Typical macronutrient content of the major generic classes of alcoholic beverage commonly consumed in the UK**

	Nutrient Content†‡					
	Wine		Beer		Spirit (40% ABV)§	
	/125 ml	/liter	/284 ml	/liter	/25 ml	/liter
Water (g)	100–114	810–910	260–270	920–950	17	680
Alcohol (g)	10–14	80–110	8–14	30–50	8	320
Carbohydrates**						
Total (g)	0.25–7.5	2.0–60	4–12	15–37	Trace	Trace
Free sugars (g)	0.25–7.5	2.0–60	Trace–8.5	Trace–30	Trace	Trace
Fiber (g)	Trace	Trace	Trace–2.8	Trace–10	Trace	Trace
Nitrogenous matter – proteins, amino acids, nucleotides etc. (g)	0.02–0.4	0.2–3.0	0.6–1.7	2.0–6.0	Trace–0.08	Trace–3.0
Lipid (g)††	Trace	Trace	Trace	Trace	Trace	Trace
Caloric value (kJ)	335–523	2678–4184	335–536	1172–1883	234	9373
(kcal)	(80–125)	(640–1000)	(80–128)	(280–450)	(56)	(2240)

*Typical guideline values are presented, drawn from a large number of sources considered to represent the range of each generic product most usually consumed in the UK (e.g. *McCance and Widdowson's The Composition of Foods* – Food Standards Agency, 2002). For comparative purposes, values are presented per serving of 125 ml wine, 284 ml (half pint) beer and 25 ml spirits, and also per liter. Although these volumes were originally considered to represent approximately one unit of alcohol, regular wines range in alcohol content from about 8% alcohol by volume (ABV) for some white wines, up to about 14% ABV, or more, for some red wines, and beers from about 3% ABV for some light beers and mild ales up to about 6% ABV, and more, for strong ales and barley wines.

†As well as the nutritionally significant micronutrient content, there are residual components of the production of alcoholic beverages that have no known nutritional value, and some may even be toxic but, in general, these compounds appear to make negligible contribution: e.g. during analysis of regular wines and beers less than 0.5g/serving may be specifically identified as 'ash' or nonspecific residues.

‡Wherever the value indicated is 'trace,' negligible or low amounts may be present that could influence the character of the beverage but that may be insignificant in nutritional terms.

§Spirits of 40% ABV (about 80% proof) are presented for comparison.

**Carbohydrate levels in alcoholic beverages may be very variable indeed, according to desired sweetness. Dessert wines, in particular, may have sweetness of character due to specific production techniques and liqueurs are, essentially, spirits that have sugar infusions according to taste required.

††Trace lipids in alcoholic beverages are derived from plant cell wall material.

(carbohydrates, proteins and lipids – or fats) content of the major classes of commonly consumed alcoholic beverage. However, it is important to be aware that the alcohol and nutrient content for individual products within each class and style of alcoholic beverage may occasionally extend far beyond the typical range presented. The macronutrients present in these major classes of beverage and their biological function are described individually in the following sections. The relative importance, in terms of absolute amounts likely to be provided of the various nutrients, that is the nutrient load, towards nutritional requirements, will also be appropriately considered. For information regarding key vitamins and minerals, see the micronutrient content of alcoholic beverages (Chapter 5.4).

Alcohol content is usually a significant determinant of how much of a particular alcoholic beverage will be consumed and, therefore, the contribution that its total intake is likely to have on dietary energy intake overall. Current recommendations are that alcohol should provide no more than about 5% of total dietary

energy intake, which should otherwise be provided by a balance of the macronutrient sources of dietary energy (carbohydrates, lipids, and proteins) during habitual consumption of food and drink (British Nutrition Foundation – http://www.nutrition.org.uk). Recommendations for a healthy balanced diet for an adult who does not consume alcohol would be expected to provide at least 50% of energy from carbohydrates (of which 11% would derive from free sugars, excluding those free sugars provided by milk), up to 35% from fat (of which 11% is provided by saturated fatty acids and 2% trans fatty acids) and 15% from protein. Therefore, for adults consuming alcohol at 5% of their daily energy intake these figures need to be adjusted slightly, with carbohydrates then representing 47% (10% free sugars excluding milk derived sugars) and fats contributing 33% (10% saturated fatty acids). Currently, the average UK diet generally conforms very closely to these recommendations with the *trans*-fatty acid levels being below recommendations, but saturated fats and nonmilk free sugars both above targets. For more details, see the British Nutrition Foundation website (http://www.nutrition.org.uk) and Department of Health's 'COMA' Report (Department of Health, 1991).

Fermentation products, such as wines, beers and ciders, usually comprise relatively small, but variable, amounts of carbohydrates, and nutritionally insignificant amounts of protein and fats (lipids). Spirits, being distilled products, essentially do not contain carbohydrates, proteins or fats. The addition of mixers to alcoholic beverages modifies the alcohol concentration and carbohydrate content, and may even add lipids or proteins to the beverage, according to content of the mixer; examples of mixers include fruit juices, which add primarily carbohydrates, with some vitamins and minerals (see Micronutrients, Chapter 5.4); cream, predominantly providing calories from lipid; and eggs, that contribute protein to the beverage. In addition to possible health consequences associated with ingestion of alcohol per se, preferential consumption of beverages of higher carbohydrate content may, therefore, have further implications for optimum health in terms of possible acutely raised blood glucose levels and chronic weight gain due to increased caloric intake, from carbohydrate (16 kJ/g or 3.75 kcal/g) as well as alcohol (29 kJ/g or 7 kcal/g).

The principle constituents of a vast majority of alcoholic beverages are water and alcohol and, essentially, these vary with each other inversely according to alcoholic strength; that is, as the proportion of alcohol increases, the proportion of water decreases. The alcohol content of beers, ciders and wines is essentially determined by the amount of fermentable sugars in the original mix and length of time of the fermentation processes, although some additional sugars may be added for a secondary fermentation, e.g. some sparkling wines and some bottled beers. Yeasts commonly used for producing most traditional alcoholic beverages are only capable of working in alcohol concentrations less than about 15% ABV and so alcohol levels do not exceed this value during fermentation. For spirits and liqueurs, the relative amounts of water and alcohol are determined by the extent of distillation, which concentrates the alcohol, with stronger distilled beverages being produced by additional distillation processes. With the exception of the occasionally significant, but very variable amounts of carbohydrates that might be present, other ingredients are usually too small in quantity to significantly affect the relative amounts of alcohol and water in a serving of these usual styles of alcoholic beverages. The addition of mixers, such as fruit juices, reduces the relative alcohol concentration proportionally as water is increased.

5.3.1 Water

Often overlooked in terms of its contribution to nutrition, water is usually the major constituent of any alcoholic beverage, including more than half the content of most spirits. There are multifarious sources of water used for aiding production of the raw ingredients or added during processing that contain a large variety of desirable and beneficial constituents, as well as some undesirable and possibly harmful constituents, many of which could be present in finished alcoholic beverages, depending on the particular processes adopted for

their production. For a comprehensive overview of water regulation in the USA, which is consistent with standards in the UK and Europe, see Tarver (2008). Along with raw ingredients, the source of water used in the production of alcoholic beverages is a determinant of the relative amounts of the various elements that are present in the final product of fermentation. However, further processing techniques, particularly distillation, eliminate much of the water and essentially all the minerals, leaving very small amounts of certain elements such as phosphorus, potassium and sodium, for example, in the final beverage, some of which may originate from elsewhere during continued processing, copper from distillation in copper stills (Sections 3.2.4 and 3.5.3) and as a result of aging, for instance (Section 5.10.3).

Alcoholic beverages may make a significant contribution to water intake, especially those with high water content such as beers. Indeed, to avoid ingesting contaminated water, beer has often been the beverage of choice over many centuries, especially as communities started to grow with people living in closer proximity to each other. During previous times in the UK, 'small beer' may have been consumed regularly by children for intake of water and other nutrients ('small' because of its low alcohol content compared with the stronger beers consumed by men and women, generally). However, despite being the major constituent of most alcoholic beverages, the potential hydration capability of water from these sources may be curtailed to a greater or lesser extent because of the diuretic effects of the alcohol (Kleiner, 1999), which are dependent on the relative amounts of alcohol present in the beverage combined with the quantities consumed. Therefore, additional intake of water may be beneficial at times of high alcohol consumption, especially to accompany spirits and liqueurs.

Essential to life itself and to optimum health and performance, water is a major functional component of the body, contributing very approximately 60% (42 kg) of body weight in a nominal 'reference adult man,' weighing 70 kg and in whom 15% of body weight consists of fat (or lipid), and contributes 55% (33 kg) body weight in a nominal 'reference adult woman,' weighing 60 kg, of which 25% body weight comprises body fat. It is essential that water lost from the body through the kidneys, lungs, skin and intestine is balanced by intake from food and drink to maintain a healthy equilibrium. Water output is extremely variable, and increases with such factors as high environmental temperature and intense physical activity. A typical daily water turnover for a sedentary/moderately active individual living in a fairly temperate region such as the UK may be around 2.5 l (perhaps 2.9 l for men and 2.2 l for women; Kleiner, 1999), which is *equivalent* to about eight (300 ml) glasses of water. However, it is important to understand that that the amount represented by these eight glasses is just that, an equivalent. A significant amount of water is obtained from all drinks, not just glasses of unadulterated water, and a very large proportion is also obtained from food, especially fruit and vegetables, making an average daily intake for the population of about 2.5 l in total. Over recent years the fact that this is equivalent to eight glasses of water has been mistakenly used by some health and fitness practitioners to indicate the need for a daily intake of eight glasses of water in addition to the other sources of water from both food and other drinks. This is essentially an ill considered myth, as is the often misinterpreted recommendation that beverages containing diuretic substances, such as coffee and alcoholic drinks, should not be counted towards water intake. Indeed, the effectiveness of hydration of water in a beverage is determined by the volume ingested in relation to the diuretic capacity of that beverage, i.e. although both may have the capacity for hydration, a cup of strong coffee might hydrate less than a cup of weak coffee.

Of the water required by a typical healthy, albeit sedentary, adult living in a temperate zone, about 1000 ml is provided as it exists in that molecular form (H_2O) from foods, with about another 250 ml provided by metabolic processes producing water as an end product of oxidation of mainly fats and carbohydrates (Kleiner, 1999). That is, although some foods (principally fruits and vegetables) contain relatively high amounts of water per se, other foods that contain sources of metabolic intermediary compounds, the fats and carbohydrates (and even alcohol), also contribute to water balance because they undergo catabolism to liberate water and CO_2 as end products of metabolism during energy production. Additional amounts of water are required at times of increased physical activity and in high environmental temperatures to replace that

utilized for temperature regulation. Under normal healthy conditions and sufficient fluid availability intrinsic mechanisms will ensure that the flux of water through the body is in equilibrium by a balance of hormones such as vasopressin, also known as antidiuretic hormone. Equally in health, excess intake is also balanced by increased output and, apart from being the result of certain underlying clinical conditions, excess intake in otherwise healthy individuals has seldom, if ever, been shown to be of major detriment. However, in certain disease states, such as alcoholic liver disease or kidney disease, positive water balance may exacerbate such serious health problems.

The amount of water present in the body is also known as the distribution volume into which water soluble alcohol may be distributed once it has been absorbed and before being broken down by the liver. Along with the capacity and location of the enzymes responsible for the breakdown of alcohol, it is this distribution volume that determines the effectiveness of the toxicity of alcohol and its intoxicating properties. This may be one of the reasons that women with lower total body water appear to be more susceptible to the effects of alcohol than men. An understanding of factors such as levels of body water is, therefore, important for consumers and practitioners, especially when treating the effects of alcohol per se in acute poisoning, for example, or the longer-term health consequences of chronic alcoholism, particularly alcoholic liver disease (cirrhosis). As well as its relative distribution into the total body water pool, the full intoxicating and metabolic potential of alcohol consumption is ameliorated by its rate of consumption, its concentration and the presence or absence of bubbles, the amount and type of food taken concurrently, and the effectiveness of the alcohol dehydrogenase enzymes present in the stomach, as well as in the liver (Crabb *et al.*, 2004).

The body mass index (BMI; kg body weight/height^2) is often, and frequently erroneously, used as indicative of the amount of alcohol that an individual may consume before reaching a level considered to increase health risk. However, BMI is dependent to a large extent on the relative amount of body fat, and it is also important to realize that the proportion of body weight accounted for by water is extremely variable due to the large variation in fat content between individuals (fat in its pure chemical form does not absorb alcohol). Body fat may represent perhaps only about 4% of body weight typically in body builders, but up to potentially 50% or more in morbidly obese individuals. Such considerations would result in proportions of water in the whole body of up to 70% (49 kg water in a 70 kg man of 4% fat) and down to 36% (26 kg water in a 70 kg man of 50% body fat). Therefore, because this level of variability in body fat is confounding, ineffective and uninformative for clinical use, the concept of dividing the composition of the whole body into its chemically distinct fat and fat free mass (FFM) compartments was applied for functionally standardizing body composition independently of the various levels of body fat. In body composition modelling (for example, Fuller *et al.*, 1992), all chemically extractable fat (all lipids) are assigned to the fat fraction of the body and FFM represents a conglomerate of every other chemical component of the body. The FFM is predominantly composed of water, protein and mineral in fairly consistent relationships with each other and it is considered to be the metabolically active body compartment.

$$\text{Body weight} = \text{fat} + \text{fat-free mass (FFM)}$$

$$\text{FFM} = \text{water} + \text{protein} + \text{mineral} (+ \text{ relatively smaller amounts of other components such as DNA, urea etc.})$$

This construct of body composition is modeled at the chemical level where all water is assigned to the FFM and is useful for many purposes of research and clinical practice, and so any alcohol absorbed by the body only appears in the FFM, not in the chemically distinct fat. This model is not to be confused with the conceptually valid and alternative tissue level model of body composition (Wang *et al.*, 1992) in which the various tissues, such as muscle and adipose tissue and organs, are composed of differential amounts of the body components according to structure and function. Water is distributed predominantly in tissues that have

relatively low fat content, such as muscle (consisting perhaps about 80% water), and less so in adipose tissue (about 20% water) that is designed for fat storage and metabolism. Therefore, ingested alcohol is distributed throughout the body tissues, but more so in muscle and organs and less so in adipose tissue.

This standardization of hydration fraction of FFM is of value to health practitioners in general because in a normal healthy adult there is a fairly constant hydration fraction of the FFM (mean, about 0.73 or 73%) that falls consistently within a very narrow range (perhaps about 70–75%). Importantly for practitioners involved in certain disease states, such as alcoholic liver disease (cirrhosis) and kidney disease, the fraction of water in FFM shows a much greater variability and may fall substantially outside the normal reference range. Cirrhosis patients, for example, may present with a hydration fraction outside either extreme with overhydration (edema and ascites) or underhydration, both of which are potentially very problematical (Morgan *et al.*, 2006). Total body water is distributed between the intra- and extracellular fluids, but in overhydration the main expansion occurs in the extracellular fluid. Aberrant body water may be of serious detriment to the normal physiological functionality of the body. The key functions of water are: (a) the efficient transport of nutrients, gases (O_2, CO_2), internal secretions (e.g. hormones) and waste products, both within and between the cells, helping to maintain the critical acid/base balance of the body for optimum organelle and enzyme performance, and (b) thermoregulation, particularly the elimination of excessive heat from the body, that may have been generated by metabolic processes or be the result of high environmental temperatures, and the dissipation of this heat into the surrounding environment through the skin (sweat) and lungs (water vapor). Although water insufficiency and imbalances may be responsible for some of the symptoms of the alcohol hangover (Wiese *et al.*, 2000), excessive alcohol intake can have far more serious health consequences than a transient hangover, due to its effects on water balance alone, with severe dehydration enhancing the toxic effects of alcohol and its intermediary metabolites, and exacerbating the effects of any xenobiotics that may be present. Also, dehydration significantly lessens the ability of the body to regulate its own temperature, which may lead to severe complications, even death, especially in extreme climatic conditions. However, it is also important to remember that excessive alcohol intake over the long term can lead to a number of serious health sequelae independently of issues of water balance, for which compensatory mechanisms are fairly effective overall.

5.3.2 Alcohol

After water, alcohol is predominantly the second major constituent in the usual styles of alcoholic beverages (Table 5.3.1), typically contributing about 3–4 g (or more)/100 ml (about 5% ABV or more) in beers and ciders, 6–11 g/100 ml (about 8–4% ABV) in wines and 15–30 (or more)/100 ml (20–40% ABV, or more) in fortified wines and spirits. The representation of alcohol levels as g/100 ml alcohol is an informative alternative to the UK 'unit' or other 'standard' amounts for comparative purposes depending on preference and required application (Section 5.1.1). However, the variability in alcohol content within each type of alcoholic beverage is substantial. For example, beers can be produced that are well below 3% ABV (2.4 g/100 g), with others up to about 12% ABV (9.5 g/100 g). Wines may be below 8% ABV (6.3 g/100 g), for some white wines usually, up to about 14% ABV (11 g/100 g) for some reds. The generally higher concentration of alcohol in spirits is also quite variable and spirits are often measured in proof as an alternative to ABV; a 40% ABV spirit is about 70 degrees of proof with a 25 ml serving containing 10 ml (or 7.9 g) alcohol. Alcohol content is a significant determinant of how much of a particular alcoholic beverage will be consumed according to taste and character of the beverage concerned as well as the capacity of the individual and, therefore, the contribution that its total intake is likely to have on dietary energy intake overall (see Chapter 5.5).

Alcohol may be considered as a nutrient in some respects as it provides a source of metabolic energy at 29 kJ/g (7 kcal/g). Otherwise, alcoholic beverages are generally perceived to be the source of so called 'empty

calories,' which essentially means provision of calories alone, from both alcohol and the simple sugars that are often present (carbohydrates provide 16 kJ/g or 3.75 kcal/g), with no further benefit as in many, but not all, alcoholic concoctions there are no other constituents with any significant nutritive benefit. Also, alcohol and its metabolites are essentially toxic to the human body, although it does provide a relatively sterile dietary environment for consumption of other nutrients, including water at times when alternative supplies of water may be contaminated. Alcohol may have health promoting benefits if taken in low to moderate amounts via any beverage style, as ethanol per se appears to lower cardiovascular risk, as suggested by certain fundamental and epidemiological studies (see Rimm *et al.*, 1996; Puddey *et al.*, 2003). Although there may be mixed influences on lipid metabolism depending on such factors as subject variability and amount consumed (Feinman and Lieber, 1999), moderate alcohol consumption may increase plasma high density lipoprotein (HDL) relative to low density lipoprotein (LDL) and promote cholesterol transport away from arterial walls, which appear to be beneficial, along with other clinically important effects such as promoting anticoagulant properties and direct effects on reducing platelet aggregation (Puddey *et al.*, 2003; Ruf, 2004). However, there are severe detrimental health effects of alcohol consumption possible in high consumers and alcoholics (Table 5.1.1 in Chapter 5.1; see also Chapter 5.5 and Sections 5.1.2 and 5.6.2).

Certain types of alcoholic beverage, especially those with relatively low alcohol concentration, are potential sources of important nutrients such as vitamins, minerals and antioxidants. In general, these sources include the less refined fermentation products of wines and beers, but not spirits, as distillation and concentration of alcohol tends to eliminate many of the less volatile compounds, including potential nutrients. However, the nutritive value of such beverages is uncertain because the amounts consumed may be too small to be effective, relative to dietary requirements, especially if the alcohol intake is low to moderate. Additionally, for those nutrients that might appear to be consumed in significant quantities from alcoholic beverages, there may be confounding effects of lower intakes from the otherwise usual dietary sources as, in high consumers and alcoholics especially, regular meal patterns, and food quantity and quality may be disrupted, reducing nutrient consumption overall. This may be because of either conscious or insensitive social changes or perhaps insensitive compensatory mechanisms to account for increased alcohol consumption. The alcohol ingested also interferes with absorption, assimilation and metabolism of a large number of important nutrients (Lieber, 2000), effectively increasing dietary requirements. High alcohol intake may also contribute towards significant imbalance of water in the body, as it is known to influence release of antidiuretic hormone, causing increased loss of water in the urine, potentially altering the flux of nutrients through key metabolic pathways.

5.3.3 Carbohydrates (Including Fiber)

Carbohydrates, also called saccharides, are simply composed of carbon, hydrogen and oxygen atoms (as is alcohol, incidentally; C_2H_5OH – the formula for ethanol), but exist in multiple and complex forms, with many important functions in the human body, and in plants, where they are integral to the provision, transport and storage of energy and constitute major structural elements. Monosaccharides (simple sugars such as fructose and glucose, amongst others) are the basic monomeric units of carbohydrates that may exist independently or may be used as the building blocks of disaccharides (two units), oligosaccharides (between two and nine units) and polysaccharides (10 or more units). Available, or glucogenic, carbohydrates are the free sugars (e.g. glucose, fructose and the oligosaccharides) and complex carbohydrates (glycogen, starch and dextrins) that collectively constitute the glycemic carbohydrates (see *McCance and Widdowson's, The Composition of Foods* – Food Standards Agency, 2002). Dietary carbohydrates are usually one of the major sources of total metabolic energy requirements providing 16 kJ/g (3.75 kcal/g) and representing typically 45–65% of energy requirements globally with the UK recommendation that carbohydrates should provide about 50% of energy

intake (see British Nutrition Foundation website – http://www.nutrition.org.uk). Based on a nominal daily energy intake of 8368 kJ (2000 kcal) for an 'average' individual in the population, the UK recommendation for daily carbohydrate intake would be about 250 g. Fats and protein, and any alcohol consumed, can also be broken down to provide intermediates for catabolism and energy production through acetyl coenzyme A and entry into the tricarboxylic acid cycle.

However, not all carbohydrates are utilized in the same way, with some of the more complex compounds (e.g. certain oligosaccharides, nonstarch polysaccharides and resistant starches, also known collectively as dietary fiber) being indigestible in the upper gastrointestinal tract and so pass into the lower gut where some may be utilized by gut flora and some perhaps eliminated essentially unchanged. Therefore, the oxidation of certain ingested carbohydrates may not be complete, indicating a difference between the potential fully combustible energy ingested, the digestible energy and the net metabolic energy that is actually realized (Elia and Cummings, 2007).

The ubiquitous carbohydrate, glucose, is central to intermediary metabolism and is especially important as the usual source of energy for the brain, which cannot utilize fats. In animals, glucose is stored as the polysaccharide glycogen molecule. In plants, glucose is stored as multiple units to form the polysaccharide molecule starch, which must be broken down to individual glucose units for utilization by yeast during fermentation. The malting and mashing processes used in beer production facilitate the enzymatic breakdown of the complex carbohydrates present in barley and other grains, predominantly the polymers starch and β-glucans, into simple sugars that are then more available for fermentation into alcohol (Sections 2.6.2 and 5.7.4). Dextrins are relatively low molecular weight carbohydrates that are produced by hydrolysis of starch, the higher linear and branched forms of which cannot be readily utilized by yeasts and so appear in varying amounts in some finished beers (Section 2.6.2). Fruits, particularly grapes, provide the simple sugars glucose and fructose that, as such, can be fermented to produce wines, in particular, without the need for further processing. A number of different carbohydrates are present in raw ingredients and find their way unchanged or modified into the final fermentation product in variable amounts, depending on the source of raw materials used and the type of alcoholic beverage produced. Analysis by various methods, such as HPLC, ion chromatography (Section 4.3.3) and gas chromatography (Section 4.3.2) and electrophoresis (Section 4.6.1), indicates the presence in beers of fairly sizeable amounts of the carbohydrate malto-oligosaccharides, consisting of maltose, maltotriose, maltotetraose and maltopentaose (maltohexose, maltoheptose and the higher malto-oligosaccharides are also found in amounts that decrease inversely according to molecular size), with slightly smaller amounts present of glucose, sucrose and fructose, as well as the sugar alcohol glycerol. Similar analyses for wines indicate varying, but often sizeable amounts of glycerol, glucose, fructose and tartrate, with lesser amounts of lactate, citrate, malate, succinate, acetate and others. It is difficult to be sure of exact quantities present in alcoholic beverages, as detection of individual carbohydrates and their respective levels are very much method dependent and, of course, actual levels vary considerably, being very much product dependent. Behind alcohol and CO_2, glycerol is usually the third largest product of yeast fermentation, but may be added artificially for smoothness, especially to some wines. In contrast to wines and beers, distillation processes ensure that spirits have effectively negligible carbohydrate content, unless perhaps a mixer might be added or sugars infused for producing liqueurs (Chapter 3.9).

Individually, the carbohydrates and alcohols other than ethanol are not thought to make important contributions to the nutritional value of beers and wines, as none of them are essential (i.e. carbohydrates can be produced within the body from breakdown of fats and proteins). However, collectively they may contribute significantly to metabolic energy at times of high alcoholic beverage consumption. Also, the contribution of carbohydrates (16 kJ/g or 3.75 kcal/g) to the energy content of many other types of alcoholic beverages could be considerably higher in, for example, sweet wines, liqueurs and fortified wines, and others that may have simple sugars added during the final stages of preparation, especially specific market orientated products such as 'alcopops.'

Beers and dry wines may provide relatively low amounts of carbohydrates in terms of dietary requirements, but which again vary greatly according to the particular product, and, to reiterate, these could be relatively significant at times of high consumption, providing energy at 16 kJ/g (3.75 kcal/g) of carbohydrate. A half pint of beer (284 ml) of about 3.6% ABV, although only about one unit of alcohol (7.89 g of alcohol at 29 kJ/g or 7 kcal/g, contributing 229 kJ or 55 kcal), may typically provide a total of 376 kJ (90 kcal), about 146 kJ (35 kcal) from carbohydrates. A serving of wine containing one unit of alcohol (125 ml of 8% ABV) may provide about 418 kJ or 100 kcal (about 188 kJ or 45 kcal from carbohydrates). In contrast, a serving of spirits containing one unit (25 ml of 40% alcohol) is not so product dependent and provides a consistent 229 kJ (55 kcal), as essentially all energy is provide by its alcohol content and no carbohydrates of metabolic utility are present. However, some beers, wines, fortified wines, dessert wines and infusions in liqueurs may be quite high in carbohydrates through the addition of flavoring and preference for sweetness (either by controlled fermentation or simply added in the final stages of manufacture). This extra carbohydrate content may significantly increase relative energy value even further. For example, a 38 ml serving of coffee liqueur (60–65% proof, about 30% ABV and 9 g alcohol) may provide 502 kJ or 120 kcal (264 kJ or 63 kcal from alcohol and 238 kJ or 57 kcal from carbohydrates) and a glass of sweet sherry (one 100 g serving of 15% alcohol) may provide up to about 711 kJ or 170 kcal (439 kJ or 105 kcal from alcohol and 272 kJ or 65 kcal from carbohydrates). The addition of mixers to alcoholic beverages effectively reduces their alcohol concentration (total alcohol quantity is the same) but, in part at least, any potential nutritional gains may be effectively diminished by added carbohydrates, again often in the form of free sugars. Examples of mixers include fruit juices, e.g. orange juice for sangria, which adds primarily water and carbohydrates (mainly as free sugars), with some vitamins and minerals (Sections 5.4.1 and 5.4.2). Certain concoctions may have carbohydrates added in conjunction with the addition of other ingredients such as cream, which predominantly provides calories from lipid (37 kJ/g or 9 kcal/g), and even eggs that contribute protein (17 kJ/g or 4 kcal/g) to the beverage. In view of all of these considerations, preferential consumption of such beverages of higher carbohydrate content may, therefore, have implications for optimum health in addition to those associated with the ingestion of alcohol per se.

Some of the less refined alcoholic beverages, especially certain beers and ciders rather than wines, also contain significant amounts of more complex carbohydrates, in contrast to the simple sugars, which may serve two functions with potentially significant health benefits. Those which are slowly digestible by the enzymes of the small intestine ensure that the absorption and concomitant rise in blood glucose is less rapid and with a lower peak concentration than that produced by the more simple monosaccharides and disaccharides. This means less demand on the pancreas for insulin production, along with its possible rebound effects, and lower blood glucose helps to avoid the possible damage inflicted on important functional proteins, including haemoglobin (Hb1Ac), and structural proteins in the vasculature, when glucose becomes attached to them (Ceriello *et al.*, 2008).

The second benefit of complex carbohydrates comes from those that are essentially indigestible in the upper human intestine, colloquially and collectively known as *dietary fiber*, which consists of insoluble and soluble plant derived nonstarch polysaccharides (e.g. cellulose), dextrins, β-glucans and many other molecules. Fiber is a key component of the diet because it is indigestible by enzymes in the upper intestinal tract, a property that is important in terms of potential health benefits, as it passes essentially unchanged into the large bowel. Some of the nonstarch polysaccharides, resistant starches and oligosaccharides that pass into the large intestine are utilized by the bacteria that reside there to produce other compounds of nutritional value, such as the volatile short-chain fatty acids, acetate, propionate and butyrate. Acetate and propionate are absorbable through the intestinal wall and are utilized for energy production within the usual pathways of intermediary metabolism, acetate being available to all cells, such as muscle, propionate usually being metabolized by the liver, and butyrate is a major source of energy for the colonic epithelium (Cummings and Macfarlane, 1997). The nutritional importance of complex carbohydrates is also due to the provision of

energy for bacterial growth. Any complex carbohydrates that remain unchanged or unused by the gut or the gut bacteria help to bulk the intestinal contents, along with greater levels of bacterial matter, reducing transit time and the potential gut contact time of any toxic compounds, which may be expelled from the gut with greater expedience.

As bacteria are provided with the energy to grow by such carbohydrates, they are also able to utilize certain potential toxins based on sulfur and nitrogen, which are detoxified by incorporation into bacterial proteins and excreted (Cummings and Macfarlane, 1997). Apart from a few alcoholic beverages that are relatively unrefined, e.g. some beers and ciders, or that may have ingredients added to the final product, fiber is not really present in any significant amounts due to clarifying and filtering processes etc. Fiber is absent from spirits due to distillation, negligible in wines and only present in small amounts in most of the more commonly consumed beers. However, some beers and ciders, especially those of higher strength alcohol and some specialist products, have significant amounts of fiber; perhaps 6 g/l in some strong UK beers, when considered in perspective of a daily intake of about 20–40 g fiber in a typical Western type diet and perhaps 50 g or more in less developed countries, or in regions in which cereals and fruits represent greater predominance in the diet. For example, some locally produced beers that are made from various grains, such as wheat or sorghum, may be consumed as the simple fermentation product without further processing to remove spent ingredients (Chapter 2.7). Certain cocktails and mixers may have added fiber in the form of fruit pieces, but again this is probably not of any major nutritional significance.

5.3.4 Nitrogenous Compounds: Proteins, Peptides, Amino Acids, Amines and Nucleotides

Proteins are large polymers made up of their constituent building blocks known as amino acids. RNIs for protein are 55.5 g/day and 45.0 g/day, respectively, for men and women of up to 50 years of age, with slightly adjusted values for those over 50 years (Department of Health's 'COMA' Report – Department of Health, 1991; British Nutrition Foundation website – http://www.nutrition.org.uk). These values are based on requirements of 0.75 g/kg body weight/day, slightly above the estimated average requirement of 0.6 g/kg body weight/day, in order to ensure inclusion of a majority of the population. However, people in the UK generally exceed guidelines for protein intake, with such levels constituting 10–35% of the total recommended daily energy requirements, with a target balance of 15% of total energy intake provided by protein (17 kJ/g or 4 kcal/g) – see website of the British Nutrition Foundation (http://www.nutrition.org.uk). With very few exceptions, the levels of protein in most alcoholic beverages are relatively insignificant in terms of their contribution to the diet. Beers may contain some proteins derived from the original ingredients, essentially malt (or fruits if used), and from residual yeasts, but many proteins are fully or at least partially hydrolyzed during production. Proteins are desirable in beers, as they make a predominant contribution towards maintaining the head of the beer. However, nutritionally significant amounts of protein may only be found in very few of the more commonly consumed beers in which levels of protein may reach about 1.7 g in half a pint (284 ml). This is equivalent to about 3–4% of the daily protein requirement in a 'reference' man weighing 70 kg and represents sufficient intake for up to 2–3 kg of the body weight. In the very early versions of beers and the less processed beers currently being produced there may be greater amounts of protein, but these styles are not as readily available as the more refined products of greater clarity demanded by market forces. Protease enzymes are responsible for the breakdown of many of the proteins in beer into peptides and amino acids. In wines, the more lengthy secondary fermentation breaks down proteins so that negligible protein content usually remains in the finished product. The levels of protein in spirits are also negligible, essentially because of elimination by distillation of any protein that is present in the fermentation product. However, there are certain residual proteins of significance to health that may be found in finished beers, especially those that are ethanol soluble storage proteins, or prolamins, of cereal grain origins and some of which may cause autoimmune reactions

in genetically susceptible individuals, a condition known as celiac disease (McGough and Cummings, 2005). Wheat and barley contain the glycoprotein, gluten (specifically constituted of gliadin and glutenin), and barley contains hordein, also a glycoprotein, of which significant amounts are quantifiable in certain beers (Ellis *et al.*, 1990) (Section 5.11.3). Although gluten may be used as a clarifying agent in wines, residual gluten, if any, may not be present in sufficient amounts to cause significant immunoreactive responses (Cattaneo *et al.*, 2003).

Peptides are smaller polymers of amino acid building blocks than proteins that may exist in relatively small amounts in beers and wines. Like proteins, ingested peptides are digested in the intestine releasing easily absorbable amino acids that may be used to construct functional proteins and peptides once assimilated into the body, such as structural elements, enzymes and hormones. One such tripeptide is glutathione (formed from the amino acids glycine, L-cysteine and L-glutamate), which occurs in grapes and is also produced by the action of yeast, and so is present in small amounts in finished wines and beers. Although ingested through wine and beer consumption, glutathione is unlikely to be absorbed as such into the body without first being degraded into its constituent amino acids and so, as it can be readily synthesized *de novo* by the body, it is not considered to be an essential nutrient. However, glutathione is a very important molecule in human biology because of its antioxidant properties that protect the cells directly against the effects of certain toxins, especially free radicals or highly reactive oxygen and nitrogen species (Wu *et al.*, 2004; Franco *et al.*, 2007) (see Chapter 5.8). There is a cellular equilibrium between the oxidized (GSSG) and reduced (GSH) forms of glutathione that is the key to its antioxidant potential since this balance greatly favors GSH, especially at times of oxidative stress due to increased levels of free radicals. Alcohol catabolism is itself responsible for production of certain highly reactive free radicals. It has been suggested that because wines and beers contain significant levels of multifarious antioxidants (Section 5.4.3) they may be of significant benefit in maintaining the important GSH/GSSG equilibrium by helping to keep glutathione in its reduced GSH form. Glutathione also appears to have an antioxidant role in the beers and wines themselves, by preventing chemical oxidation of vulnerable components and preserving the taste and character of such beverages, but glutathione derived from these sources is likely to be of limited nutritional value, making negligible contribution to daily requirements.

The building blocks of proteins and peptides are *amino acids* of which many, such as alanine, glutamic acid with glutamine, arginine, cysteine, methionine, asparagine, threonine, lysine and γ-aminobutyric acid, are present in variable amounts in wines, beers and ciders as free amino acids. Proline appears to be the most abundant, probably because it is not utilized by yeast, as are other amino acids. Because of the distillation processes, spirits contain negligible amounts of amino acids. Although some of the amino acids in beers and wines, e.g. methionine and lysine, are considered to be essential amino acids (those that cannot be synthesized within the body and so must be obtained from exogenous sources), alcoholic beverages are not considered to be nutritionally significant sources. For amino acid content of typical alcoholic beverages, see *McCance and Widdowson's, The Composition of Foods* (Food Standards Agency, 2002).

Biogenic *amines* and *polyamines* are present in fermented beverages at variable levels, depending on the original ingredients, including potential bacterial contamination, and processing techniques, and may be produced by decarboxylation of free amino acids in the beverage itself, beer for example (Kalac *et al.*, 2002; Kalac and Krizek, 2003). Different amines are produced in varying amounts according to the strain of yeast used for fermentation and are usually present in relatively small quantities in wines, beers, ciders and some spirits, perhaps just a few milligrams per liter up to about 130 mg/l (Ancín-Azpilicueta *et al.*, 2008). Essentially of negligible nutritional significance, some bioactive amines may be considered to be effective at the micronutrient level with concomitant health implications. Certain of these amines, including histamine, tyramine and phenylethylamine, are associated with some biological functionality, including potential toxicity and other health exacerbations such as allergenic and asthmatic reactions (Vally and Thompson, 2003), although the extent of these affects remains uncertain (Jansen *et al.*, 2003) (Section 5.11.3). Red wines appear

to contain far greater amounts than white wines, which may account for greater perceived occurrence of such reactions from red wine (Ancín-Azpilicueta *et al.*, 2008). Recently, there has been increasing interest in melatonin (*N*-acetyl-5-methoxytryptamine), which is an indoleamine synthesized from 5-hydroxytryptamine (serotonin) and is perceived to be of particular importance in many aspects of human biology, including key hormonal and antioxidant functions. For example, the cyclical nature of melatonin levels enables control of circadian rhythms, including the regulation of sleep (Danel and Touitou, 2004). Melatonin and its metabolites also have very powerful antioxidant properties as hydroxyl radical scavengers, perhaps five times greater than vitamin C, which is especially beneficial in maintaining the integrity of DNA (Coghill, 2007). As well as being synthesized by various tissues in the body, particularly the pineal gland, melatonin is also produced by plants, the products of which include alcoholic beverages. Of particular relevance, grapes (especially the skins of red grapes that contain melatonin in amounts that are variable depending on family of grape – Iriti *et al.*, 2006), apples and barley appear to be natural sources of melatonin (e.g. Badria, 2002; Iriti and Faoro, 2006). It is assumed, therefore, that melatonin and some of its bioactive metabolites might be present, probably in small and variable quantities, in the wines, ciders and beers produced from such sources, but relatively little definitive information on this subject is currently available. Alcohol itself is known to alter sleep patterns (Roehrs and Roth, 2001) and secretion of melatonin from the pineal gland, and this effect may be due to chronic exposure to alcohol or a shift in secretion patterns altering circadian synchronization (Danel and Touitou, 2006).

The nucleotides, purines, particularly guanine and adenine, and pyrimidines, particularly thymine, cytosine and uracil, are produced when yeast cells multiply during fermentation because these nucleotides are synthesized and incorporated into the nucleic acids DNA and RNA (thymine is usually found in DNA and uracil is exclusively found in RNA). As well as being integral to nucleic acids, phosphorylated purines (ATP, GTP) and phosphorylated pyrimidines (UTP, CTP) are important sources of energy generation for driving cellular reactions. Although humans do not need exogenous sources of these nucleotides because they can be synthesized *de novo* or salvaged and reutilized by the body, the ingestion of high levels of purines may impose increased health risks. The catabolism of both adenine and guanine proceeds via the common intermediary, xanthine, which is then converted into the end product uric acid and this relatively insoluble compound is then usually excreted in the urine. However, especially in conditions that produce unusually high levels, excess uric acid is implicated in some metabolic disease processes, such as gout, where it is deposited on joint surfaces and in other tissues. In addition to the potential effects of exogenous purines, ethanol per se is suggested to increase blood levels of uric acid, particularly as it promotes degradation of adenine coupled with increased production of lactate and concomitant hyperuricaemia, which may predispose to gout in the same way (Section 5.6.2). In contrast, the pyrimidines are reduced and cleaved to produce ammonia, CO_2 and β-amino acids, none of which are currently implicated in increased disease risk. Beers are more heavily implicated in increased risk of gout than wines because of their greater purine content. For a comprehensive review, see Yamamoto *et al.* (2005).

Generally, however, the amounts of proteins, peptides, amino acids and other nitrogenous compounds involved in alcoholic beverages in general probably make only small contributions to dietary requirements, unless added at the final stages of preparation, for example the addition of eggs, milk and tomato and orange juices for certain cocktails.

5.3.5 Lipids

Plant wall lipids from the raw ingredients used in the fermentation process may contribute to the character of an alcoholic beverage, but may be oxidized when exposed to certain prevailing conditions to cause undesirable changes in taste and character. Lipid levels are generally so low in wines, beers, ciders and especially spirits

that they are of negligible nutritional significance. However, they may be present as ingredients added at the final preparation stages of a particular beverage, e.g. cream liqueurs, and the addition of these sources may also contribute small quantities of fat soluble vitamins and minerals, as well as being a source of energy (lipids provide 37 kJ/g or 9 kcal/g).

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5.4

Micronutrient Content of Alcoholic Beverages

The micronutrient content of an alcoholic beverage may be substantially different from that which might be expected based on the original ingredients and may also be extremely variable, even within each generic type of beverage, reflecting the similar situation encountered when considering macronutrients (Chapter 5.3). Therefore, chemical analysis of the final product is required to be certain of the micronutrient content and, therefore, potential micronutrient intake from the many alcoholic beverages currently available. Some summary information is readily available for the generic products (e.g. *McCance and Widdowson's, The Composition of Foods* – Food Standards Agency, 2002 and websites such as http://www.nutritiondata.com/). It is also important to be fully aware that micronutrient information may exist for various serving measures (e.g. wine as 125 ml, 175 ml or 250 ml glass), as concentrations in standard amounts (g/l or g/100 g, for example) or even as percentage of the RNI per serving, and so adjustments for actual serving sizes may be needed in order to provide a reasonable estimate of intake. The RNI is the dietary reference value normally assigned to micronutrients (as it is for proteins) and is the value applied to ensure that nutrient intake is sufficient for a vast majority of any population group of interest (see Nutritional fundamentals, Section 5.1.4, above, and the website of the British Nutrition Foundation – http://www.nutrition.org.uk).

Alcoholic beverages may contain sufficient amounts of many essential elements (also referred to colloquially as minerals and trace elements), electrolytes, vitamins and other compounds to make significant contributions to nutritional status, but these contributions could be quite variable. In nutritional terms, 'minerals' are considered to be those chemical elements other than carbon, oxygen, hydrogen and nitrogen, that are found to exist mainly in the form of ions and are essential for: (a) maintaining the electrolyte balance of the body, both as independent entities and in chemical combination with other elements, (b) utility in certain structural roles, such as calcium in bone, and (c) for acting as cofactors in some enzymatic reactions, copper in redox enzymes such as cytochrome oxidase, for example. Minerals are often classed as 'macrominerals' if they are required in relatively large amounts, such as calcium, which is integral to bone structure, or potassium for maintaining electrolyte balance. 'Microminerals,' that are required in relatively lesser amounts than the macrominerals, are also known as trace elements, and include copper, for example, which is integral to redox reactions of the oxidation and reduction pathways or electron transport chains, or iron, which is important for hemoglobin function, but required intake is relatively low on a daily basis. The simple ingredients, particularly the juice of the fruit for wines or malt and source water for beers, have variable, but significant, levels of

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

valuable elements and electrolytes that may survive processing and so persist into the finished product with potentially important nutritional benefits. Spirits, however, appear to contain very small amounts, if any, due to losses through the distillation processes. Alcoholic beverages generally contain relatively lower amounts of sodium compared to higher amounts of potassium than found in the usual diet, with potential health benefits, especially regarding cardiovascular function. For general information, see the website of the British Nutrition Foundation – http://www.nutrition.org.uk.

Other micronutrient constituents of alcoholic beverages, particularly the vitamins and phytochemicals, are also very variable. For example, certain of the water soluble vitamins and many of the different classes of plant derived antioxidants originating from either the raw ingredients or fermentation processes, or both, are found in variable yet potentially nutritionally significant quantities in wines, beers and ciders. However, although these particular compounds are essentially removed during the distillation of spirits, other potential micronutrients (and also some toxins) may be introduced at a later stage into distilled alcoholic beverages. In particular, for those beverages which are allowed to age in various types of wooden casks, certain phenolic compounds may become infused during storage and so present substantially altered nutrient profiles that may be specific to the individual product. Some such compounds appear to have potential health benefits, whereas others may be toxic or carcinogenic (Goldberg *et al.*, 1999). Dietary antioxidants, essentially consisting of vitamin E (tocopherols), vitamin C, vitamin A and β-carotene (the vitamin A precursor, provitamin A), with integral involvement of the mineral selenium and the vast array of polyphenols (Chapter 5.8), increasingly appear to have major significance for human nutrition and health status. Working independently or interacting in synergy, collectively these antioxidants help prevent oxidative damage by free radicals. Consisting mainly of reactive oxygen and nitrogen species, free radical damage of key molecules involved in cellular proliferation (e.g. DNA), transport (e.g. low density lipoproteins) and structure (e.g. cell wall membrane proteins) may be ameliorated through complex interactive reducing environments within tissues and cells provided by these dietary antioxidants (Eastwood, 1999). Alcoholic beverages may be an important, if variable, source of some of these antioxidants in their own right, but also if certain mixers, such as fruit juices, are added when making cocktails.

Due to the considerable variation in the types, composition and amounts consumed of the vast numbers of different alcoholic beverages, only the most common types will be considered here. However, the characteristics of any nutrients likely to be added to the basic beverage style will be presented in order to provide information on their nutritional value that is as comprehensive as possible. To reiterate, this is important because, although the intake of vitamins, minerals and other phytochemicals may be, on an individual basis, apparently too low in amount or concentration to be functionally effective, it has been suggested that certain members of these nutrient classes may act synergistically to provide significant nutrition and health value. However, there are particular confounding factors that may be encountered when trying to fully define the importance of specific nutrients, either individually or in combination, as they are usually consumed as part of the habitual diet along with a multitude of other bioactive compounds. The 'French paradox' phenomenon is particularly appropriate in this respect, as consumption of red wine has been implicated in reducing the apparent risk to cardiovascular health of the French population, despite relatively high intakes of animal fats in the diet. However, as such health benefits have been suggested mainly on the basis of associations evident from epidemiological studies, definition of any underlying mechanisms of individual nutrient effectiveness or synergistic activity must await more fundamental studies. Also, an important question remains as to whether or not it is the red wine per se that proffers some of these suggested health benefits; i.e. is it the effects of the alcohol content or some properties of phytochemicals in the red wine that confer cardiovascular protection (Rimm *et al.*, 1996), or simply perhaps that consumption of red wine is associated with some other health promoting practice? Although this is clearly a topic of some controversy, with many unanswered questions remaining, attempts have been made to view existing evidence in a balanced perspective (e.g. de Lorgeril *et al.*, 2002; Goldfinger, 2003; Ferrières, 2004). Furthermore, it is also important to consider the potentially

detrimental health implications of excessive alcohol ingestion on the intake, absorption, metabolism, excretion and, therefore, overall equilibrium, of a number of key micronutrients, including, for example, vitamin A, carotene, zinc, folic acid, iron and selenium (Lieber, 2000; and several chapters in Watson and Preedy, 2003).

5.4.1 Vitamins

Raw ingredients, such as fruit and malt, used in the production of alcoholic beverages are good sources of water soluble vitamins with the fermentation process potentially adding significant amounts due to the actions of yeast, some B vitamins such as riboflavin, for example. However, the vitamin content of the final product may vary considerably from expected levels because of further processing and storage conditions. For example, vitamin C is susceptible to the effects of heat and oxidation, and thiamine may be taken up and utilized by the yeast, so these vitamins may feature less significantly in finished beers or wines. Distillation processes ensure loss of essentially all water soluble vitamins. Fat soluble vitamins are not a significant nutritional consideration in any alcoholic beverage, unless a mixer such as cream is added to particular types of beverage for making specific cocktails. Typical concentration ranges of vitamins for generic styles of alcoholic beverages are presented in Table 5.4.1.

Table 5.4.1 *Typical water soluble vitamin content* of representative wines and beers† commonly consumed in the UK‡*

	Water soluble vitamin content§			
	Wine		Beer	
	/125 ml	/liter	/284 ml	/liter
Thiamine (B1; mg)	Trace–0.01	Trace–0.08	0.009–0.026	0.03–0.09
Riboflavin (B2; mg)	Trace–0.05	Trace–0.4	Trace–0.11	Trace–0.4
Niacin (B3; mg)	0.1–0.3	0.8–2.1	0.6–2.3	2.0–8.0
Pantothenate (B5; mg)	Trace–0.23	Trace–1.7	0.1–0.28	0.3–1.0
Pyridoxine (B6; mg)	Trace–0.1	Trace–0.8	0.03–0.26	0.1–0.9
Biotin (μg)	Trace–2.5	Trace–20	Trace–2.8	Trace–10
Folate (μg)	Trace–1.5	Trace–12	11–34	40–120
Cyanocobalamin B12; μg)	Trace–0.33	Trace–1.0	Trace–0.23	Trace–0.8
Vitamin C (ascorbic acid; mg)	Trace	Trace	Trace	Trace
Choline (mg)	6–8	48–64	22–35	77–123
Betaine (mg)	0.26–0.4	2.4–3.2	17–25	60–88

*Fat-soluble vitamins are usually only found in nutritionally insignificant or trace levels at best in alcoholic beverages and are, therefore, not presented.

†Spirits are essentially devoid of nutritionally significant vitamins due to distillation processes during production and, therefore, are not presented.

‡Typical guideline values are presented, drawn from a large number of sources considered to represent the range of each generic product most usually consumed in the UK (e.g. *McCance and Widdowson's The Composition of Foods* – Food Standards Agency, 2002). For comparative purposes, values are presented per serving of 125 ml wine and 284 ml (half pint) beer, and also per liter.

§Wherever the value indicated is 'trace,' negligible or low amounts may be present but which are insignificant in nutritional terms.

Water Soluble Vitamins

As individual nutrients, water soluble vitamins are essential for a number of roles in the human body. However, they are also thought to be able to act synergistically with other vitamins, minerals and phytochemicals for increased health benefits. The required levels of dietary intake for each vitamin is variable and depends on demands that are driven by the number and extent of the functions of the body in which the vitamin is involved, and these may be increased in times of physical activity, stress, trauma and injury. The water soluble vitamin content of alcoholic beverages in relation to the RNIs set in the UK to cover the body requirements for these vitamins is discussed here (see also Nutritional Considerations, Section 5.1.4). As well as being present in significant amounts in whole grains and lesser amounts in grapes and grape juice that are prepared and used for fermentation, B vitamins in particular are produced in significant amounts by yeast and may be present in nutritionally significant levels in the final products of fermentation. However, as with other less volatile compounds present in fermentation products, water soluble vitamins are essentially eliminated during distillation processes.

The important B vitamins, folate, pyridoxine, B12 and betaine (trimethylglycine), appear to be critical for the maintenance of cardiovascular health, primarily protecting against the exacerbating effects of homocysteine in recurrent cardiovascular events. Appropriate amounts and proportions of these particular B vitamins reduce blood levels of homocysteine, whereas deficiencies cause raised homocysteine levels. Homocysteine is a natural homolog of the amino acid cysteine that is formed from methionine and the intermediate *S*-adenosyl methionine in the body (i.e. it is not obtained from any dietary sources). It has a short period of existence before being recycled into methionine or irreversibly converted to cysteine. During its existence, homocysteine is highly reactive with the capability of detrimentally binding to structural and functional proteins, including those integral to the cardiovascular system increasing the risk to cardiovascular health. For a perspective on homocysteine and health, see de Bree *et al.* (2002), and for the major implications for both the health and treatment of high alcohol consumers, especially alcoholics, due to alcohol related nutritional disturbances exacerbating these effects, see Lieber (2000).

Thiamine (vitamin B1) is essential for healthy growth and development, the functioning of the nervous system and heart and it enables intermediary metabolism of carbohydrates to proceed effectively. Thiamine occurs in beer and wine products as it is present in the whole grains and fruit used in production, but due to losses occurring during processing (because it is both heat labile and is utilized by yeast), these levels are not necessarily nutritionally significant. The RNI for thiamine for adult males up to 50 years of age is 1.0 mg/day with 0.9 mg/day for those 50+ years, and 0.8 mg/day for all adult females (Department of Health's 'COMA' Report – Department of Health, 1991; British Nutrition Foundation – http://www.nutrition.org.uk), and so beer products especially could make a small contribution towards these recommendations. A 330 ml glass of regular lager may provide up to about 2% of the daily requirement of thiamine, with other types of beer potentially providing greater amounts of thiamine (perhaps certain beers that are less refined or those that have higher alcohol content). However, a glass of wine (any wine) appears to provide only about 0.01–0.02% of the daily recommendation. There is no measurable thiamine in distilled products. Thiamine deficiency leading to impairment of brain function may be particularly important in alcoholics, as thiamine intake may be very low and alcohol may interfere with thiamine absorption, but this has only been shown in animal models (Lieber, 2000).

Riboflavin (vitamin B2) is required for catabolism of carbohydrates, fat and protein in the production of energy, and also for iron metabolism and transport. The RNI of riboflavin is about 1.3mg/day for adult males and 1.1mg/day for adult females (Department of Health's 'COMA' Report – Department of Health, 1991; British Nutrition Foundation – http://www.nutrition.org.uk). Riboflavin is present in small amounts in grapes and grape juice, but is not present in finished wine in nutritionally significant levels, providing less than about 0.1% up to perhaps 3% at most of the RNI per individual serving. Beers, however, contain relatively greater

amounts of riboflavin; a can (330 ml) of regular beer might provide about 0.1 mg, with some less refined and stronger (ABV) beers providing greater amounts, perhaps up to about 10% or more of the RNI per serving. Distilled products usually contain no measurable riboflavin. However, alcoholic beverages that are made by the addition of milk or egg products (Section 3.9.5) may make a significant contribution to the required dietary intake of riboflavin as these particular products are recognized as good sources of this vitamin (British Nutrition Foundation – http://www.nutrition.org.uk).

Niacin (or nicotinic acid; vitamin B3) is present to a greater or lesser extent in most foods, but with very small amounts in grapes and grape juice, and is not found in any great quantities in wines, with a single serving providing up to about 0.3 mg (2% of the RNI). Beers, however, are richer sources of niacin with a 330 ml serving of regular beer or lager providing about 1.8 mg, which is slightly over 10% of the RNI. The RNI for niacin (nicotinic acid equivalents) is 17 mg/day for males and 13 mg/day for females, reducing to 16 mg/day and 12 mg/day, respectively at 50+ years. Distilled alcoholic beverages contain no measurable quantities of niacin. Certain mixers, such as milk, eggs and tomato juice, used to make alternative types of alcoholic beverages may contain small amounts of niacin, but their contribution to nutritional value overall is thought to be relatively insignificant when consumed in this form. Niacin is required for structural integrity in the skin and membranes, for functioning of the nervous system and for catabolism of nutrients to provide energy (for details see Department of Health's 'COMA' Report – Department of Health, 1991; British Nutrition Foundation – http://www.nutrition.org.uk).

Adult men and women require between about 3 and 7 mg/day of *pantothenic acid (vitamin B5)* which is considered the safe intake, but, because deficiency diseases are very rare, specific RNIs are not provided for this particular vitamin (Department of Health's 'COMA' Report – Department of Health, 1991). Pantothenic acid is used to synthesize coenzyme A, which enables cellular transport of carbon atoms that are important in respiration and the biosynthesis of fatty acids and cholesterol, amongst many other compounds, and coenzyme A initiates the all important tricarboxylic acid (TCA) cycle. Small quantities at least of pantothenic acid appear to be present in almost all foods. Relatively small amounts are present in grape and grape juices, and from negligible to relatively small concentrations in wines, perhaps only 0.1 mg (possibly about 1–3% of the safe intake) per serving. Similarly, levels found in beer are not particularly significant nutritionally, perhaps 0.1–0.23 mg per 330 ml serving of regular beer (about 1–8% of the safe intake), despite whole grains being a relatively good source of pantothenic acid. Distilled alcoholic beverages contain no measurable quantities of pantothenic acid. Therefore, generally, alcoholic beverages cannot be considered especially good or appropriate sources of pantothenic acid unless other ingredients, such as eggs, that are high in pantothenic acid are added, but, with one or two notable exceptions (Section 3.9.5), such beverages are not very common.

Vitamin B6 or pyridoxine (also pyridoxal) is a cofactor for a number of enzymatic reactions in protein and amino acid metabolism and in the breakdown of glycogen to produce glucose. Pyridoxine is also involved in iron transport and metabolism. The UK RNI for men is 1.4 mg/day and 1.2 mg/day for women (Department of Health's 'COMA' Report – Department of Health, 1991; British Nutrition Foundation – http://www.nutrition.org.uk). Good sources of pyridoxine are wheat germ and yeast, and there are potentially significant quantities that are retained in finished beers; for example, a 330 ml bottle of regular lager may contain 0.1–0.2 mg of pyridoxine which is about 8–15% of the RNI. Grapes contain small, but significant, amounts of B6 (perhaps about 7% RNI in 100 g of the fruit; National Agricultural Library (NAL) at the United States Department of Agriculture (USDA): http://www.nal.usda.gov/), with pyridoxine also present in finished wines, which may also contribute nutritionally significant quantities, as a standard glass of red or white wine can contain about 0.1 mg or about 8% of the RNI, although much less may be present, depending on the extent of processing of the wine. Distilled alcoholic beverages appear to contain no measurable quantities of pyridoxine.

Although *biotin (vitamin B7*) is present in high levels in brewer's yeasts and is relatively stable chemically, the levels in alcoholic beverages are relatively small. Adequate dietary levels, that are also considered to be within the safe intake limit, are somewhere between about 10 to 200 mcg per day and so the actual contribution of alcoholic beverages is difficult to ascertain, but may be nutritionally significant under certain circumstances. Biotin is a cofactor integral to a number of enzymatic reactions involved in growth, development and maintenance of cellular integrity of tissues such as nerves, skin and bone.

Folic acid (pteroyl glutamic acid; vitamin B9) is a product of yeast and is present in high levels in some fruits and vegetables, such as oranges and orange juice (National Agricultural Library (NAL) at the United States Department of Agriculture (USDA): http://www.nal.usda.gov/) that may be used in the production of certain alcoholic beverages. Folate is required for cellular reproduction and growth, and for forming blood cells and in the nervous system. There is some difference in dietary recommendations for folate intake between the UK and USA; the UK RNI for adult males and females is 200 μg/day, whereas the USA recommendation is considerably higher at 400 μg/day. Grapes and grape juice contain relatively little (about 1% or less of requirements per 100 g) and wines likewise (less than 1% of requirements per serving). However, sangria, which may have orange juice as a major ingredient and which has folate levels at perhaps 180–200 μg/l, may provide significant amounts of folate, depending on relative amounts of wine and juice in the mixture and quantities consumed, and whether or not folate absorption is compromised, perhaps during periods of excessive intake of alcohol. Beers contain relatively high levels of folates with 'can' sized servings (330 ml) of regular lager providing perhaps 20 μg (10% UK RNI). Whilst some beers may contain negligible folate (possibly due to conditions such as storage), others, especially stronger beers, may contain significantly more (Owens *et al.*, 2007). No measurable quantities of folate have been reported for distilled alcoholic beverages.

Cyanocobalamin (vitamin B12) is involved in normal cellular metabolic functioning and DNA synthesis and is particularly important for the nervous system and blood formation. The means by which the body obtains and utilizes vitamin B12 is complex. It is obtained from animal sources almost exclusively and requires a gastric intrinsic factor to be effectively absorbed. Essentially, B12 can only be formed from the activity of certain bacteria and, unless there is some sort of bacterial action, there is essentially no provision of B12 from the raw ingredients of alcoholic beverages or the fermentation processes. Therefore, under normal circumstances there is no measurable vitamin B12 in wines and distilled alcoholic beverages and only small amounts in beers; a 330 ml serving of regular lager may provide up to a possible 1 μg B12. The UK RNI is 1.5 μg/day for both men and women (the US DRI is considerably higher at 2.4 μg/day for adults). As vitamin B12 is present in dairy products and eggs, it may be found in significant levels in alcoholic beverages that are produced from these, such as fermented whole milk products or cocktails that have eggs added or emulsion liqueurs that are made with eggs (Section 3.9.5). However, alcoholic beverages made from the addition of cream, e.g. coffee cream liqueurs, appear to have negligible amounts of vitamin B12.

Now considered as an essential water-soluble B vitamin, *choline* is required for structural functions, neurotransmission and as a provider of methyl groups, through its *betaine* metabolite, a function that contributes along with other key B vitamins (folate, pyridoxine, vitamin B12) to the lowering of blood levels of homocysteine. As sufficient intake of choline is suggested to be around 425 mg to 550 mg per day, beer intake appears to be able to contribute significantly to this requirement (typically about 4–8% in a half pint), but wine intake is more marginal (possibly 1–2% from a 125 ml glass) and distilled products have negligible quantities, if any.

The UK RNI for *ascorbate (or ascorbic acid; vitamin C)* is 40 mg/day for both men and women (interestingly, the USA DRI is far higher at 75 mg/day for women and 90 mg/day for men). Ascorbate is required for connective tissue, blood vessels and the nervous system, being a key component in the synthesis of collagen in these tissues and in wound repair. It is present in varying quantities in the fruits and vegetables that may be used to produce alcoholic beverages. Some such fruits, especially those that are used for home beverage production, have substantial amounts of ascorbate (e.g. rose hip at 2000 mg/100 g of fruit), and others have

relatively high levels (e.g. blackcurrants at about 180 mg/100 g) when compared to daily requirements. Grapes are a significant source of ascorbate (about 10 mg/100 g). However, in the final products of fermentation and any subsequent distillation there is none or relatively little ascorbate because it is susceptible to degradation by heat (boiling alone is not totally effective) and exposure to air (oxygen), especially in combination. In those tested, most wines made from grapes, regular beers and spirits contain no measurable vitamin C. However, vitamin C may be added to some wines and some bottled beers (perhaps to 30 mg/l) in order to stabilize them against possible oxidation spoilage (e.g. oxidation of the trace lipids that originate from plant cell wall material) (Section 2.6.12). In alcoholic beverages that are mixed with fruit juices to produce certain cocktails or mixers, there are significantly higher levels of ascorbate. Fruit juices most usually added to alcoholic beverages include orange juice (typically 50 mg per fruit, and about 33 mg/100 g orange juice) and tomato juice (about 10 mg/100 g of fruit, and about 20 mg/100 g tomato juice) that may contribute significantly to daily requirements provided that any processing of the juice does not adversely affect the ascorbate levels.

Fat Soluble Vitamins

The several forms of the fat soluble *vitamin A* and the many members of the related family of *carotenoids (carotenes and xanthophylls)* are not considered to make any significant contribution towards the nutrient value of alcoholic beverages per se as they are present in low concentrations in both the raw materials and fermentation products (Section 5.11.2). However, vitamin A and its carotenoid precursors, pigments that confer colour to yellow and orange fruits (e.g. apricots) and vegetables (e.g. carrots) and egg yolk, may also be present in some of the ingredients, such as tomato, apricot and melon juices, cream and eggs, that could be added to certain alcoholic beverages to produce various cocktails. Vitamin A is essential for maintaining vision, immunity and bone health. Lycopene and lutein are carotenoids that have particular antioxidant properties protective of vision and against atherosclerosis. It is very unlikely that alcoholic beverages would make a major contribution towards dietary requirements, as the UK RNI for vitamin A is 700 μg/day (700 μg is equivalent to 2300 International Units – IU) for adult males and 600 μg/day for females. However, certain specialist alcoholic beverages may provide nutritionally significant quantities of vitamin A and carotenoids; a single serving of a coffee cream liqueur (Section 3.9.5) contains about 100 μg (300 IU) of vitamin A and related compounds. The key ingredient of these beverages for providing vitamin A is cream as, for example, coffee liqueurs made without cream (Section 3.9.4) contain negligible or no vitamin A. The other fat soluble vitamins (D, E and K) are not generally present in sufficient quantities in alcoholic beverages to warrant further consideration.

5.4.2 Electrolytes and Functional Elements (Minerals and Trace Elements)

There is some inconsistency with regard to the classification and terminology involved in nutritional aspects of the elements. From geology, the traditional understanding of 'mineral' is a naturally occurring, physically constant, highly ordered and structured substance or compound that is formed from geological processes, and is distinct from 'animal' and 'vegetable.' However, in nutritional terminology, minerals have become synonymous with inorganic elements that are essential for maintaining human life. Therefore, the nutritional contribution of those essential inorganic elements most likely to be found in the more commonly consumed styles of alcoholic beverage will be presented according to their predominant biological functionality; electrolytes and elements will be considered as discrete nutritional entities (see website of the National Agricultural Library (NAL) at the United States Department of Agriculture (USDA): http://www.nal.usda.gov/. Sodium (Na^+ ion), potassium (K^+ ion), chloride (Cl^- ion) and inorganic sulfate (i.e. sulfur in association with oxygen to form the SO_4^{2-} ion) are considered the essential electrolytes and are present in the body in

relatively large amounts. The remaining elements of nutritional importance may be considered to fall into two classes representing those required in large and small amounts, in macro (or bulk) and micro (or trace) levels, respectively. Elements required in relatively large amounts include calcium, phosphorus and magnesium. Certain trace elements, such as copper, may only be required in small quantities, but are essential for the proper functioning of particular enzymes, for example in the electron transport chain. Iron and selenium are of particular interest currently because deficiencies in specific vulnerable population groups are becoming a potential threat to optimum health (British Nutrition Foundation – http://www.nutrition.org.uk).

Inorganic elements and electrolytes in beers originate essentially from the malt with significant contributions likely from the source water and also from any salts that may be used to adapt the mineral content of this water for taste preferences and character. In wines, the mineral content is determined predominantly by the fruits and juices used as raw ingredients, but some salts may be added during processing, such as K^+ or Na^+ and SO_4^{2-} from oxidation of sulfites (Section 2.5.2). The various amounts and ratios of a number of inorganic elements and electrolytes are influential in the production of alcoholic beverages and on their nutritional content because of a number of possible factors: flavors and character may be affected directly; the performance of yeast is optimized by the presence of essential minerals such as magnesium, manganese, copper, zinc and sulfur; and, pH (measure of acidity) is dependent on certain ions, such as calcium, that help to precipitate some proteins and phosphates, producing an increase in hydrogen ion concentration (lower pH; greater acidity) and improving key enzyme activity as a result (Section 2.2.3). Additional zinc is often needed for promoting fermentation, as there may be insufficient zinc present in the raw ingredients for optimum yeast activity (Vecseri-Hegyes *et al.*, 2005; 2006). Much of the calcium naturally present in the source water, or perhaps added during processing, is precipitated before the product is finished. Distillation tends to eliminate a majority of minerals and trace elements so that spirits and liqueurs may have insignificant amounts, if any, of the key elements needed for optimum health. Therefore, distillation products will not be considered in great detail as sources of minerals, despite there being measurable quantities present of some elements, as these are generally negligible or too low to significantly affect daily nutritional requirements even if excessive amounts of such alcoholic beverages are consumed. Typical concentration ranges of electrolytes and elements for generic styles of alcoholic beverages are presented in Table 5.4.2. General information regarding recommended dietary requirements can be found at the British Nutrition Foundation website – http://www.nutrition.org.uk; and in the Department of Health's 'COMA' Report – Department of Health, 1991).

Electrolytes

Potassium is an essential electrolyte, which helps to optimize balance of fluid across the cell membranes to ensure proper cell functioning. Adult males and females require about 3.5 g of potassium per day (RNI), ideally provided by fruits and vegetables, which are relatively good sources. A single serving of wine may typically provide about 100–190 mg (3–5% RNI). Similarly, a regular beer (330 ml – can sized serving) provides only about 75–100 mg (2–3% RNI), and perhaps slightly more in some products. However, as it is the relative amounts of potassium to sodium that are important to health (potassium is thought to counteract the potential high blood pressure exacerbations of high sodium) and as sodium is relatively low in wines and beers, the consumption of wines and beers have been purported to have some nutritional benefits or, at least, may not be detrimental to health in this particular respect.

The RNI for *sodium* is 1.6 g/day for adults in the UK. The average Western diet contains a great excess of sodium partly due to the addition of salt to foods during processing and consumption. However, not all foods and drinks are high in sodium, and these include wines and beers. A serving of wine may contain only a negligible amount, about 5–7 mg or 0.3–0.4% daily RNI, a regular beer provides about twice this amount, but this is still nutritionally insignificant, and distilled beverages much less so (approximately10 times less).

Addition of mixers to spirit and liqueur bases can influence the relative amounts of sodium and potassium provided. Cream liqueurs, for example, may provide negligible potassium, but about 2% of the sodium RNI in

Table 5.4.2 *Typical nutritionally significant electrolyte and element content of representative wines and beers* commonly consumed in the UK*[†]

	Electrolyte and Elemental content[‡]			
	Wine		Beer	
	/125 ml	/liter	/284 ml	/liter
Electrolytes				
Sulfate[§]	Variable	Variable	Variable	Variable
Potassium (mg)	75–160	600–1280	85–140	300–500
Sodium (mg)	5–15	40–120	17–45	60–160
Chloride (mg)	4–14	30–110	60–100	170–350
Elements**				
Calcium (mg)	9–18	70–140	14–26	40–90
Copper (mg)	Trace–0.08	Trace–0.6	Trace–0.2	Trace–0.7
Iron (mg)	0.3–1.3	2.7–10	Trace–0.6	Trace–2.0
Magnesium (mg)	9–15	70–120	14–28	50–100
Manganese (mg)	0.05–0.17	0.4–1.3	Trace–0.09	Trace–0.3
Phosphorus (mg)	8–29	60–230	31–57	110–200
Selenium (μg)	Trace–0.3	Trace–2.4	Trace–3.4	Trace–12
Silicon (mg)	Trace	Trace	30–80	90–240
Zinc (mg)	Trace–0.17	Trace–1.4	Trace–0.9	Trace–3.0

*Spirits are essentially devoid of nutritionally significant elements due to distillation processes during production and, therefore, are not presented. However, some electrolytes may be present as water may be added back to the distillation product in order to achieve a certain alcohol concentration.

[†]Typical guideline values are presented, drawn from a large number of sources considered to represent the range of each generic product most usually consumed in the UK (e.g. *McCance and Widdowson's The Composition of Foods* – Food Standards Agency, 2002). For comparative purposes, values are presented per serving of 125 ml wine and 284 ml (half pint) beer, and also per liter.

[‡]Wherever the value indicated is 'trace,' negligible or low amounts may be present, but which are insignificant in nutritional terms.

[§]Sulfate content can be quite variable, but alcoholic beverages are not a necessary source of sulfate.

**See text for details of other potentially significant elements.

one serving. Although, in Mexico, Tequila (Section 3.5.5) is drunk neat (possibly accompanied by sangrita – see Section 5.2.4), elsewhere it may be consumed with large amounts of the crystalline mineral salt (table salt, NaCl) and lime, favoring sodium intake relative to potassium. In contrast, unprocessed fruit juices, such as grape, orange and tomato juices, added to alcoholic beverages provide additional potassium (maybe 3–5% of the RNI per serving, depending on mix), with negligible amounts of sodium.

The UK RNI for *chloride* is 2.5 g/day for adults. As nutritional entities, chloride and sodium are normally considered together since they are the constituents of common or table salt (NaCl, comprising about 60% by weight of chlorine). Therefore, because of its equivalence to sodium, wines and regular beers contain very little chloride (less than 10% RNI), the levels of which are relatively insignificant when considered in perspective of the usual intake of 'salt' in the diet (8.1 g/day for women and 11 g/day for men in the UK which are far higher than daily recommended levels of no more than 6 g/day). The very much lower amounts of chloride in spirits, predominantly due to distillation, are nutritionally negligible.

There are no recommendations for intake of inorganic *sulfate* because of the ready availability of sulfates from foods and water. Inorganic sulfates are complemented by organic sources of sulfur provided by the

catabolism of sulfur containing compounds, such as the amino acids methionine and cysteine. Nevertheless, wines and beers may be major sources of inorganic sulfates, especially those that have sulfur compounds added for modifying flavors or for imparting preservative properties during processing. Some beers are produced from water that contains naturally high levels of calcium sulfate, which has a particularly desirable property as it draws out hop oils to impart a characteristic firm bitterness. This property has been extended to beers from areas where the source water lacks calcium sulfate by introducing this, or perhaps magnesium sulfate, into the brewing procedure, a process known as 'Burtonization,' to mimic the source water from Burton-on-Trent in England, which is naturally rich in sulfates (Section 2.6.2). Since the times of the Greeks and Egyptians, and still in widespread use, sulfur based compounds have been added to wines, beers and ciders as preservatives. Collectively termed 'sulfites,' being based on sulfur dioxide (SO_2), there are a number of such preservatives (Sections 2.5.2 and 2.5.3) that are generally considered to be relatively safe when added to foods and drinks, as they can be readily detoxified by the liver, to produce the electrolyte sulfate for example.

Although sulfur compounds from alcoholic beverages are not of any major nutritional significance in terms of the amounts provided when compared to those needed for maintenance of optimum health, they may be important because of their potential effect on the balance of bacteria present in the anaerobic environment of the lower intestine (Gibson *et al*., 1993). Essentially, in the absence of sulfate in the diet, the bacteria of the lower intestine are predominantly those that produce methane; these are the methanogens. However, sulfate reducing bacteria predominate in the lower intestine when sufficient sulfurous compounds are made available and so sulfates and sulfites provided by alcoholic beverages, along with requisite hydrogen, may reach the lower gut and be utilized by these sulfate reducers under the prevailing anaerobic conditions to produce the extremely toxic compound hydrogen sulfide. Therefore, it is these sulfate reducing bacteria that have been implicated as potential causative agents in a number of disease states, especially certain conditions of the gut such as colon cancer and inflammatory bowel diseases, which may be exacerbated in susceptible individuals. However, further work is needed to ascertain which, if any, of the many sulfate reducing bacteria are specifically responsible for causing these diseases, or whether or not other gut bacteria may also be implicated. Indeed, there is also uncertainty as to whether or not sulfate reducers are causative of these conditions or merely opportunistic, inhabiting an already inflamed gut, or there may also be synergistic actions with other microbes and, of course, the balance of such microbes may also be important (Gibson *et al.*, 1993). Also, the absolute amounts of sulfate actually derived from alcoholic beverage ingestion and actually reaching the lower intestine and, therefore, the overall nutritional significance is largely unknown.

Functional Elements

Calcium is required by the body in relatively large amounts, as it is important in bone structure and function, for maintaining the acid base balance of body fluids and in blood clotting. RNIs set in the UK are about 700 mg/day for adult men and women although adequate intakes for optimum bone health are considered to be higher in the USA, at about 1000 mg/day for both men and women from age 18 to 50 years, rising to about 1200 mg for those of 50 years and above (Institute of Medicine of the National Academies – http://www.iom.edu/). Calcium is present in relatively small amounts in wines (perhaps 8–15 mg per serving) contributing only about 1% of the daily dietary requirement per serving. Similarly in beers, about 1% of the required daily calcium intake is present in a serving of 330 ml of regular beer or lager. Higher amounts of calcium are possible in beers made from water naturally rich in calcium sulfate or to which calcium sulfate has been added (known as 'Burtonization'), although much of this calcium may be precipitated out as insoluble calcium phosphates, for example, resulting in lower amounts in the final product. Calcium is absent from distilled beverages unless ingredients containing calcium are added from mixers, e.g. milk products added to coffee flavored liqueurs or fruit juices added to spirits. However, the amounts provided are very low, perhaps only about 1% of daily recommendations from a serving of cream liqueur or 1–2% of the RNI from a tomato

juice mixer depending on amount used. Orange juice that is supplemented with calcium may possibly be added as a mixer to some beverages to provide nutritionally significant amounts of calcium (perhaps 150 mg per 100 ml), although this is not such a common practice. Incidentally, the fat soluble vitamin D may also be used as a supplement in some orange juices; vitamin D and its metabolites aid in the efficient absorption and utilization of calcium to maintain the integrity of bone, for example. However, there is negligible vitamin D, if any, in a vast majority of alcoholic beverages compared to the amount of vitamin D usually obtained from the action of sunlight on vitamin D precursors in the skin that occurs mainly between the months of March and September in the UK.

Obtained from whole grains and seeds, *copper* is present in finished beers up to perhaps 0.07 mg in a half pint (6% of UK RNI; 1.2 mg/day for adults). Wines may contain relatively more, up to about 0.12 mg per regular serving (10% RNI). However, certain authorities have in the past recommended greater amounts, up to 3 mg/day for optimum health, in which case any copper present in wines and beers would, clearly, be far less significant nutritionally. Less refined products containing more of the original ingredients, such as some locally produced beers, may contain relatively greater amounts of copper, and the use of copper vessels for production may allow copper to infuse the beverage to a small extent. However, the question remains as to whether or not such levels reach nutritional significance. Indeed, it is also uncertain as to what extent copper may be biologically available in the forms present in alcoholic beverages (Section 5.10.3). Copper is required for the proper functioning of a number of enzymes, especially those involved in oxidation and reduction processes, i.e. the electron chain (Section 5.8.2).

Almost all of the *iron* contained in the body is found in the red blood cells, where it is an integral part of the haemoglobin molecule that binds oxygen for transport around the body. Requirements for adult women up until the menopause (RNI 14.8 mg/day) are far higher than for men or postmenopausal women, both of which require 8.7 mg/day. Therefore, the amounts of iron found in wines (0.4–0.7 mg; 3–8% RNI depending on requirement) and in most regular beers (0.1 mg, about 1%) are not considered to be of nutritional importance, especially as iron from such sources (nonheme iron) may not be as biologically available as iron obtained from animal sources (heme iron). Some beers, stouts for example, are claimed to have higher levels of iron but, again, their nutritional value is uncertain mainly for this reason. If nonheme iron is ingested with vitamin C then it is suggested that greater amounts of iron are potentially absorbed through this interaction. However, some iron may be bound to certain other dietary constituents, preventing absorption in this form and so may be passed from the body without being absorbed.

Magnesium is an important cofactor for many key enzymes in the pathways of the body involved in energy balance and also has important electrolyte functionality. Relatively large amounts of magnesium are required to fulfill these functions. Adult males require about 300 mg/day RNI and adult females 270 mg/day, depending on age. Therefore, the provision of 10–18 mg in a serving of wine provides about 3–7% of the RNI and 20–25 mg in a serving of regular beer contributes about 7–9% of the RNI.

Phosphorus is a key element with a number of biological roles, usually in the form of its phosphate moiety, for example: (a) as a major component of hydroxyapatite, phosphorus is an integral chemical component of the skeleton, (b) phosphorus is essential in the transport and storage of energy mainly because of the ability of phosphate to bind to adenosine to form the high energy compound adenosine triphosphate (ATP), which is used for quick release of utilizable energy in times of threat, stress and trauma, and (c) phosphates contribute to the structure of the nucleic acids, RNA and DNA. The RNI for phosphorus in adult males and females is 550 mg/day, so that a serving of wine providing 15–35 mg contributes about 3–6% RNI and a regular beer might provide about 50 mg (9% RNI); wines and beers may, therefore, be nutritionally significant sources of phosphorus. However, negligible amounts (perhaps 1–2 mg) have been measured in distilled beverages. Milk and egg products are considered to be good sources of phosphorus and so may provide significant amounts if used as mixers for some types of alcoholic beverages or as ingredients of emulsion liqueurs (Section 3.9.5).

The levels of *selenium* in food products are, generally, very much determined by the amount of selenium present in the soil from which the plant food sources are harvested, and any additional processing may influence these levels even further. Selenium supports antioxidant functionality through its integration with certain enzymes, thyroid and immune function. The RNI for adults for selenium is 75 μg/day for men and 60 μg/day for women. Therefore, the 0.2–0.3 μg in a serving of wine is negligible nutritionally and 1–2 μg in a serving of beer (1–3% RNI) has only marginal significance.

Although *silicon* is implicated in the maintenance of healthy bone, particularly in the prevention and treatment of osteoporosis in older people (especially postmenopausal women), connective tissue and wound healing, there are currently no definitive guidelines for dietary intake. Silicon may have an important protective role against the effects of aluminium, which possibly include dementia and bone exacerbations. Alcoholic beverages do not appear to contain aluminium, but beers and ciders, especially, may contain significant levels of silicon. Silicon is found in foods such as cereals, of which barley is of particular interest in brewing, whole grain products (especially beer), some vegetables, some fruits, notably apples, and hard water. It has been speculated that the amount of silicon required may be as low as 5 mg to 10 mg per day, provided it is in a form in which it may be readily absorbed e.g. silicic acid, whereas others suggest 20–50 mg daily because much silicon may be ingested in the form of insoluble silicates that are absorbed relatively poorly. Beers may contain silicon in levels between about 10 mg and 40 mg/l and so apparently may make a very significant contribution towards this requirement, especially as the silicates in beer seem to have relatively greater bioavailability. Wines do not appear to have nutritionally significant levels of silicon and levels in spirits are negligible.

Daily *zinc* RNIs are set at 11 mg for adult males and 8 mg for adult females. Therefore, the low levels of zinc found in a serving of wine (up to about 0.2 mg; 1–2% RNI) do not appear to make a nutritionally significant contribution to dietary intake. Although there is also some zinc in whole grains and zinc congeners may be added, further processing may reduce its levels in beers to virtually nothing, perhaps by binding to the phytates present and that are removed during processing. Spirits appear to have no measurable zinc present because of the distillation process, even though zinc may be added during production to modify the character of the beverage. However, small amounts of zinc may be present in some cocktails such as cream liqueurs, but again these are not thought to be nutritionally significant (perhaps about 1% RNI per serving).

Amid much debate regarding the need for fluoridation of water, as it is needed to prevent dental caries, fluoride does not otherwise appear to be an essential nutrient and it may be toxic in excess. Alcoholic beverages are not considered important sources of fluoride, although levels may be marginally significant if fluoridated water is used in their production, e.g. tap water or ice added to spirits.

5.4.3 Phytochemicals

Knowledge regarding the vast number and diversity of the phytochemicals, compounds derived from secondary plant metabolism, particularly the bioactive phytoestrogens and antioxidants, is ever increasing, with major interest focused on their purported nutritional and health benefits, including antibiotic and anti-inflammatory potential (Section 5.11.2) (Bravo, 1998; Eastwood, 1999; Aron and Kennedy, 2008). In addition to the antioxidant capacity of vitamins, other naturally occurring antioxidants, largely phenolic compounds, are present in relatively high levels in a great majority of wines, especially red wines, beers and ciders, which may have a tangible influence on the antioxidant status of consumers (Chapter 5.8) (Soleas *et al.*, 1997; Ghiselli *et al.*, 2000; Tsang *et al.*, 2005; Zern and Fernandez, 2005). There are also potentially significant amounts of antioxidants in some spirits, notably those spirits that are matured in oak casks (see for example, Sections 3.2.5, 3.3.4, 3.5.3 and 3.6.2). Much of the antioxidant character of spirits originates from the wood via infusion of antioxidants into the beverage over time (Goldberg *et al.*, 1999; Madrera *et al.*, 2003). Some

wines and beers may also undergo extended maturation processes in which the nature and amounts of plant derived bioactives, notably the antioxidants, may be modified. Within each category of alcoholic beverage there is substantial variability in the type and amount of each of the component antioxidant molecules, depending on factors such as source of ingredients and processing techniques that lead to antioxidant capacity being considerably product dependent (e.g. Landrault *et al.*, 2001). A large number of different casks are used in the aging of spirits that may be made from a variety of woods and with different levels of charring of these woods, and such factors mainly determine the amount and diversity of phenolic compounds present. Indeed, some furans may also be present in beers and spirits that have both carcinogenic and anticarcinogenic potential, as well as antioxidant and anti-inflammatory capacity (Goldberg *et al.*, 1999).

Generally, the color and the intensity of color of both the raw ingredients and the final product are potential indicators of the relative content of antioxidants (Hancock *et al.*, 2007). Average dietary intake of polyphenols from all foods is about 1 g per day and major sources include alcoholic beverages, mainly wines and beers, along with tea, coffee, fruit juices and other miscellaneous plant derived foodstuffs (Paganga *et al.*, 1999; Scalbert and Williamson, 2000).

In addition to their antioxidant capacity, certain phytochemicals are also considered to have other potential biological effects and health benefits, particularly in relation to maintaining cardiovascular and bone health and with anticarcinogenic activities. Although these benefits are due in large part, but not entirely, to their ability to quench free radicals or reactive oxygen and nitrogen species (Bravo, 1998), additional effects appear to include the estrogenic or antiestrogenic properties of phytoestrogens, characterized by chemical structures closely mimicking those of the naturally occurring sex hormones of the human body, which enables binding to key receptors and enzymes involved in metabolism of these hormones, especially at lower doses (Rosenblum *et al.*, 1993; Milligan *et al.*, 2000). Some of these effects are implicated in protective roles but others are not.

Although not ubiquitously considered essential to sustain human life, these multifarious compounds are of growing interest, as their consumption has been implicated in many and various benefits to health, especially in reducing cardiovascular health risks and in combating other degenerative diseases. The use of 'essential' in the nutritional sense implies that morbidity and mortality are inevitable without an exogenous source of such compounds in the diet – as would be the case with vitamin deficiencies, for example. In this particular sense, phenolic compounds and other phytochemicals are generally considered to be nonessential in nature and there is still some controversy surrounding the validity of claims that these compounds actually do have such an extensive range of health benefits. Indeed, in some quarters they are not considered to be nutrients on this basis and have even been labeled as 'antinutrients,' despite having recognized nutritional value in other ways (Bravo, 1998). According to dictionary definitions, nutrients are nourishment and as nourishment sustains 'proper condition,' perhaps beneficial phytochemicals should be considered as nutritionally significant, if not essential. In contrast, it is because of their ability to bind and precipitate proteins that some polyphenols, especially the larger molecular weight tannins, have been termed 'antinutrients,' as they appear to have potential adverse effects on digestion and absorption of proteins and may deactivate important functional proteins. For example, digestive enzymes may be impaired, which may interfere with digestion and absorption of other key dietary nutrients (Bravo, 1998). Polyphenols also have metal chelating capabilities (Section 5.8.7) (Bravo, 1998), which may be of detriment if such chelation interferes with the normal absorption and assimilation of valuable elements. In contrast, these binding capabilities may also extend to certain potential toxins, which may be of benefit if they can be eliminated from the body before being absorbed (Section 5.8.9).

Natural derivatives of the shikimate and acetate pathways in plant metabolism (Soleas *et al.*, 1997), there are a vast number of diverse and often complex phenolic phytochemicals that may be present in those plants consumed by humans (well in excess of 8000 presently described in the literature, of which greater that 5000 are flavonoids), considerable numbers of which may be found in alcoholic beverages. Each one of the phenolic compounds has in common at least one aromatic ring and at least one hydroxyl group, which are responsible for the antioxidant properties of these molecules. It is the molecular similarity to estrogen, particularly the

essential phenolic ring, which provides phenolic compounds with their estrogenic or antiestrogenic activities. More complex esterified, methylated and other derivatives may also be found naturally occurring and include phenol/coumaric acid and pyrocatechol/caffeic acid derived lignins, for example. Not all of these phenolic compounds find their way into alcoholic beverages and not all that are present in the final product have been measured as yet.

Polyphenols (consisting essentially of flavonoids and phenolic acids with smaller amounts of stilbenes and lignans, for example) (see Chapter 5.8) are the predominant sources, perhaps up to about 90%, of the antioxidant capacity present in the raw ingredients of alcoholic beverages, particularly the skins and seeds of grapes and also in malt and hops. Most polyphenols are extracted effectively during fermentation and may be quite well preserved in the final product, even if perhaps some are chemically altered or lost during later stages of production (Fantozzi *et al.*, 1998). Indeed, the production of some alcoholic beverages, notably certain red wines, may effectively concentrate these antioxidants to greater levels in the final product; the level in red wine could be up to four or five times the levels found in the ingredient grape juices. However, some potential antioxidants may be lost through precipitation. Once ingested, the bioavailability – absorption and metabolism – of polyphenols is determined by their particular chemical structures, molecular size, degree of polymerization and solubility, combined with levels of glycolyzation, acylation and conjugation, and possible synergism with other dietary components (Section 5.8.9). Indeed, in a specific example, anthocyanins were shown to be better absorbed when administered in grape juice than red wine, that led the authors to suggest a possible synergism with the glucose component of the grape juice (Bitsch *et al.*, 2004), although lower rates of absorption due to interactions with other components of the wine is possible. However, many polyphenols may not be ingested in an absorbable form and also may be indigestible by enzymes in the upper gastrointestinal tract, so pass into the colon where some of them may be metabolized by colonic bacteria (Section 5.8.9) (Bravo, 1998).

The nutritional importance to human health of phenolic compounds present in alcoholic beverages is uncertain for a number of reasons. As the antioxidant capacity of phenols and polyphenols is known to be variable between the different structural classes and the different compounds within these classes, the levels needed to have a significant effect on the human body is difficult to elucidate. The total antioxidant capacity may be a key factor; this is illustrated by the fact that red wine, with a mean concentration of total phenolic acids and polyphenols of about 1200 mg/l (typical range 900–2500 mg/l), has a greater purported potential health benefit than white wine, with a typical range of 190–290 mg/l. Interestingly, white wines that have been fermented with the skins of the grape apparently have greater antioxidant capacity than those that do not have the grape skin present so that the polyphenols cannot be extracted from it (Fuhrman *et al.*, 2001). However, even though certain classes or individual antioxidant compounds may be present in greater amounts in some alcoholic beverages than others, they may not have the same level of antioxidant capacity and be relatively less effective biologically, as well as having different levels of bioavailability (Soleas *et al.*, 1997; Bravo, 1998). Certain antioxidants in beers are purported to have as great or greater antioxidant capacity when compared to those present in wines, with greater potential for health benefits implied (for discussion see 'Beer and Health' in Hornsey, 1999 – Chapter 9). Also, although, in general, concentrations of antioxidants in beers may be lower than in wines, beer is usually consumed in greater quantities than wine so that the available antioxidant load may be at least comparable. The presence of alcohol may also be significant, as it appears to aid absorption of some compounds and may also cause chemical changes that increase antioxidant potential, or simply help to preserve it. The antioxidant potential of beer was suggested to be due, at least in part, to enhancement by ethanol per se (Ghiselli *et al.*, 2000). However, experiments comparing the antioxidant enhancing properties of ethanol consumption alone with alcoholic beverages containing antioxidant phytochemicals showed that there was little or no effect of ethanol per se and, as there was no apparent major effect of antioxidants from some nonalcoholic beverages, such evidence indicates that ethanol may act by simply aiding antioxidant absorption. In contrast, higher levels of absorbed anthocyanins were observed with grape juice ingestion compared to equivalent amounts taken in red wine, which the authors attributed to a synergistic effect of

glucose (Bitsch *et al.*, 2004). Interestingly, ferulic acid uptake if administered in beer is consistent with absorption from other sources in the diet, whole tomatoes for example (Bourne *et al.*, 2000).

The ability of the human body to digest, assimilate and effectively utilize the different phenolic antioxidants is also a little uncertain, as is the relative importance of their interactions with other nutrients. For example, polyphenols are known to be capable of binding with nonheme iron, a factor that could influence the digestion of both these important nutrients, potentially preventing proper absorption and thereby compromising both nutrition and health status. A number of polyphenols, especially some of the larger and more complex flavonoids (e.g. the tannins) are not readily digested and are, therefore, not readily absorbed in large amounts. However, many of the multifarious phenolics present in alcoholic beverages, provided that they are in certain forms, may be absorbed by the human intestine in quantities sufficient for health benefits; these include nonflavonoids, such as stilbenes and anthocyanins, and some flavanols are also capable of being absorbed. The active antioxidant group of polyphenols is the hydroxyl moiety which makes possible metabolic processing by the intestines as these compounds are being absorbed, and then by further metabolism in the liver and kidneys. By these processes, various conjugates may be formed, some of which retain and even enhance the original antioxidant capabilities of these phenolic compounds (Section 5.8.9). However, regular and continuous consumption of a sufficient amount and wide variety of phenolic compounds may be needed to maintain antioxidant potential for real and lasting benefit to health because of their rapid turnover, in terms of relatively high rates of absorption, assimilation and excretion.

As individual compounds, the antioxidant potential and other nutritional properties of polyphenols may not be sufficient to be nutritionally effective, but collectively there may be nutritional and health benefits (Eastwood, 1999). Indeed, the possible synergism of polyphenol actions may extend to include other antioxidants, such as some of the vitamins and minerals, to maintain a reducing environment. Glutathione is a very important molecule in this role because it has strong antioxidant properties when in the reduced form (GSH) that protects the cells against toxins, especially free radicals or highly reactive oxygen species (Wu *et al.*, 2004; Franco *et al.*, 2007). The equilibrium between the oxidized (GSSG) and reduced (GSH) forms of glutathione favors GSH when antioxidants are sufficient to maintain such a reducing environment. Alcohol catabolism is itself responsible for production of highly reactive free radicals. It has been suggested that because wines and beers contain significant levels of multifarious antioxidants they may be of significant benefit in maintaining this important balance by helping to keep GSH in its reduced form. Glutathione also appears to have an antioxidant role in the beers and wines themselves because its presence prevents chemical oxidation of vulnerable components and preserving the taste and character of such beverages, but glutathione ingested in this form is probably of limited nutritional value, as it is broken down in the intestine and so makes only a negligible contribution to daily protein requirements (see Section 5.3.4).

Plant derived bioactives extend to include many other classes of compounds. For example, certain members of the furan class of chemicals have antioxidant capacity and some have anticancer properties, although others may be toxic and carcinogenic (Goldberg *et al.*, 1999). Furans are derived from thermal decomposition of pentose based compounds (cellulose) and may be present in beers, wines and spirits, adding to the antioxidant capacities of vitamins and polyphenols also present.

Phytochemicals in Wines

Wines, especially red wines, are rich sources of bioactive phytochemicals (Soleas *et al.*, 1997; López-Vélez, *et al.*, 2003). There are well in excess of 2000 phenol based compounds found in wines, derived predominantly from the grape (van de Wiel *et al.*, 2001), the antioxidant properties of which are reputed to be responsible for the 'French paradox' because they confer cardioprotective effects through mechanisms opposing development of atherosclerosis, i.e. by helping to minimize oxidation of cholesterol (Puddey *et al.*, 2003). These phytochemicals come mainly from the seeds, skin and stems of the grape and there are generally greater levels in red wines than in white wines because such extraneous materials are allowed to remain in the

fermentation mixture for red wines, but which are usually removed for white wine fermentation (with some exceptions). The anaerobic environment of the fermentation process in wine manufacture not only produces alcohol but also modifies the phenolic content of the juice to alter antioxidant potential and enhance product stability. A comprehensive discussion and description of the numerous polyphenols found in red and white wines is presented by German and Walzem (2000; with an informative summary comparison presented by these authors in their Table 2, page 570). Furans may be present in wines that have been aged in wooden casks e.g. charred oak, due to leeching out.

Phytochemicals in Beers

The array of antioxidants originating from barley and malt has significance to both manufacturer and consumer, as they help to prevent the development of staleness in beer (e.g. Lu *et al.*, 2007), and many of them may be found in significant levels in finished beers. These and other plant bioactives, such as active phenolic derivatives or those originating from hops and added ingredients, may have importance because of their potential contribution to health and nutritional status of the moderate consumer through antioxidant (Ghiselli *et al.*, 2000) and estrogenic effects (Rosenblum *et al.*, 1993; Milligan *et al.*, 2000), some of which may be undesirable, especially in men. Potentially significant bioactive phytochemicals reported to be present in beer and mainly derived from hops include humulone (an α-acid), lupulone (a β-acid), phytoestrogens such as the relatively potent 8-prenyl-naringenin (hopein), daidzein and genistein, xanthohumol, a polyphenol of the chalcone group, flavonones, *iso*-xanthohumol, 6-isoprenylnaringenin (for discussion see 'Beer and Health' in Hornsey, 1999 – Chapter 9). The pooled concentration of polyphenols may decrease during the various stages of the brewing process, mainly because of precipitation of insoluble tannins and flavonoids (Fantozzi *et al.*, 1998). However, there are significant levels present in the final product, especially in the darker beers, and these are linked with alterations in metabolism leading to improved health, particularly lessening cardiovascular risk (Denke, 2000; Baxter and Hughes, 2001; Kondo, 2004; Gorinstein *et al.*, 2007).

Phytochemicals in Spirits (and Liqueurs)

Significant antioxidant capacity may be found in certain spirits and liqueurs, the levels of which depend on the particular production methods and aging processes involved during manufacture (e.g. Goldberg *et al.*, 1999; Madrera *et al.*, 2003). Some phenolic compounds derived from the original ingredients and those produced through the fermentation processes may possibly find their way into the products of distillation, but probably only in trace amounts at best. Other polyphenols and some furans, which are derived from thermal decomposition of cellulose based matter formed from charring of the wood, may be leeched out of casks used for maturing distilled beverages, producing significant antioxidant and estrogenic properties in some whiskeys, brandies and rums (e.g. Rosenblum *et al.*, 1993). Spirits produced without processes of aging in these wooden casks, such as vodka, gin, many fruit spirits and grappa, have been found to be largely devoid of antioxidant capacity, to such an extent that an effective negative oxidation capacity may be demonstrable (Goldberg *et al.*, 1999).

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5.5

Alcohol Ingestion, Absorption and Catabolism: Metabolic and Nutritional Consequences

Consumed as a component of the diet or produced by the action of microorganisms present in the gut on the contents passing through the gut, alcohol, as a small water soluble molecule, may be absorbed into the blood along the whole length of the gastrointestinal tract. The rate of absorption is determined by a number of factors including: (a) alcohol concentration, (b) the type of alcoholic beverage consumed, (c) how quickly it is consumed, (d) the presence of any food and the amount and type of this food taken at and around the same time as the alcoholic drink, (e) the point of alcohol absorption in the gastrointestinal tract and (f) the extent to which any alcohol present has been broken down by the enzyme alcohol dehydrogenase (ADH) before it can be absorbed. Such factors are known to be interactive and variable and so contribute to differential rates of absorption both within and between individuals (Morgan and Ritson, 2003; Paton and Touquet, 2005; Crabb *et al*., 2004).

The rate of alcohol absorption is dependent on its concentration, being the highest from beverages containing about 20% to 30% alcohol, and on the speed of its consumption. Alcohol consumed in aerated beverages is more readily absorbed, as is alcohol taken on an empty stomach (Paton and Touquet, 2005). The amount and type of food taken at or around the same time as the alcoholic beverage contributes to the rate of alcohol absorption, predominantly because the rate of emptying of gastric contents into the small intestine is reduced by the presence of food. Spirits also reduce the rate of gastric emptying. Therefore, as the stomach absorbs alcohol relatively slowly compared to the faster rate of absorption from the small intestine, any alcohol that is retained in the stomach for longer periods effectively slows its rate of absorption. A number of drugs may act by either increasing or decreasing the rate of gastric emptying. Additionally, although a large proportion of alcohol in the stomach may be metabolized by very active ADH enzymes in certain individuals with such capacity before being absorbed into the blood (Crabb *et al.*, 2004), other individuals may not have this capability. In such susceptible individuals, a relatively greater proportion of the alcohol ingested may be absorbed, with the potential for greater intoxication and harm, although the amount ingested overall may be lessened as a result.

Once it is absorbed, alcohol passes via the portal vein to the liver where it is metabolized, the extent of which depends on the capacity of the enzyme systems that it encounters relative to the amount of alcohol

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

present. Any alcohol that passes through the liver without being metabolized distributes within the water compartments of the various body tissues through the systemic circulation.

The human body has no capacity for the storage of alcohol per se and so, as it is also highly toxic, alcohol needs to be eliminated from the body or detoxified with expedience. The largest part, by far, of any alcohol ingested by the body or, indeed, any that is produced by microorganisms in the intestinal tract, is metabolized by the liver, with much lower levels metabolized in other tissues, and perhaps only about 2–5% being eliminated unchanged in urine, breath and sweat. However, the liver has a limited capacity for alcohol breakdown resulting in increasing blood alcohol levels during periods where ingestion exceeds this capacity, with concomitant increased risk of organ and tissue damage. Typical alcohol metabolism is about 6 g/h, but the actual rate for individuals varies considerably. Blood alcohol concentrations after consumption of alcoholic beverages in the absence of food reach their maximum quite quickly, perhaps within 30 min for a single drink with a much higher peak occurring later as consumption increases (Wilkinson *et al.*, 1977), with maximum levels and the times of achieving these peaks depending on quantity ingested and prevailing conditions of absorption and assimilation. Following cessation of alcohol consumption, blood levels gradually decrease with alcohol breakdown in the liver and other routes of elimination as the blood circulates the body, with the extent of alcohol exposure varying with total amount and rate consumed. It is important, especially for moderate and 'sensible' drinkers, to be fully aware that blood alcohol levels reach a lower maximum peak and remain lower if an equivalent quantity of alcohol is consumed over a longer time period.

Alcohol consumption and catabolism has potentially serious implications for health, especially in terms of liver disease (Section 5.6.2), by generating oxidative stress, particularly through the cytochrome P450 CYP 2E1. In addition, alcohol intake may effectively displace the intake of other nutrients and alcohol and its metabolites may interfere with certain metabolic pathways of a number of key nutrients, even if dietary intake of these nutrients may be apparently adequate (supposedly meeting those levels of nutrients required for optimum health in the absence of alcohol). As CYP 2E1 also converts other xenobiotics into highly toxic intermediates, alcohol may also act synergistically on CYP 2E1 with such xenobiotics (e.g. paracetamol), and especially in the fasted state, to deplete glutathione (GSH) levels (Section 5.3.4) and significantly contribute further to oxidative stress. Provision of the amino acid cysteine may be used to replenish GSH therapeutically.

5.5.1 Alcohol Catabolism

The pathways of alcohol metabolism result in certain intermediary compounds that may be more or less toxic than alcohol itself and which, along with other nutrients ingested at the same time or perhaps as integral parts of alcoholic beverages themselves, impact in various ways on nutrition and health, either detrimentally or beneficially. The first biochemical reaction of alcohol breakdown, or catabolism, is catalyzed by the enzyme ADH, which is a zinc metalloenzyme that exists in multiple forms, or isoenzymes. The presence of some of these isoenzyme polymorphisms, although they may have the potential to oxidize alcohol at different rates, does not appear to have a major effect *in vivo* under normal circumstances in most populations. However, in certain populations, relatively inefficient or nonfunctional ADH polymorphisms may limit the ability to tolerate even low levels of alcohol. An isoenzyme of ADH, along with cytochrome P450, with potential for oxidizing alcohol is present within the gastric mucosa, but, although it has been suggested that up to about 20% of that ingested may be oxidized in the stomach by this route, the exact structure and role of this particular isoenzyme in alcohol metabolism overall is relatively unknown.

In order to prevent or minimize any possible damage caused by ingested alcohol reaching susceptible cells in key organs and tissue systems supplied by the systemic circulation, it is important to understand the potential extent of action of the ADH enzymes essentially present in the stomach and liver. Therefore, the concept of 'first pass metabolism' (FPM, or presystemic metabolism) has been applied. The FPM is the amount of alcohol broken down by ADH enzyme action in the stomach and liver (the predominant organ

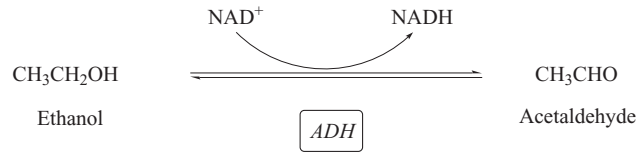

Figure 5.5.1 *The conversion of ethanol ('alcohol') to acetaldehyde. ADH is alcohol dehydrogenase; NAD is nicotinamide adenine dinucleotide*

for alcohol detoxification) to attenuate the potential systemic effects of alcohol. The extent of FPM may be determined practically by administration of ethanol either orally or intravenously followed by a sequence of blood alcohol level measurements which are used to: (a) construct a graph of alcohol concentration against time for each route of administration, (b) obtain by integration the areas under the curves for each of these routes and (c) calculate the difference between these areas under the curves to provide a measure of FPM. It is interesting that large numbers of individuals in certain populations do not have active gastric ADH enzymes; in particular, about 30% of some Asian populations lack active ADH and women generally have lower ADH levels than men (e.g. Frezza *et al.*, 1990). A particularly good descriptive account of the implications of different expressions of ADH polymorphisms (and acetaldehyde dehydrogenase) is that given by Crabb *et al.* (2004).

Alcohol takes precedence in the hierarchy of macronutrients to be oxidized, followed sequentially by carbohydrates, proteins and fats, with the oxidation of these other macronutrients, particularly fats, being suppressed temporarily during the disposal of alcohol, which occurs to a greater or lesser extent, depending on the amount of alcohol present. There is also some controversy regarding the efficiency of utilization of the energy provided by alcohol oxidation (Prentice, 1995). The products of alcohol catabolism are variously utilized by the usual pathways and cycles of intermediary metabolism, potentially creating an imbalance in the normal metabolic flux through such pathways, especially in times of excessive alcohol consumption (see Section 5.5.4).

In healthy individuals with a moderate alcohol intake, the first stage in the catabolism and detoxification of alcohol, predominantly in the liver, involves the cytosolic enzyme ADH and chemical reduction of the oxidized form of the cofactor nicotinamide adenine dinucleotide (NAD^+) to NADH (Figure 5.5.1); this reaction catalyzes the conversion of alcohol to acetaldehyde, an intermediary product that is more toxic than alcohol, but which is rapidly converted to acetate and then to other harmless products.

In consumers of higher levels of alcohol and in alcoholics, there is the alternative mixed function oxidase pathway of the smooth endoplasmic reticulum, a cellular organelle, that also produces acetaldehyde from alcohol. The microsomal ethanol oxidizing system (MEOS) of the cytochrome CYP 2E1 isoform of P450 catabolizes alcohol in conjunction with the reduced form of the cofactor nicotinamide adenine dinucleotide phosphate (NADPH), hydrogen ions and oxygen to form acetaldehyde, the cofactor $NADP^+$ in its oxidized state and hydrogen peroxide (Figure 5.5.2). The potential activity of this enzyme may be increased

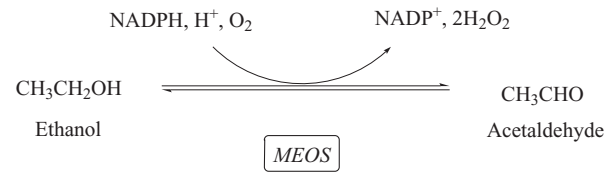

Figure 5.5.2 *The conversion of ethanol to acetaldehyde. MEOS is microsomal ethanol oxidizing system; NADP is nicotinamide adenine dinucleotide phosphate*

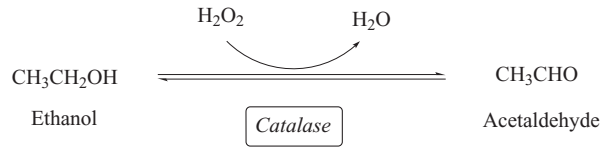

Figure 5.5.3 *The conversion of ethanol to acetaldehyde using the enzyme catalase*

significantly as a result of chronic use or abuse of alcohol. The MEOS system uncouples oxidative phosphorylation to release some of the energy contained in alcohol for dissipation as heat rather than storage. Oxidative phosphorylation would normally be the pathway involved in converting metabolic energy released through alcohol catabolism to formation of high energy phosphate bonds in ATP. Such uncoupling and energy release may be partially responsible for limiting the potential weight gain from excess alcohol intake (Prentice, 1995).

The ADH pathway is dominant at low levels of alcohol because the reaction constant or K_m for this enzyme reaction (the K_m of a reaction is a constant that indicates the potential rate of the reaction) is much lower than that for the MEOS pathway, which may become more significant at higher alcohol levels, and this rate may be increased up to about 10-fold. Alcohol may also be oxidized by the enzyme catalase, which is located in the peroxisomes (Figure 5.5.3). However, this particular pathway is not believed to make a significant contribution to alcohol catabolism under normal physiological conditions (Morgan and Ritson, 2003). Whichever pathway has been involved in the first stage of breakdown of alcohol, it is essential that the acetaldehyde produced is further metabolized to limit its toxicity, as it has the potential to damage both functional and structural components of cells by forming adducts with a number of important molecules such as DNA, RNA and proteins.

Acetaldehyde is converted to acetate by two isoenzymes of acetaldehyde dehydrogenase, located in the cytosol and in mitochondria, and involves the integral conversion of the cofactor NAD^+ to NADH (Figure 5.5.4). Polymorphisms of acetaldehyde dehydrogenase are known to exist which are associated with interindividual differences in the efficiency of alcohol breakdown and this may be, in part at least, the basis of differences between the sexes in dealing with the after effects of intake of alcoholic beverages. It is believed that individuals lacking sufficient acetaldehyde dehydrogenase activity may show the characteristic signs of reddening in the face due to build up of acetaldehyde.

Alcohol catabolism has, at this point, produced the nontoxic derivative, acetate; this acetate is now condensed with coenzyme A in a reaction in the cytosol that also involves ATP to form acetyl coenzyme A (acetyl Co-A), with the production of the side products pyrophosphate (PPi) and AMP (Figure 5.5.5). Acetyl Co-A can now enter the tricarboxylic acid (TCA) cycle (also known as the citric acid cycle or Kreb's cycle), the enzymes of which are situated in the mitochondrion, where acetyl Co-A is used in aerobic respiration, to be eventually broken down to produce CO_2, water and energy. The TCA cycle is more normally instigated

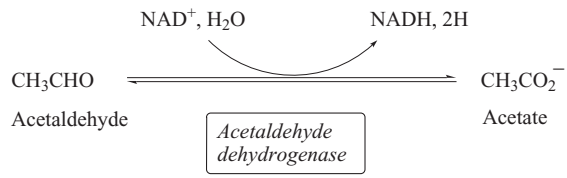

Figure 5.5.4 *The conversion of acetaldehyde to acetate. NAD is nicotinamide adenine dinucleotide*

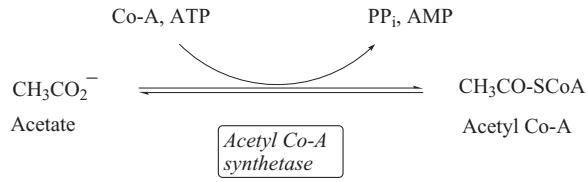

Figure 5.5.5 *The conversion of acetate to acetyl Co-A. Co-A is coenzyme A; ATP is adenosine tri-phosphate; PP_i is inorganic phosphate; AMP is adenosine mono-phosphate*

by acetyl Co-A derived from pyruvate and its interaction with the pyruvate dehydrogenase complex. Alternatively, in some circumstances, such as those that might occur after alcohol consumption, acetyl CoA can also be used as a substrate for lipogenesis, the synthesis of molecules of fatty acids that can be stored in the adipose tissue of the body.

It is evident that alcohol ingestion may have a number of metabolic sequelae that could have major implications for health, as described in Chapter 5.6.

5.5.2 Methanol Catabolism

Methanol is another primary alcohol found in alcoholic beverages in varying amounts according to type of beverage, with the highest concentrations, up to about 2% by volume, being present in dark wines and spirits, such as red wines, brandies and whiskies. The metabolism of methanol follows a similar pathway to that of ethanol (Figure 5.5.6), but is reported to be much slower, by about 10-fold. The catabolism of methanol by ADH produces formaldehyde, which is very toxic indeed.

Formaldehyde is converted to another highly toxic intermediary, formate (formic acid), using enzymes with aldehyde dehydrogenase activity (Figure 5.5.7); formaldehyde dehydrogenase and acetaldehyde dehydrogenase with this capability both appear to be active in this process in the liver, but some evidence suggests that formaldehyde dehydrogenase and alcohol dehydrogenase are so similar that they may essentially be the same enzyme.

Formate is a metabolic intermediate of one carbon compounds, with its carbon atom appearing in methyl groups for transmethylation, eventually being oxidized to carbon dioxide, although some formate may be excreted unchanged in the urine.

5.5.3 Effects of Alcohol on Intake of Foods and Energy

Amounts and patterns of food intake are differentially influenced by alcohol consumption, the extent of which depends on acute or chronic use or whether it is low to moderate or excessive consumption during particular

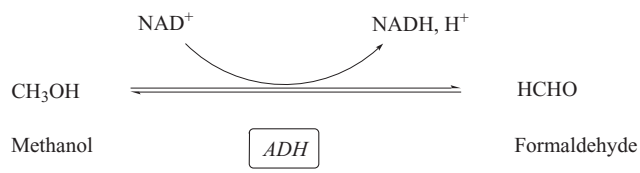

Figure 5.5.6 *The conversion of methanol to formaldehyde. ADH is alcohol dehydrogenase; NAD is nicotinamide adenine dinucleotide*

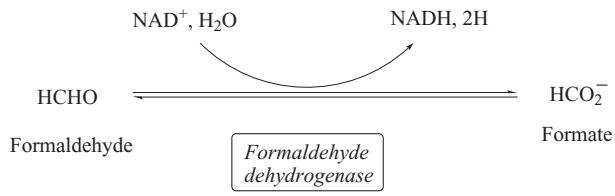

Figure 5.5.7 *The conversion of formaldehyde to formate. NAD is nicotinamide adenine dinucleotide*

time frames (Forsander, 1998; Yeomans *et al.*, 2003; Yeomans, 2004; Caton *et al.*, 2007; Kokavec, 2008). It appears that alcohol consumed on a single occasion has a short term stimulatory influence on appetite, the 'aperitif effect,' which may increase food intake in the short term, provided that any food is taken within one hour, otherwise such stimulatory effects may not be maintained. Food intake in the mid- to longer-term may then be reduced as a compensatory response. Increases in orosensory mechanisms and/or impaired satiety mechanisms have been implicated in these appetite stimulatory effects of alcohol, or the disinhibition of restrained eating (Yeomans, 2004). Primarily, alcohol acts centrally by stimulating γ-aminobutyric acid receptors, as well as having other pharmacological effects implicated in appetite acting through, for example, leptin, serotonin, glucagon-like peptide-1, neuropeptide Y and endogenous opioids. There may also be the effects of expectation. The timing of alcohol intake is also important, as its pharmaceutical actions occur sooner than its nutritive effects. However, any energy consumed in the form of alcohol (7.1 kcal/g) is considered per se to be an additional passive intake in excess of energy requirements as, from a limited body of evidence in humans, there is no compensatory response to any additional intake of energy from alcohol. In animals it appears that any additional energy intake obtained from alcohol is compensated for by a reduction in food intake, primarily in the form of carbohydrates if all macronutrients are made freely available. Therefore, regular consumption of alcohol in moderation may effectively increase habitual energy intake because of the additional provision of calories from the alcohol and the induced increase in food intake, which is not subject to compensation due to the continued alcohol intake.

Alcohol intake in humans and animals is influenced by relative consumption of the macronutrients; in experimental conditions high protein and low carbohydrate diets tend to lower alcohol consumption, whereas high carbohydrate and low protein tends to increase intake. Such factors may be less influential in high consumers and alcoholics as satiety mechanisms may be outweighed by other more compelling factors. The overall effects of these factors on energy balance and maintenance of body weight is uncertain and varies considerably between individuals because of additional factors such as possible compensatory metabolic mechanisms and changes in physical activity. These issues are reviewed by Prentice (1995) and Yeomans (2004).

5.5.4 Consequences for Intermediary Metabolism of Alcohol Catabolism

The products of complete alcohol detoxification are now indistinguishable from regular intermediary substrates and cofactors universally involved in various reactions within the normal metabolic processes. However, such substrates and cofactors may be produced in disproportionate amounts to those normally encountered in intermediary metabolism and their excess may adversely influence the normal functioning of key enzyme processes in certain pathways and cycles. This leads to changes in the normal flux of intermediary substrates, diverting them into alternative pathways, which may then lead to further metabolic exacerbations.

Alcohol catabolism may result in metabolic imbalances because of the increased reduction of NAD^+ to NADH through reactions catalyzed by the enzymes alcohol dehydrogenase and acetaldehyde dehydrogenase. The generation of reducing equivalents may exceed capacity for redox homoeostasis, leading to these metabolic disturbances. In the cytosol, the rate of conversion of glucose to pyruvate during glycolysis may be impaired because of the decreased availability of NAD^+ for the step that converts glyceraldehyde-3-phosphate to 1-3-biphosphoglycerate within the 'pay off' phase of glycolysis. The pay off phase is so called because of the net gain of ATP and NADH from this phase, as opposed to the 'preparatory' or 'investment' phase of glycolysis, which initially consumes energy for the pathway to generate appropriate substrates for this pay off phase. Therefore, lower levels of energy rich products will result in conditions that favor the reduced state of the cofactor, i.e. the NADH form.

Although NADH is also a normal product of glycolysis, it is essential that adversely high levels, perhaps derived from alcohol catabolism, are oxidized back to NAD^+ to prevent metabolic complications, such as acidosis. In the cytosol, effectively facilitating this oxidation can occur through, primarily, the malate–aspartate shuttle or, of secondary importance, the glycerol–phosphate shuttle, both of which shunt these reducing equivalents to the mitochondria where electrons are eventually coupled to the high energy containing molecule adenosine triphosphate (ATP), which is produced through the process of oxidative phosphorylation. These shuttles are essentially dependent on substrate concentration. However, the extent to which the whole process can occur is reliant on the rate of functioning of the TCA cycle within the mitochondria, as this may be constrained by build up of cellular energy levels and NADH. Increased levels of cellular energy and NADH, derived from the mitochondrial conversion of acetaldehyde to acetate by acetaldehyde dehydrogenase during alcohol catabolism, may limit the rate of further mitochondrial conversion of NAD^+ to NADH, thus slowing flux of substrates through the malate–aspartate and glycerol–phosphate shuttles. Reactions within the TCA cycle that involve the NAD^+/NADH cofactor and, therefore, those that are potentially constrained by increases in the ratio of NADH:NAD^+ are: isocitrate to oxalosuccinate, α-ketoglutarate to succinyl-CoA and malate to oxaloacetate. Thus, three molecules of NADH are generated from NAD^+ for every acetyl group entering the TCA cycle.

Increased amounts of NADH may also impact on the process of gluconeogenesis. The normal metabolic fate of the substrate pyruvate is the production of glucose. Pyruvate is derived from usual metabolic processes such as transamination of amino acids. NADH affects the rate of interconversion of pyruvate and lactate by favoring lactate production through its influence on the reversible reaction involving the enzyme lactate dehydrogenase, so effectively reducing gluconeogenesis from pyruvate, as this pathway is inhibited by low concentrations of pyruvate. The consequent lactate build up generates acidosis and potential hypoglycaemia occurs with the lower rate of glucose synthesis. Lactate also interferes with the ability of the kidney to excrete uric acid, and uric acid build up by this route may contribute towards hyperuricaemia leading to gout. This may also occur through ketosis and acetate production induced by excess alcohol combined with ATP catabolism or through purine production. Of all factors that contribute to hyperuricaemia, the influence of alcohol on metabolism of the purine bases hypoxanthine, xanthine and uric acid is one of the most significant (Yamamoto *et al.*, 2005 and Section 5.3.4).

Alcohol induced lipid synthesis may occur through two metabolic pathways that produce glycerol and fatty acids, with the result that heavy drinkers and alcoholics may gain body weight initially through increased fat deposition. The electron transport chain may be an alternative beneficiary of NADH, synthesizing ATP as a ready source of energy, a reaction that inhibits the normal oxidation of fats in the fatty acid spiral and TCA cycle and allowing fats or acetyl Co-A to accumulate with the concomitant production of ketone bodies. Additionally, positive lipid balance may also be enhanced as the rate of fatty acid oxidation may be reduced through its reliance on sufficient concentrations of the oxidized form of the cofactor, NAD^+. Although ethanol stimulates hepatic lipogenesis to raise plasma triglycerides, it also increases metabolism of triglyceride rich

lipoproteins in the periphery, because of induced lipoprotein lipase activity, and increases apolipoprotein synthesis (ApoA1 and ApoA11) in hepatic cells with the net effect of increasing the plasma high density lipoprotein (HDL) fraction; raised levels of HDL are considered to be a negative risk factor for coronary heart disease. For general discussion of cardiovascular implications of alcoholic beverages, see Puddey *et al.* (2003).

It is the toxicity of alcohol and its intermediates in conjunction with its effects on metabolic imbalance that contribute to the health risks and, indeed, hangover associated with consumption of alcoholic beverages (Wiese *et al.*, 2000), especially excessive consumption. For example, the aldehydes are toxic because they are capable of forming adducts with proteins and nucleic acids, as well as interfering with other key biochemical compounds and reactions.

It is argued that the suppression of fat oxidation, in particular, by alcohol consumption, on top of the caloric intake of the normal diet, should lead to weight gain mainly through increased storage of fat. However, other changes in metabolism (e.g. futile cycles that essentially 'waste' energy through the dissipation of heat), dietary intake and physical activity levels may help to balance energy intake and expenditure, and so maintain consistent body weight in moderate consumers of alcoholic beverages. This seems to be an important factor resulting from induction of the MEOS pathway for alcohol catabolism in individuals consuming high levels of alcohol, especially alcoholics. So far, experimental and epidemiological evidence is inconclusive and the debate is ongoing as to whether or not calories from alcohol count significantly towards energy balance and obesity (Prentice, 1995; Foster and Marriott, 2006).

5.5.5 Effects of Alcohol and its Metabolites on Absorption, Metabolism and Utilization of Key Nutrients

Alcohol and its toxic metabolites have major effects on absorption, metabolism and activation of a number of other important nutrients (Lieber, 2000; and various chapters in Watson and Preedy, 2003). Although the nutrient intake of low or moderate consumers of alcohol may not be significantly affected, in high alcohol consumers and alcoholics nutrient supply may be limited to the detriment of nutritional status with major clinical exacerbations. This may occur not only because of relatively low intake of nutrient rich foods, either by inadequate amounts of food consumed in total or by poor selection of available foods, but also because of major interference with the normal digestion, assimilation and metabolism of nutrients. In heavy consumers and alcoholics especially, alcohol ingestion may be associated with bouts of diarrhea, possibly because of changes in gut motility in which the impeding waves of the jejunum are decreased and the propulsive waves of the duodenum are increased with exposure to alcohol. Alcohol may also exacerbate any existing lactase deficit. However, even in low and moderate consumers, alcohol may have detrimental effects on the digestion and absorption of other nutrients, which usually occur at the subclinical level in most healthy individuals, but there may be a significant health risk to the more susceptible. Alcohol interferes with digestion of macronutrients (fat, protein, carbohydrate), especially by acting to decrease the secretion of digestive enzymes released from the pancreas. Even if food is digested, absorption of all nutrients may also be impaired due to damage to the cells of the stomach and intestines. In those alcoholics with apparent concurrent nutrient deficiencies, nutrient absorption may be impaired to an even greater extent. For example, alcohol has been implicated in impairing absorption of folate because it inhibits expression of a folate carrier; folate deficiency variously created by this and other factors, such as low folate intake and increased renal excretion of folate may reduce further the effectiveness of the folate transport proteins. Furthermore, folate deficiency also appears to compromise nutritional status by reducing absorption of thiamine.

Even if nutritionally significant amounts of nutrients are absorbed, alcohol and its metabolites may interfere with utilization and storage of important nutrients. The effect of alcohol may be through a direct influence on the nutrient concerned, or through modifying enzyme actions or actual enzyme damage in the enzymes of pathways involving that particular nutrient. For example, in erythrocytes the rate of degradation of pyridoxal-5′-phosphate (vitamin B6) is greater in the presence of acetaldehyde; this may occur through its displacement from protein and subsequent hydrolysis by phosphatases. Alcohol exposure also inhibits hepatic folate uptake and increases renal excretion of folate, creating changes in methionine metabolism in the liver, while also exacerbating oxidative hepatic damage with further implications for carcinogenesis. Vitamin B12 status is also reduced with exposure to alcohol. Therefore, inadequate levels of folate and vitamin B12 occurring independently or concurrently could be significant in metabolism of one carbon compounds with potential procarcinogenic effects due to loss of maintenance of integrity of DNA, potentially through such factors as aberrant base incorporation, and its possible increased strand break vulnerability, impaired DNA methylation and reduced DNA repair mechanisms. Loss of such capabilities is especially important in view of the additional risk to the integrity of DNA (and RNA and protein) by increased levels of free radicals created by catabolism of alcohol and its metabolic consequences. Alcohol also interferes with protein and nitrogen metabolism. Even the transient effects of isolated exposure of alcohol interferes with hepatic uptake of amino acids, elevating their blood levels and impairing synthesis of key proteins and their secretion e.g. albumin, fibrinogen and some key lipoproteins. As discussed above, alcohol also influences metabolism of the purine bases leading to hyperuricaemia and gout (Yamamoto *et al.*, 2005; and Section 5.3.4).

Such detrimental effects of alcohol and its metabolites may not be immediately evident in low to moderate consumers, but may be exacerbated considerably in high consumers and alcoholics, leading to conditions recognized as symptomatic of deficiency diseases and other seriously debilitating conditions associated with compromised metabolism, and these are described in greater detail in the following chapter, which considers the health implications of alcoholic beverage consumption (see particularly Section 5.6.2).

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5.6

Health Consequences of Alcohol Intake

Even in your pleasures you should be wise.

—Voltaire

5.6.1 Overview: Alcoholic Beverages and Society

In addition to complementing the intake of water and nutrients provided by essential nonalcoholic drinks (Wolf *et al*., 2008), alcoholic beverages have the biphasic effects of stimulation and sedation, the relative sensitivities of which are determined subjectively by the concurrent blood alcohol levels and may be perceived as desirable outcomes of alcohol consumption (Addicott *et al*., 2007; Morean and Corbin, 2010). Alongside taste, these factors help to explain why consumption of alcoholic beverages such as wines, beers and spirits has long been integral to various cultures throughout the world, although alcohol use may be prohibited on religious grounds in many others. The extent of both the significant health consequences and potential benefits associated with alcoholic beverage intake is determined predominantly by the amount of alcohol consumed, possibly tempered by other factors such as the time period over which drinking occurs, concurrent ingestion of food, relative concentration of alcohol and the presence or absence of enzymes that break down alcohol and its toxic by-products into nontoxic end products. It is not surprising therefore, that major societal, cultural and environmental dynamics may influence the fine balance between health maintenance and detriment. For the essentials of health implications associated with alcohol intake see the general texts of Morgan and Ritson (2003), Paton and Touquet (2005) and Smart (2007).

In order to better understand the multifarious health consequences and possible benefits of ethanol intake in cultural perspective, a sketch is provided here of the major influences on alcoholic beverage consumption. There are a number of reasons why alcoholic beverages are consumed, including:

- As sources of safe uncontaminated water; drinks containing low or moderate levels of alcohol were much safer than local water in many places throughout the world, especially as more heavily populated communities started to develop, which is still the case in some parts, and, for example, low alcohol beers were often provided for manual workers, such as in the steel industry.

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
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- As significant sources of food; the cloudy maize and sorghum based drinks of South America and Africa, in particular, are considered significant sources of nutrition.
- As accompaniments to meals, including aperitifs, drinks with the meal and digestifs; these practices are common throughout the world and somehow or other involve most of the beverages described in this book.
- As adjuncts integral to formal and informal meetings, ceremonies of some religions, such as baptisms, funerals and weddings, parties and other celebrations and social gatherings and events.

In societies in which alcohol is tolerated, moderate consumption is associated with most of these practices or occasions and so is considered quite acceptable on a social level. However, excess intake is a ubiquitous social problem, which exacerbates at different levels within communities that are otherwise accepting of alcoholic beverages, and may be part of the reason that some other communities prohibit the use of alcohol. Differential levels of consumption are determined by a number of factors, with young people particularly vulnerable to the temptations of drink, and those in certain social groups more so, through the influence of advertising, role models and peer pressures. The lure of alcohol may be at its strongest when there are also established legislative or social restrictions, sometimes taking this to excess as seen with weekend binge drinking, for example. It is quite clear that alcohol and some of its catabolic products are extremely toxic, particularly in the young and other vulnerable groups, causing many acute and chronic conditions to exacerbate, especially if alcohol is ingested to excess, whereby the substantial increases in morbidity and mortality then become major public health concerns. For public health perspectives on alcohol intake in the UK, see the websites of the Department of Health (http://www.dh.gov.uk/) and the Faculty of Public Health (http://www.fph.org.uk/). The extent of damage caused at different levels of alcohol intake is differentially determined by individual characteristics, such as the capacity of enzymes in the stomach and liver to break down alcohol and the capability of the body to neutralize or deactivate highly reactive intermediary compounds that may be formed during catalysis of alcohol.

Alcohol intake is completely forbidden on religious grounds for millions of people and many populations across the world. Within many societies, particularly those of Anglo Saxon, Celtic or Nordic origin, where alcohol is not necessarily illicit, temperance movements have variously existed over the years that seek to limit the drinking of alcoholic beverages by legislative (political) means or social pressures. Although prohibition, as experienced by Canada and the USA (~1919–1933) and Scandinavia (except Denmark), for example, is an extreme measure taken to try prevent any alcohol consumption at all, it has quite often had the opposite effect by fostering the illegal private and 'commercial' production and smuggling of alcoholic beverages, leading to expanding crime and excessive drinking in certain sections of society. Perhaps the great jazz cornetist Bix Beiderbecke might not have died in 1931, at the age of just 29, if prohibition had not been in force in the USA at that time. Most temperance movements are based on religious beliefs and have had (at least temporary) influences on alcohol consumption in many countries over many years, usually by legislation that restricts availability in some way. Still today, on the one hand there are those who would say, 'I hope the day is near at hand, When strong drink will be banished from our land.' (William McGonagall). But on the other hand, there are many who are skeptical of extreme measures. 'Prohibition has made our president a dictator, executing an unpopular law by force of arms. It has made our congressmen cowards and hypocrites, passing more and more oppressive laws, while themselves carrying whisky flasks in their hip pockets. Prohibition has divided our people into factions almost as bitterly hostile to each other as the factions that existed before the Civil War' – the opinion of William R. Hearst in the 1930s.

Governments have imposed various restrictions on alcohol, especially in times of war, quite often with the aid of influential abstemious individuals or groups. During World War I the British Government introduced legislation to limit consumption of alcoholic beverages, part of which was the setting up of restricted opening hours for licensed premises (public houses, inns and hotel public bars). In Germany and Austria, certain

quantities of barley for brewing were redirected into the making of bread, and the French Government requisitioned the cider orchards of Brittany and Normandy for the production of industrial spirit and banned the production and sale of absinthe in 1915. In the UK, in 1916, the government requisitioned (nationalized) the breweries and licensed premises of several breweries around the important munitions factories in the Carlisle, Gretna and Annan areas. By strictly overseeing the production and drinking of beer in that area, the government sought to control the perceived heavy drinking of munitions workers (Hornsey, 2003).

The relationship between alcohol consumption and health is complex (Mukamal and Rimm, 2008; Rehm *et al.*, 2010), being detrimental with high intake, but apparently beneficial at low to moderate intake at the population level (Grønbaek, 2009), but with no definitive consensus as to whether or not to drink in moderation or to abstain for maximum health benefits for individuals (Mukamal *et al.*, 2006; Kloner and Rezkalla, 2007). When relative morbidity and mortality rates are plotted against increasing levels of alcohol intake, the result is a j shaped curve (Figure 5.6.1). This appears to be due mainly to the effects of low to moderate alcohol consumption reducing the incidence of coronary heart disease to slightly below that of abstinence, but then the risks associated with increasing alcohol intake rise significantly in a largely dose related manner (Gurr, 1996). Generally, there is little or no advantage of alcohol per se on public health overall. The dividing lines between possible health benefits and causing no harm, minimal harm and significant harm are diffuse for populations because of considerable variability in response to alcohol between people, but for any individual this divide may be quite well defined. This is confounded by the fact that some modern societies have seen the rise of binge drinking or excessive consumption of alcohol over relatively short periods of time, interspersed with periods of low level intake or abstention. Binge drinking is a particularly worrying trend in some societies (e.g. Britton and McKee, 2000), even more so in young people (Plant *et al.*, 2009) and especially children (e.g. Stolle *et al.*, 2009). Furthermore, although health problems generally become more pronounced with increased ethanol intake per se (i.e. dose related), they may be compounded by other concurrent factors, such as poor diet, smoking, pharmaceuticals (prescription and so called 'leisure' drugs) and lack of exercise. Therefore, for the individual, these factors help to explain why there is no strict definition of low, moderate and excessive intake when applied to alcohol consumption. Even intakes consisting of less than a 'moderate' weekly intake of alcohol may be far more detrimental than beneficial for some individuals, depending on

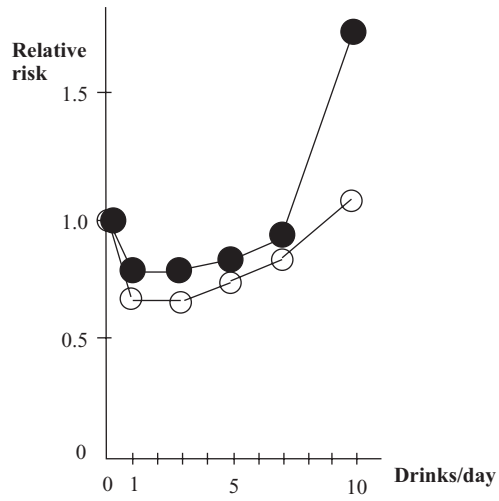

Figure 5.6.1 *Relationship between alcohol consumption, all cause mortality (●) and cardivascular disease mortality (◯)*

distribution of intake over the week. Indeed, binge drinking may be worse for health than exactly the same amount of alcohol consumed over longer periods, but in moderation. Again, general texts (Gurr, 1996; Morgan and Ritson, 2003; Paton and Touquet, 2005; Smart, 2007) provide further insights into alcohol and health, with a comprehensive review of moderate drinking provided by Gunzerath *et al.* (2004), and the more complex nutrition and health issues are considered in greater detail in Watson and Preedy (2003).

Epidemiological evidence and some mechanistic studies suggest that low to moderate alcohol intake imparts specific health benefits to certain populations in general (e.g. in the Mediterranean – Athyros *et al.*, 2007/8) and particularly to individuals at risk from metabolic syndrome (Alkerwi *et al.*, 2009), cardiovascular disease (with the possible exception of stroke), diabetes (Koppes *et al.*, 2005; Baliunas *et al.*, 2009) and neurological disorders (Collins *et al.*, 2009). The reductions in risk may or may not be due entirely to alcohol per se, since there are many other substances in alcoholic beverages that have been implicated in health benefits, such as polyphenols (especially in red wine; see Chapter 5.8) and β-glucans (especially less refined beers). There could also be a possible synergism between alcohol and other entities, which also gives rise to some apparent health benefits. For example, there may be some protection against stomach infection by *Helicobacter pylori* (which has been linked with dyspepsia and ulcer formation) by both alcohol and the antioxidants found in some alcoholic beverages (Lin *et al.*, 2005), although there is some doubt about this (see below). Alcohol consumption has also been linked anecdotally with some protection against the formation of kidney and gall bladder stones and as a measure of protection against osteoporosis. However, whether or not some or all of these purported multifarious effects of moderate alcoholic beverage consumption are actually beneficial is still largely speculative. However, there is also some question as to the health value overall and strong alcohol per se may be a direct cause of many inflammatory conditions.

The exact effects of alcohol experienced by an individual depends on body size, the relative amounts of metabolically active tissues, sex, heritability, ethnicity and some concurrent factors, such as general health, fatigue and state of mind. Clearly, there is, for even the average individual, a certain minimum risk associated with low to moderate consumption, which is considered to be up to about three drinks daily. Exactly what is meant by 'a drink' (i.e. reflecting the amount of alcohol actually consumed) has been the subject of much debate, giving rise to the concept of a standardized unit of alcohol. The fact that the definition of a unit of alcohol varies from country to country indicates (Table 5.6.1) general uncertainty amongst health researchers, health practitioners and policy makers. Nonetheless, broad policy guidelines for recommended maximum

Table 5.6.1 *Units of alcohol from around the world*

Country	Unit of alcohol or standard drink (g ethanol)	Official recommendations for daily maximum drinks for men (M) and women (F) (units/day)*
Australia	10	2 (M), 2 (F)
Austria	6	4 (M), 2.67 (F)
Canada	13.6	2 (M), 2 (F)
Denmark	12	3 (M), 2 (F)
Japan	19.75	1-2
New Zealand	10	3 (M), 2 (F)
UK	8	4 (M), 3 (F)
USA	14	2 (M), 1 (F)

*Where daily limits are the same for men and women (e.g. Canada), the weekly limits often allow more drinks for men (e.g. in Canada, maximum weekly intake for men is $2 \times 14 = 28$ units, whereas for women it is $2 \times 9 = 18$).

daily intakes have been laid down (e.g. Department of Health website – http://www.dh.gov.uk/), even if they do differ in policy and approach (Crombie *et al.*, 2007).

The factors that cause certain individuals to cross the dividing line between low or moderate and excessive drinking are not fully understood and whether or not a moderate drinker will progress to a state of alcohol abuse is not possible to predict with any certainty from current knowledge (Lucas *et al.*, 2005). For these and other reasons, the American Heart Association advises people who have medical conditions that may supposedly benefit from low alcohol intake, not to take up drinking alcohol if they do not already do so (O'Keefe *et al.*, 2007).

Acute health problems due to excess alcohol include increased risk of accidents and injury, transient gastrointestinal problems, cardiovascular effects, toxicity and behavioral changes, along with the risk of death from vomit inhalation in the extreme (Vonghia *et al.*, 2008). Accidents occur as balance and judgments of distance and speed are generally impaired after consumption of more than about three or four drinks and large excesses may cause further impairment due to transient hypoglycemia. Chronic problems include addiction, cardiovascular disease, damage to the central nervous system and other neuropathies, liver disease, cancers, osteoporosis, arthritis and psychological disorders. Malnutrition, presenting either as undernutrition or overweight and obesity, is a possible outcome of long term alcohol misuse with various disease exacerbations that this may cause due to the added burden of poor diet and poor nutritional status. Of course there are possible consequences for every member of the community of another person's excessive alcohol consumption, acute or chronic, e.g. accident or intrafamily issues (Greenfield *et al.*, 2009).

Based on the differential implications of alcohol consumption, whether for benefit or harm and at the individual or population health levels, various methodologies are used to assess the effects of alcohol intake by measuring physiological responses in individuals through to the prevalence of alcohol related diseases in populations. In order to establish the true effects of alcohol, it is essential to design statistically sound research studies that are based on techniques appropriate to the research in question and, therefore, avoid obtaining erroneous or misinterpreted data. The dissemination of misleading information obtained through poorly planned and executed research may impact significantly on recommendations for public health promotion strategies, etc. There are a number of ways of classifying research study designs according to the question being asked and resources available, but they essentially fall into two main categories, experimental and observational.

Experimental studies, including powerful randomized controlled trials, are statistically powerful designs that may be prospective and longitudinal and measure the differential effects of interventions in a treatment group against a control group, similarly examined, but without the intervention or administered with a placebo instead. These studies are likely to provide the most convincing data on causation of alcohol related disease. However, it would be unethical and expensive, amongst other things, to subject a group of healthy individuals to years of excessive alcohol intake, or indeed any of its toxic metabolites, to see if their occurrence of disease was worse than in a control group of abstainers (e.g. Ren and Wold, 2008). In contrast, if the short term differential effects of intervention using a specific nutrient in alcohol, say a particular polyphenol in wine, are intended, then this is less of an ethical issue. It is important for study validity that the intervention and control groups are matched as consistently as possible in all other factors, diet for example, so that any difference between them is as certain as possible to be due to the intervention of alcohol itself and not to confounders, such as differential levels of smoking between the groups.

Observational studies can largely be divided into prospective and retrospective subcategories, each of which can be further subdivided into longitudinal and cross sectional designs. Of particular importance to evaluating the effects of alcohol on population health is the science of epidemiology, the use of statistical methods specialized for making inferences that reflect experimental study design as far as possible, but cannot be used to replace interventions or to draw the same conclusions regarding disease etiology. Observational studies can only infer that associations exist between cause and effect or outcome, they cannot conclusively

prove causation like the more powerful experimental studies. However, the use of case control studies is particularly valuable in assessing the possible effects of alcohol in later disease onset and again essentially uses two groups retrospectively, or even prospectively, one with exposure to alcohol and one without (the control group), and examines the difference between them in terms of outcome, thereby enabling stronger inferences of association to be drawn. However, there are potential difficulties with such studies, including the reliability of recall and the presence of uncontrolled confounding factors (diet, exercise, smoking etc.).

For more information regarding study design in general, see Altman (1991) and Nelson *et al.* (2004).

Of course laboratory studies are also important, particularly for determining the mechanisms of how alcohol affects health, and may be performed either *in vivo* in individuals, or groups of subjects, or *in vitro* on cell lines and organelles, and on isolated biochemicals; they have the advantage that some of the short-term direct effects of alcohol can be studied in controlled conditions with little risk of lasting harm. Human studies include analyses of biological fluids, such as plasma and urine, and physiological measurements, such as heart rate, blood pressure and cognition. Studies using animals have the advantage of including whole tissue analyses, but they may not be acceptable, as the findings may not be appropriate to physiological responses in humans.

5.6.2 Harmful Effects of Excessive Alcohol Intake

Although a low to moderate intake of alcohol may perhaps be of some health benefit, levels above about three to four standard UK units lead to significantly increased risk of mortality from circulatory disease, and several other conditions, as well as to increases in morbidity generally, especially liver, gastrointestinal and neurological diseases. Such risks are essentially dose dependent, with women more susceptible than men to alcohol overall (e.g. Nolen-Hoeksema and Hilt, 2006; Lang *et al.*, 2007; Ferreira and Willoughby, 2008; Taylor *et al.*, 2009). Athletic performance is variously, mostly adversely, affected by alcohol consumption (El-Sayed *et al.*, 2005; Shirreffs and Maughan, 2006). A summary of significant conditions generally associated with alcohol intake is given in Table 5.1.1 of Chapter 5.1. The development and progression of certain alcohol related conditions, especially those promoting some cancers, are known to be modified by individual genetic make up and epigenetic effects (Shukla *et al.*, 2008), but this complex area of investigation is still largely in its infancy (Druesne-Pecollo *et al.*, 2009; and see 'Hepatic (Liver) Disease' section). As the following section covers the most important or defined health implications, which is not exhaustive, useful perspectives may be found in texts by Paton and Touquet (2005), Morgan and Ritson (2003) and Smart (2007), and more specific nutrition related issues are presented in more depth in Watson and Preedy (2003). Also, there is such a vast amount of information currently available on alcohol and health that only certain references have been selected as the most interesting and/or appropriate for either providing direct factual matter or as a means to sourcing further details, i.e. through the use of comprehensive reviews (e.g. Gunzerath *et al.*, 2004).

Accidents and Injury

Both acute and chronic misuse of alcohol increase the risks of accidental injuries and death, such as from mishaps in the home or at an otherwise secure event, road traffic accidents, industrial accidents, fires (very often associated with smoking) and even drowning. Indeed, the relative risks of morbidity and mortality directly associated with accidents and injuries influenced by alcohol intake are higher than other alcohol related sequelae (Morgan and Ritson, 2003) and, as for other consequences of excess intake, the risk is dose dependent (Taylor *et al.*, 2010).

As well as increasing the risk of injury, excessive alcohol consumption also increases the risk of morbidity and mortality following trauma or surgery, and recovery time is greater, because wound healing, including

collagen production, epithelial restoration and blood vessel repair is impaired by exposure to alcohol consumed before sustaining injury (Radek *et al.*, 2009).

Alcohol Addiction (Alcoholism, Alcohol Abuse or Alcohol Dependence)

This addictive syndrome is characterized by compulsive alcohol abuse leading to possible long term psychological and physical dependence, with significant deteriorations in health and social consequences. As well as excessive abuse of alcohol, the alcoholic: (a) may be unable to control its use, which is usually persistent despite recurrent health and social implications, (b) may neglect other activities and social interactions whilst concurrently fostering alcohol related behaviors or succumbing to their sequelae, (c) may develop increasing levels of tolerance and (d) may suffer serious physical and psychological symptoms upon withdrawal (McKeon *et al.*, 2008). Its causation and pathophysiology remain largely unclear, but are known to be multifactorial (Moussas *et al.*, 2009). Alcohol addiction may be at least partially under the influence of genetic (Stacey *et al.*, 2009) and environmental interactions with alcohol consumption (van der Zwaluw and Engels, 2009) and may involve a number of neurotransmitters (Heinz *et al.*, 2009).

Chronic alcohol abuse may also have indirect implications for health. For example, there is evidence of increased alcohol mediated incidence of acute respiratory distress syndrome and severity of multiple organ dysfunctions in critically ill patients (Moss and Burnham, 2003; Boé *et al.*, 2009).

The individual physical and psychiatric consequences associated with alcohol dependence syndrome are described in the appropriate sections within this chapter.

Cardiovascular Complications

The association between alcohol and cardiovascular health detriment or benefit is particularly complex and may depend on the balance between antioxidant and prooxidant status and its relative contribution to the development of atherosclerosis (Puddey *et al.*, 2003). Although low to moderate levels of alcohol consumption appear to be of some cardiovascular benefit, there is little to question that excessive amounts have truly detrimental effects, the severity of which worsen with increasing alcohol intake in an essentially dose-related manner (Gurr, 1996). However, there is even debate as to whether or not alcoholic beverages do actually provide any true cardioprotective effects at all (e.g. Fuchs and Chambless, 2007) as there are methodological issues that may confound the validity of many of these studies. Evidence that low to moderate alcohol consumption is relatively cardioprotective and that, above a certain point, increased levels of alcoholic beverage consumption significantly raise the risk of cardiovascular morbidity and mortality, is predominantly taken from epidemiological studies, some of which present quite strong associative evidence, but are not conclusive of causation, as the relationship of alcohol and health is so complex (e.g. Mukamal, 2007). Clearly alcohol should never be recommended as a substitute to lifestyle and other therapeutic agents (Vogel, 2002) based on epidemiological evidence alone. Ideally randomized controlled trials are needed to confirm whether or not alcohol is actually the predominant causative agent per se, or if alternative etiological factors are central to cardiovascular health detriment or benefit, such as the by-products of alcohol catabolism, the other constituents of alcoholic beverages or the pattern of consumption (O'Keefe *et al.*, 2007). As such studies may be extremely difficult and unethical to undertake in humans as serious harm may be inevitable, animal models are often used to suggest both causation and possible mechanisms of action (e.g. Zhang *et al.*, 2004), but findings in animal models may not be entirely appropriate to the human condition. Therefore, in order to build a complete picture of how alcohol impacts on human health, a number of different, but integrated, approaches are needed (Lucas *et al.*, 2005). Possible benefits of alcohol per se on cardiovascular health and other components of alcoholic beverages are discussed in the next section (Section 5.6.3).

Metabolic challenges created by both greater acute and chronic alcohol intake clearly predispose to cardiovascular exacerbations (Kawano, 2010), including cardiac arrhythmias, cardiomyopathy, vascular hypertension and coronary heart disease, with the particular disease presentation determined by the relative amount and pattern of alcohol consumption and length of time of exposure (see general text of Morgan and Ritson, 2003). The heart itself is acutely affected by excessive alcohol intake which causes reduced physiological function through diverse origins involving alcohol and many of the metabolic products of alcohol catabolism, particularly acetaldehyde (Ren and Wold, 2008). Chronic alcohol use may cause cardiac myopathy characterized by feelings of fatigue and breathlessness in the first instance, which may alleviate on abstinence, otherwise it may exacerbate as arrhythmias, cardiomegaly, dysfunction of cardiac myofibrillar architecture and contractility, and also as hemorrhagic stroke, noncoronary artery disease related heart failure, edema and increased venous pressures, and other symptoms of heart failure with continued alcohol abuse (Klatsky, 2009; 2010).

Increases in systolic and diastolic blood pressure, or hypertension, are associated with alcohol intake, again in a dose related manner and dependent on individual (e.g. male versus female) and population (e.g. Asian men) characteristics (Taylor *et al.*, 2009), although definitive mechanisms have not yet been fully identified (Klatsky and Gunderson, 2008). Hypertension may be caused, at least in part, by vascular endothelial dysfunction mediated through alcohol induced oxidative stress, insulin resistance and increased lipoproteins (Bau *et al.*, 2007). Chronic heavy drinking apparently leads to a higher risk of hemorrhagic stroke, but not of ischemic stroke generally (Klatsky, 2007), although again this is complicated because a number of factors have been implicated with risk of stroke and there are disparate outcomes in terms of the different subdivisions of ischaemic and hemorrhagic stroke that may present due to differential amounts and patterns of alcohol consumption (Klatsky, 2005). Although high density lipoprotein cholesterol (HDL-C) levels are increased by alcohol intake, this purported cardiovascular risk benefit observed in many other situations is offset by raised low density lipoprotein cholesterol (LDL-C) levels and particularly by increased concentrations of serum triglycerides (Böhm *et al.*, 2004; McBride, 2008). Also, platelet aggregation, which may be decreased by low intakes (Ruf, 2004) is increased by chronic alcohol consumption (Böhm *et al.*, 2004).

Drug/Alcohol Interactions (Pharmaceuticals and Drug Abuse)

There are multifarious interactions of drugs and alcohol that may have differential implications for drug efficacy and health sequelae according to whether or not alcohol intake is concurrent and is acutely excessive or chronically abused (Weathermon and Crabb, 1999). For a very informative but general perspective, see Morgan and Ritson (2003, pages 48–50). Older adults may be particularly vulnerable to such interactions as there is increased usage of drugs and there may be complications with compliance (Adams, 1995; Moore *et al.*, 2007).

In the first instance, drug ingestion may be limited because of poor compliance, anorexia or vomiting and the presence of alcohol in the gut may interfere with absorption of drugs, physically limiting or even enhancing the amounts available to target tissues (e.g. Lennernäs, 2009).

Alcohol and its products may reduce the capacity of the body to detoxify certain pharmaceuticals and xenobiotics, e.g. through various detrimental interactions with cytochrome P450 (CYP2E1) during catabolism of alcohol through the microsomal ethanol oxidizing system (MEOS; Leiber, 2000). Efficacy of prescription drugs may be also inhibited by alcohol through competition at the site of action and other actions such as compromising lipid membranes and the enzymes of some biochemical pathways.

The interactions of alcohol and drugs may be additive or each may potentiate the effects of the other. For example, alcohol taken in addition to particular drugs, such as sedatives and some analgesics, may cause drowsiness and so there may be increased risk in the operation of machinery and driving etc. The toxic effects of some drugs may be exacerbated by alcohol, causing local lesions in the gut and liver, for example.

Alternatively, some drugs may inhibit the enzymes responsible for alcohol catabolism leading to significant build up of the toxic product, acetaldehyde.

As well as alcohol itself, alcoholic beverages also contain additional components that are likely to interact with drugs, potentially modifying their efficacy, including antioxidants (possible actions on CYP2E1 and the redox system), tyramine (acts on monoamine oxidase inhibitors producing hypertension) and other constituents of red wines and beers, such as congeners (e.g. Jang and Harris, 2007).

Gastrointestinal Disorders

Excessive alcohol intake has many acute and chronic effects throughout the gastrointestinal tract, impairing its ability to digest and absorb nutrients which may initiate a cyclical progression of further damage to the functional reserve and regenerative capacity of the gastrointestinal tract, which exacerbates malnutrition even further (Rajendram and Preedy, 2005). Alcohol related mucosal damage may also predispose to malignancy, especially if associated with smoking (Pelucchi *et al.*, 2006; Seitz and Cho, 2009). Although alcohol is not carcinogenic, or at least has very low carcinogenicity, its toxic metabolite, acetaldehyde, is a purported carcinogen, as it forms adducts with DNA potentially causing cancers, such as those of the gastrointestinal tract (Seitz and Meier, 2007; and see 'Malignancies,' below).

Chronic alcohol abuse may lead to alcoholic liver disease, often in association with other liver lesions and hepatitis C (Wakim-Fleming and Mullen, 2005), with the resultant increase in portal pressures eventually leading to esophageal varices, a life threatening condition requiring immediate and effective management (Garcia-Pagan *et al.*, 2008).

Alcohol stimulates gastric acid secretions and impairs sphincter pressures and esophageal peristalsis causing gastroesophageal reflux with consequent esophageal mucosal damage, including esophagitis, mucosal ulceration and Barrett's Syndrome (an epithelial metaplasia). Such changes are partly due to the alcohol itself and occur irrespective of the type or amount of beverage involved (Bujanda, 2000), in large part because of the acid and gastrin present in the reflux. Animal models suggest that alcohol sensitizes the esophageal mucosa to damage via changes in epithelial transport, intercellular junction disorder and weakening of the mucosal barrier, all of which permit hydrogen ion penetration of the mucosal barrier (Bor *et al.*, 1999). However, although animal models provide important information and pointers, they do not necessarily represent how human gastrointestinal systems respond to alcohol (Siegmund *et al.*, 2005). In extreme cases, severe vomiting can cause tearing of the distal part of the esophagus, known as Mallory–Weiss syndrome, which presents in a majority of alcoholic patients who have consumed excessive quantities of ethanol in the hours preceding examination (Bujanda, 2000).

Even in less extreme cases, there may be increased secretion of gastrin and gastric acid, which is partially associated with the nature of beverage consumed. Fermented drinks (beer, cider, wine, etc.) appear to stimulate gastric acid and gastrin release, whereas distilled beverages apparently do not (Teyssen *et al.*, 1997). It has been suggested that small acidic and carbohydrate molecules, such as succinic and maleic acids, may be more potent gastric stimulants than ethanol (Teyssen *et al.*, 1999). Such molecules are found in far greater amounts in fermented than in distilled beverages.

Gastric emptying is also affected by intake of alcoholic beverages, although differentially according to amount and type consumed. Low alcohol concentrations facilitate gastric emptying, whereas it is delayed by high alcohol concentrations, and fermented beverages accelerate gastric emptying (see Bujanda, 2000). Acute and chronic gastritis, in large part a result of increased acid secretion in the stomach, are common amongst heavy drinkers, especially alcoholics, and gastric and duodenal lesions caused by alcohol appear to be dose related (Knoll *et al*., 1998). Although consumption of alcohol itself has been implicated in gastric ulcer, this link is considered tenuous, and there now appears to be no association between excessive alcoholic beverage consumption and the incidence of gastric ulcers (Bujanda, 2000).

Acute and chronic alcohol consumption affects bowel motility, prolonging overall transit time, because it impairs contractile protein, vagal nerve and neuroendocrine functions, with orocecal transit time very much higher (156 min) in alcoholics compared to social drinkers (94 min) and abstainers (86 mins). Greater exposure to toxins is thought to be a factor in the development of malignancies in the lower bowel, and associated with lower levels in alcoholics of the protective agent *S*-adenosylmethionione, although the causes are multifactorial and complex (Bujanda, 2000). The lower bowel is also prone to hemorrhoids and colon varicosities due to cirrhosis and increased portal tension.

Other intestinal disorders observed in heavy drinkers (especially alcoholics) include diarrhea and malabsorption of nutrients, caused by alcohol induced changes in the digestion and absorption of food. Excessive alcohol intake probably decreases the activity of mucosal disaccharides and increases mucosal permeability, facilitating the output of water and saline solutes, giving rise to diarrhea. Malabsorption occurs in heavy drinkers, even those on an otherwise healthy diet, because impaired intestinal absorption processes cause lower uptake of many nutrients, including amino acids, mineral such as calcium, iron and zinc, and vitamins B_{12} and thiamine, for example. The comprehensive review by Bujanda (2000) provides greater details in perspective of the effects of alcoholic beverage consumption on gastrointestinal health and secondary malnutrition.

Hepatic (Liver) Disease

Excessive consumption of alcohol can lead to various lesions in the liver (Adachi and Brenner, 2005) and it is one of the leading causes of liver disease and liver disease mortality worldwide, already well established in developed countries and with increasing incidence in the developing world (Albano, 2008). Men whose intake is, on average, more than 7 or 8 units (about 60 g) of alcohol daily (a unit is 8 g or 10 ml of pure alcohol) and women drinking more than 5 units (40 g) a day are more likely to develop alcoholic liver disease than low to moderate drinkers. Clinical presentations of alcohol related hepatitis include jaundice, enlarged liver and fluid retention. Men, in particular, with mild to moderate disease may recover fully upon abstinence from alcohol. Severe alcoholic hepatitis is likely to progress to cirrhosis, more especially in women, with the development of hepatocellular failure and portal hypertension with a number of associated conditions, especially some blood coagulation and vascular disorders and hepatic encephalopathy (dysfunction of the brain – confusion, low levels of consciousness, etc.). Alcoholic liver disease is also implicated in the dysfunction of other systems, the innate and adaptive immune responses (Nath and Szabo, 2009), for example. Of those who develop cirrhosis, some will go on to acquire carcinoma of the liver (McKillop and Schrum, 2009). Overall, with continued use and abuse of alcohol, the prognosis for those with alcoholic liver disease, especially hepatocellular carcinoma, is poor. The progression of alcoholic liver disease and clinical signs of the various stages of its exacerbation are well summarized by Morgan and Ritson (2003) and are described in more detail by Lucey *et al.* (2009).

There are a number of key factors that contribute to alcoholic liver disease pathogenesis which is complex at the biochemical, cellular and organ levels and its etiology extends beyond simple malnutrition (Lieber, 2004; Albano, 2008). For example, alcohol readily induces oxidative stress through a number of pathways, particularly involving CYP2E1, threatening liver function (Cederbaum *et al.*, 2009). Alcohol is toxic to the liver predominantly because of its conversion to the primary catabolic product, acetaldehyde, which is a known carcinogen. Degradation processes involving alcohol dehydrogenase (oxidation of alcohol to acetaldehyde) generate NADH, the reduced form of NAD, which promotes fatty acid synthesis leading to steatosis, the abnormal build up of lipids in liver cells, and collagen synthesis through the increased production of lactate from pyruvate, amongst other factors. Alternatively, if activity of the cytochrome CYP2E1 (MEOS) is induced, there is build up of highly reactive oxygen species or free radicals that cause peroxidation of lipids

and cell membrane lesions, amongst other significant examples of the potential damage of oxidative stress created by excessive alcohol intake (Lieber, 2004; Albano, 2006; Cederbaum *et al.*, 2009). Oxidative stress is also evident in the mitochondria because the key reducing agent, reduced glutathione, is also oxidized through binding to acetaldehyde (Lieber, 2004). These events, alongside other proinflammatory events, lead to the inflammation, necrosis and fibrosis that are associated with alcoholic liver disease progression and may result in cirrhosis for a significant proportion of affected individuals.

Alcohol impairs the metabolism of methionine at various points, most critically methionine synthase which is a key in homocysteine remethylation. Excessive use of alcohol appears to reduce levels of *S*-adenosylmethionine and increase levels of *S*-adenosylhomocysteine and homocysteine, which are toxic, potentially causing severe impairment of liver function (Kharbanda, 2009). Significant health implications of this include increased fat deposition, apoptosis, damage to key hepatic proteins and oxidative stress, which progressively lead to such impairment.

Alternative pathways also exist for the detrimental effects of alcohol on liver function, some of which contribute to activation of hepatic stellate cells which is a central element in the development of fibrosis (Cubero *et al.*, 2009). This mechanism is thought to involve the liver–gut axis because elevated plasma endotoxins, derived due to reduced gut patency, cause inflammatory and innate immune responses in Kupffer cells via Toll-like receptors, among a number of other purported pathways (Nagata *et al.*, 2007; Szabo and Bala, 2010). Hepatic protein dynamics are also known to be modified by alcohol, with two mechanisms of trafficking identified (Shepard *et al.*, 2010a), and liver proteins are susceptible to hyperacetylation (Shepard *et al.*, 2010b). Other complex biomolecular pathways contributing to alcoholic liver disease have been suggested (e.g. Purohit *et al.*, 2009).

Hepatocellular carcinoma is associated with progressive liver disease (McKillop and Schrum, 2009) and synergisms that exist between alcoholic liver disease and hepatitis C are considered to increase the risk of hepatocellular carcinoma pathogenesis and mortality rates (Morgan and Ritson, 2003, page 18; Mueller *et al.*, 2009).

There are similarities in the etiology of other alcohol related conditions, such as cardiovascular diseases or cancers, in that the pathogenesis and extent of alcoholic liver disease and its possible progression to hepatocellular carcinoma may also be modified by differential responses to alcohol and its metabolites, due to variable genetic and epigenetic origins (Juran and Lazaridis, 2006; Wilfred de Alwis and Day, 2007). Polymorphisms in alcohol dehydrogenase, aldehyde dehydrogenase and CYP2E1, involved in alcohol catabolism itself, and enzymes involved in the metabolism of folate have been implicated (Druesne-Pecollo *et al.*, 2009). As well genetic predisposition to hepatic carcinoma, epigenetic alterations in DNA, including methylation, also occur, causing malignant changes, but these are less well understood (e.g. Herath *et al.*, 2006; Nishida, 2010).

Hypersensitivity, Allergy and Autoimmune Responses

Certain alcoholic beverages may have constituents implicated in some specific and nonspecific hypersensitivity, allergic or autoimmune reactions, including alcohol per se. Biogenic amines and polyamines in wines and beers may cause allergic reactions including asthma (Kalac and Krizek, 2003; Vally and Thompson, 2003; Ancín-Azpilicueta *et al.*, 2008), although some controversy exists as to their purported role in intolerance (Jansen *et al.*, 2003). The antigens gluten (wheat) and hordein (barley) that are in some alcoholic beverages, particularly beers, may exacerbate existing celiac disease (Ellis *et al.*, 1990; Dostálek *et al.*, 2006). Alcoholic beverages may induce the symptoms of irritable bowel syndrome (MacDermott, 2007), urticaria and anaphyloid reactions due to alcohol itself and many other, nonspecified, constituents (Ehlers *et al.*, 2002; Gonzalez-Quintela *et al.*, 2004).

Hyperuricaemia

Gout, the most common form of inflammatory arthritis in men, has long been at least anecdotally associated with chronic heavy alcohol consumption; hence, the stereotypical image of the aged, port drinking, retired colonel in a wheelchair with one foot heavily bandaged. Epidemiological studies have implicated alcohol consumption in hyperuricaemia (Choi *et al.*, 2004), possibly in a dose related manner, which in certain conditions leads to the painful condition known as gout. Integral to nucleic acids, the purines, guanine and adenine, are produced when the yeast culture grows during fermentation. Also, purines in their phosphorylated form (ATP, GTP) are important sources of energy generation for driving cellular reactions. Ingestion of high levels of exogenous purines perhaps from particular alcoholic beverages is implicated in hyperuricaemia because the catabolism of adenine and guanine proceeds, via xanthine, producing uric acid. Under certain conditions, hyperuricaemia may lead to gout, where uric acid crystals are deposited on joint surfaces and in other tissues. Alcohol per se is also purported to increase blood levels of uric acid, by promoting increased degradation of adenine and increased production of lactate (Yamamoto *et al.*, 2005). Beers are more heavily implicated in increased risk of gout than wines, partly because of their greater purine content, but spirits with negligible purine contents also raise urate (wine is considered to be a possible protective factor in some quarters).

Hypoglycaemia

Changes in intermediary metabolism caused by excess alcohol intake may result in lowered blood glucose concentrations, or hypoglycaemia. Both gluconeogenosis and glycogenolysis are inhibited by alcohol, which has particular implications if alcohol is consumed without food and in conditions in which glycogen levels are low, for example after very heavy exercise programs or sports. Alcohol is also known to increase insulin sensitivity, possibly lowering blood glucose even further. Potentially, there are significantly greater implications of excessive alcohol intake for people with diabetes (van de Wiel, 2004).

Malignancies

Excessive chronic alcohol consumption has been linked to a number of cancers, including those of the upper digestive tract and airways, although the relative risks and mechanisms may vary considerably, depending on location; the strongest associations appear to be between level of alcohol intake and cancers of mouth, pharynx, larynx and esophagus, with lower, but still significant, risks for stomach, colorectal, liver, breast and ovarian cancers (Bagnardi *et al.*, 2001; Pöschl and Seitz, 2004; Boffetta and Hashibe, 2006; Seitz and Becker, 2007; Seitz and Stickel, 2007). Alcohol (ethanol) per se does not appear to be directly carcinogenic, although it predisposes to malignancy, particularly in association with smoking (Pöschl *et al.*, 2004; Salaspuro and Salaspuro, 2004; Pelucchi *et al.*, 2006; Seitz and Cho, 2009). However, alcohol does have carcinogenic potential, as it can inhibit normal DNA methylation and modulate retinoid metabolism (Morgan *et al.*, 2004; Seizt and Stickel, 2007). In concentrations greater than about 20%, alcohol per se may cause local injuries to mucous membranes, leading to accelerated cell division and regeneration (hyperproliferation) that may ultimately lead to replication errors during DNA synthesis resulting in genetic changes, such as dysplasia and leukoplacia and carcinogenesis, which may be particularly significant in the pathogenesis of mouth (Ogden, 2005) and throat cancers (Riedel *et al.*, 2005) and esophageal and gastroesophageal cancers (Franke *et al.*, 2005), with gastric reflux also implicated (Pera *et al.*, 2005).

However, the primary catabolic product of alcohol, acetaldehyde, has been heavily implicated in carcinogenesis and mutagenesis, as have CYP2E1 derived reactive oxygen species through alcohol catabolism (Pöschl *et al.*, 2004), and acetaldehyde poses an independent risk as it is a natural constituent of many alcoholic

beverages (Lachenmeier *et al.*, 2009), notably hot Calvados as an interesting example (Linderborg *et al.*, 2008), and may be a direct carcinogen in some particularly vulnerable tissues and organs, according to cumulative exposures from environment and individual genetic and behavioral traits (Salaspuro, 2009). Acetaldehyde is usually broken down to acetic acid by acetaldehyde dehydrogenase. However, in many individuals the relative rate of this conversion may be limited, either because of reduced levels of the enzyme or reduced enzymatic capacity due to altered structure resulting from different genetic polymorphisms. Acetaldehyde may then accumulate, increasing carcinogenic potential. Mitochondrial type II alcohol dehydrogenase (ALDH2) is responsible for most acetaldehyde oxidation, but some Asian populations, including about 50% of Japanese, possess a hereditary deficiency in this enzyme. Although the symptoms of high levels of acetaldehyde are extremely unpleasant and may deter people from excessive alcohol consumption, a number of people with some such polymorphisms may become alcoholics. Indeed, epidemiological studies have shown that amongst Asian heavy drinkers, the risk of alcohol related cancers of the aerodigestive tract may be much increased in those with ALDH2 deficiency (Salaspuro, 2007; Asakage *et al.*, 2007).

The relative risks of developing cancer for chronic heavy alcohol consumers may be further increased for a variety of independent reasons with some possible overlap between them. Firstly, high consumption of particular alcoholic beverages may increase the likelihood of exposure to certain carcinogens, such as the possible presence in the finished beverage of congeners, mycotoxins (see Section 5.11.4), additives (Chapter 5.9), uncontrolled pesticide residues (Section 5.10.2) etc. Secondly, smoking and poor oral hygiene is often associated with alcohol abuse leading to increased exposure to acetaldehyde (Salaspuro, 2009). Thirdly, malnutrition may occur in excessive drinkers because of poor dietary practices and/or alcohol induced changes in digestion, absorption, assimilation and metabolism of key nutrients. Dietary deficiencies have been implicated in the pathogenesis of malignant diseases because DNA damage is not then preventable (e.g. Ames and Wakimoto, 2002). Some of these key nutrients, particularly those with antioxidant potential, such as certain vitamins, minerals and polyphenols, may be protective against the pathogenesis of cancer because they modulate the carcinogenicity of highly reactive free radicals, such as superoxide, peroxide and hydroxyl (see section 5.8.1), many of which may be derived from catabolism of alcohol via CYP2E1, from smoking or directly as constituents of the particular beverage concerned.

Long-term high daily levels of alcohol consumption increase the risk of hepatocellular carcinoma, with exposure to alcohol of greater than 10 years and in excess of 80 g daily leading to a fivefold increase in risk (Morgan *et al.*, 2004; McKillop and Schrum, 2009). However, alcohol consumption is only one factor contributing to the different potential mechanisms in the pathogenesis of liver carcinoma (Herath *et al.*, 2006).

Organs of the upper aerodigestive tract are particularly vulnerable to cancers due to cumulative environmental and individual behavioral exposures to acetaldehyde, as well as alcoholic beverage consumption, such as poor oral hygiene, smoking and its presence in a number of food sources (Salaspuro and Salaspuro, 2004; Salaspuro, 2009). Although relatively lower for lung cancer than other cancers, there is some suggestion that the alcohol related risk of lung cancer is differentially associated with the type of alcoholic beverage and amount consumed, but the link is not certain and is confounded substantially by smoking, and modest wine consumption may actually be protective in some way (Chao, 2007; Chao *et al.*, 2008).

Meta-analysis points to a relatively small positive association between alcohol intake and incidence of breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 2002) with a possible modulation of risk attributable to estrogen (Seitz and Maurer, 2007).

Malnutrition

Optimum nutritional status is an important mediator of the risk to health posed by excessive alcohol intake. Certain key nutrients (e.g. vitamins, minerals, polyphenols) are considered to be required as protective agents

against the detrimental effects of the toxic (e.g pesticides, methanol), carcinogenic (e.g. acetaldehyde) and highly reactive oxidation catabolic products (e.g. peroxides, superoxides, hydroxyl) that are due to alcohol consumption. However, excessive long-term alcohol consumption may result in malnutrition either through reduced dietary intake of essential nutrients or by the direct effects of alcohol and its by-products on nutrient absorption and metabolism. Detailed information on alcohol consumption and its effects nutrition can be found throughout the rest of this chapter with relevant references.

Metabolic Syndrome

Although it is not a discrete disease as such, the metabolic syndrome is a multifactorial condition associated with the development of serious noncommunicable chronic diseases, such as type 2 diabetes and cardiovascular disease. Metabolic syndrome is generally characterized by central obesity with various presentations of dyslipidaemia (increased blood triglyceride levels and reduced HDL-C levels), hypertension, raised fasting blood glucose levels and possible microalbuminuria. The underlying mechanisms are uncertain, but insulin resistance is heavily implicated. Although low to moderate alcohol intake may lower insulin resistance and the prevalence of metabolic syndrome (Alkerwi *et al.*, 2009), consumption in excess of recommendations may contribute to some of the specific components of the metabolic syndrome and its disease exacerbations, with suggestions that genetic–environmental interactions with alcohol (Corella, 2007) and pattern of drinking (Athyros *et al.*, 2007/8; Fan *et al.*, 2008) may have significant impact either way.

Methanol and Higher Alcohol Toxicity

Methanol, which is found in most alcoholic beverages in varying, but relatively low amounts, is not usually a major threat to health, as such, although the consequences may be acutely debilitating, perhaps causing headache and vomiting etc. However, it is extremely toxic and is catabolized to formaldehyde which is particularly toxic, and formaldehyde is then converted to another highly toxic intermediary, formic acid, which eventually is further broken down to nontoxic products or excreted. These toxic intermediary metabolites lead to high anion gap metabolic acidosis, increased osmolality, cellular and organ dysfunction, particularly neurologic, cardiopulmonary and renal (Jammalamadaka and Raissi, 2010). Methanol and higher alcohols may be concentrated during illegal distillation, and its ingestion may lead to blindness and even death. Along with certain higher alcohols and other toxic constituents, methanol may also be present in surrogate alcohols used by alcoholics and the risk of ingestion may be higher in communities where use of illegally produced alcohol is in more common practice, in some parts of Russia for example (Lachenmeier *et al.*, 2007).

Muscle

Alcohol consumption has major implications for both cardiac and skeletal muscle (Preedy *et al.*, 1999; Urbano-Márquez and Fernandez-Solá, 2004). Cardiac muscle may be susceptible to cardiomyopathy, characterized by progressive left ventricular dysfunction and arrythmias, arising from both acute and chronic alcohol excesses, increasing the spectrum of cardiovascular complications (Urbano-Márquez and Fernandez-Solá, 2004; and see 'Cardiovascular' section above). Likewise, skeletal muscle is susceptible to structural and functional myopathy due to acute and chronic alcohol abuse, with the extent of damage being dose dependent and the pathogenesis multifactorial, involving impaired myofibrillar protein synthesis, with perturbations of gene expression being heavily implicated, oxidative stresses, induced apoptosis and aberrant biomembranes (Adachi *et al.*, 2003; Urbano-Márquez and Fernandez-Solá, 2004; Fernandez-Solá *et al.*, 2007). Acetaldehyde has also been implicated in alcoholic myopathy (Preedy *et al.*, 2007).

Binge drinking may cause acute episodes of pain (myalgia), swelling and weakness in muscles, and more severe acute binge drinking may lead to rhabdomyolysis, with the associated myoglobinuria possibly causing renal tubular necrosis and kidney failure. Chronic myopathy is a muscle wasting condition caused by long-term excessive alcohol consumption, with progressive muscle weakness (see Morgan and Ritson, 2003).

Nervous System and Psychological Disorders

The variable neurological responses to acute alcohol intoxication are determined by a number of factors, including how quickly blood glucose levels rise and the extent of tolerance that might be developed. As intoxication builds, at relatively low levels there may be differential responses due to associated social interactions possibly causing relaxation, elation and effusive behavior in congenial surroundings, but depression and feelings of isolation, and the beginnings of aggressive and violent behavior in more hostile settings. As higher levels of intoxication develop, control of physical processes is gradually lost with reduction of stability, speech is slurred, there is increased risk taking as reasoning is prejudiced, drowsiness occurs and, depending on the individual, various responses may follow from jocularity to aggression, violence and paranoia (see Morgan and Ritson, 2003, pages 43–44). At least partial loss of memory of specific events during acute intoxication appears to be quite common.

Acute alcohol intoxication may have particular problems in adolescents, as the young may be less tolerant of its psychological and physiological effects. Alcohol consumption in adolescence may have greater significance as the brain and nervous system are still developing and, therefore, are more vulnerable to the toxic effects of alcohol, and some chronic nervous system dysfunctions and behavioral impairments may exacerbate more readily with alcohol consumption in this age-group (Guerri and Pascual, 2010).

Chronic excessive alcohol intake has long been associated with a number of disorders of the central nervous system (Charness, 1993; Greenberg and Lee, 2001), including alcoholic dementia, cerebellar degeneration, Wernicke–Korsakoff syndrome, Marchiafava–Bignami syndrome and central pontine myelinolysis, as well as possible brain damage from cerebrovascular events (e.g. accidents and hemorrhagic stroke), and peripheral neuropathy. Hepatic encephalopathy (dysfunction of the brain – confusion, impaired consciousness) is at least partially the result of alcoholic liver disease (see above). Contrary to common belief, excessive alcohol consumers and alcoholics with no overt clinical signs of neuropathology or hepatopathology may have some regional brain and cognitive impairment (Harper and Matsumoto, 2005; Oscar-Berman and Marinković, 2007; Harper, 2009).

Alcoholic dementia may occur in long-term excessive alcohol intake and is characterized by cognitive impairment with the possibility of slightly marred intellect, but the exact mechanism and level of impairment for the diagnosis is still uncertain (Hulse *et al.*, 2005). It is associated with heavy drinking (Letenneur, 2004) and may present with physical signs of cortical and cerebral white matter atrophy. However, there is some suggestion that alcohol may not have an associated increased risk of dementia (Anstey *et al.*, 2009) and may even have some protective value at low levels of intake (Letenneur, 2004; Peters *et al.*, 2008; Pinder, 2009) (see also Section 5.6.3).

Cerebellar dysfunction due to chronic alcohol consumption is characterized by reduced motor control (movement and balance) and a wide spectrum of neuropsychological impairments (Fitzpatrick *et al.*, 2008) with a number of possible causative mechanisms implicated (Jaatinen and Rintala, 2008), including thiamine deficiency (Mulholland, 2006). Thiamine deficiency associated with chronic alcohol abuse is also heavily implicated in Wernicke–Korsakoff syndrome, although the origins of Wernicke–Korsakoff syndrome may be multifactorial, and which is characterized by Wernicke's encephalopathy, consisting of confusion, eye indications and ataxia, and Korsakoff's psychosis; amnesia with severely impaired retrograde and anterograde memory (Morgan and Ritson, 2003, pages 20–21). Central pontine myelinolysis and Marchiafava–Bignami syndrome are quite rare (see Morgan and Ritson, 2003, page 21).

There are significant psychological symptoms associated with the alcohol withdrawal syndrome (see below).

Osteoporosis and Trauma: Bone Fracture Risk

Bone fractures are associated with acute excess alcohol intake due to both accidents and violent intentional injury. Although moderate alcohol consumption is associated with lower risk of hip fracture, chronic alcohol usage may lead to low bone density, osteopenia and osteoporosis, which increase the risk of bone fracture (Mukamal *et al.*, 2007; Berg *et al.*, 2008) and delays in fracture repair (Chakkalakal, 2005). Alcohol has a toxic dose dependent effect on osteoblast activity, suppressing differentiation and promoting adipogenesis, reducing the rate of bone remodeling, and in fracture, alcohol prevents synthesis of an ossifiable matrix slowing repair (Chakkalakal, 2005). The catabolism of alcohol produces highly reactive oxygen species which have been implicated in skeletal pathologies, including impaired bone remodeling (Banfi *et al.*, 2008).

Pancreatic Disease

Causative mechanisms leading to the development of alcohol related pancreatitis remain obscure (Schneider and Singer, 2005) partly because the susceptibility of individuals varies enormously with some low to moderate drinkers developing this condition, although there is evidence of a dose related risk of alcohol consumption on pancreatitis (Irving *et al.*, 2009). Also, particular constituents found in certain alcoholic beverages may have differential health benefits and detriments (Feick *et al.*, 2007). Acute inflammation episodes are characterized by pancreatic endocrine and exocrine insufficiencies, which may lead to malabsorption of key nutrients, jaundice and diabetes, and other severe metabolic disturbances and circulatory failure (Morgan and Ritson, 2003, page 18).

Poisoning

Acute ingestion of alcohol may result in intoxication when blood levels reach 1.8 g/l (0.18%, 180 mg/dl) and stupor and coma at 3.5 g/l (0.35%, 350 mg/dl), with death occurring from alcohol poisoning at when concentrations reach 4.5 g/l (0.45%, 450 mg/dl) in about half of all individuals so affected. This potentially fatal concentration is equivalent to 5–8 g/kg body weight in adults and 3 g/kg body weight in children, depending on fatness, at least in part. Extreme intoxication leads to severe hypoglycaemia, hypokalaemia, convulsions, metabolic acidosis, respiratory depression, possible cardiac arrythmias and then death; see the website of Southend NHS (http://www.southend.nhs.uk) for concise summary information.

Psychological Disorders and Social Consequences

Psychological disturbances occurring with excessive long term alcohol consumption may include depression, anxiety and psychosis, amongst others, and these may have social implications for interpersonal relationships and work attendance and performance, and they may often result in antisocial and criminal behavior, depending on the interplay of a number of factors (Morgan and Ritson, 2003, pages 30–32).

Excessive drinking and psychological disorders can have a significant bearing on family inter-relationships, and, conversely, family matters can affect the level of alcohol use and abuse, with serious implications for the development and wellbeing of children (Leonard and Elden, 2007).

Respiratory System

Pneumonia may develop through aspiration during acute intoxication. Excessive alcohol exposure has been associated with airway function pathobiology (Boé *et al.*, 2009), but its significance is not completely certain (Sisson, 2007) and there are potential health benefits and detriments from the nonalcohol constituents of alcoholic beverages (e.g. Kamholz, 2006).

Sexual Exacerbations, Infertility and Fetal Disorders

Reproductive capacity can be significantly reduced by acute and chronic alcohol intake. In men, alcohol has adverse effects on the hypothalamus, anterior pituitary gland and Leydig cells of the testes (Emanuele and Emanuele, 1998). In women, alcohol may reduce fertility, increase the risk of spontaneous abortion, produce menstrual perturbations and increase the risk of fetal harm (Bradley *et al.*, 1998). Even with moderate alcohol consumption there are differential effects on levels of various hormones (Gill, 2000). Indeed, excessive beer consumption appears to have a particular reproductive impact in both men and women, possibly due to certain estrogenic constituents of hops (e.g. Milligan *et al.*, 2000), and in lactating women beer drinking appears to impair milk production and motor functions of infants (Mennella, 2001; Rayburn, 2000).

Although there is still uncertainty as to whether or not moderate prenatal alcohol consumption has adverse outcomes in pregnancy (Henderson *et al.*, 2007), the evidence is strong that excessive intake, including binge drinking that extends into very early unrecognized pregnancy, may be detrimental to fetal development with concomitant life long impairments of physical and mental function (Jones *et al.*, 2006; Guerrini *et al.*, 2009). Throughout pregnancy, but particularly at 4-10 weeks, alcohol has serious implications for the developing fetus, with fetal alcohol spectrum disorder and the more serious fetal alcohol syndrome presenting at various levels of intake (Mukherjee *et al.*, 2006; Guerrini *et al.*, 2009). However, there is still some uncertainty concerning the exact amounts and patterns of intake required to produce specific symptoms, but, importantly, certain pregnancies appear to be more vulnerable to alcohol toxicity with serious fetal symptoms possibly developing even with low to moderate intakes (Guerrini *et al.*, 2009). Fetal alcohol spectrum disorder essentially presents with neurobehavioral deficits and the worsening impairments of fetal alcohol syndrome include growth retardation, central nervous system disorders, congenital abnormalities and craniofacial abnormalities (Autti-Rämö, 2002). However, alcohol is also implicated in other disorders such as those of the kidney, liver and gastrointestinal tract (Hofer and Burd, 2009).

In many countries, although it is mandatory to include on the label of an alcoholic beverage a warning on the dangers of alcohol consumption specifically during pregnancy, there is a general lack of awareness, especially in the UK, of the serious harm that alcohol can do to the fetus (Mukherjee *et al.*, 2006; Guerrini *et al.*, 2009).

Skin Disorders

Alcohol abuse, even at its early stages, may manifest as many diverse skin lesions or may exacerbate others (see Morgan and Ritson, 2003, page 22), and some are associated with the development and progression of liver disease (Ghosn and Kibbi, 2008); alcohol related skin lesions include spider naevi, linear telangiectasia, discoid eczema, acne rosacea, psoriasis and seborrhoeic dermatitis, with increased risk of infective skin diseases (Higgins and du Vivier, 1994; Smith and Fenske, 2000; Kostović and Lipozencić, 2004).

Withdrawal

The presentation of psychological and physical symptoms associated with the alcohol withdrawal syndrome is uncertain and may be anything from mild to severe, with variable outcomes from insomnia through to seizures and delirium tremens (McKeon *et al.*, 2008). Pharmacologic treatments are generally symptom based and are cross tolerant with alcohol, and such patients also need personalized, psychological and environmental support (Anonymous, 2007).

5.6.3 Possible Benefits of Low to Moderate Consumption of Alcoholic Beverages

So, evidently, consumption of alcoholic beverages has the potential for exacerbating a wide range of deleterious effects on health from transient minor injuries and lesions to very serious chronic harm and even death when consumed to excess (Table 5.1.1 of Section 5.1.2 and Section 5.6.2). However, alcoholic beverages do appear to have some health benefits as, for example, in the past and in some communities today, such beverages provide a relatively safe source of nutrients and especially water at times when alternative sources may be contaminated. Other particular health benefits appear to be associated with low to moderate alcohol consumption as shown by the 'j' or 'u' shaped pattern of relative risk with increasing intake, which shows that low to moderate intake levels present lower risk than either complete abstinence or higher intakes, especially those amounts ingested by excessive drinkers and alcoholics, in which morbidity and mortality rates are significantly increased (Gurr, 1996). Such epidemiological evidence is associative and, therefore, not conclusive of causation and so prospective and intervention trials, and mechanistic studies are required to confirm that there are tangible health benefits of low to moderate alcoholic beverage consumption (Freiberg and Samet, 2005; Collins *et al.*, 2009; Di Castelnuovo *et al.*, 2010). For example, it would be of value to identify mechanisms involved in neutralizing the detrimental effects of alcohol ingestion and its toxic catabolic intermediates, perhaps by the multitude of antioxidant constituents of particular beverages (e.g. Soleas *et al.*, 1997). However, the epidemiological evidence can still be considered a relatively strong foundation on which to base and test the premise that regular low to moderate alcohol consumption is causally linked to reduced risk of cardiovascular diseases; this is because the purported protective effect has been shown in several independent studies on different population groups (e.g. Yusuf *et al.*, 2004; Mukamal *et al.*, 2006; Beulens *et al.*, 2007; Djoussé and Gaziano, 2007), including type 2 diabetics (e.g. Koppes *et al.*, 2006).

Some of the health benefits of alcoholic beverage consumption may be due to the actions of alcohol per se, possibly as much through the psychological and social aspects of low to moderate drinking as any direct biochemical or physiological effect. However, regular low to moderate alcoholic beverage consumption may be associated with other more healthy lifestyles and dietary practices (Grønbaek, 2006) and, very often, the actual act of drinking may be conducted within a relaxing environment and convivial company. Alternatively, moderate alcohol consumption has been shown to increase plasma high density lipoprotein cholesterol levels relative to low density lipoprotein cholesterol, thereby essentially on balance preventing cholesterol deposition and plaque formation at arterial walls, and also to promote anticoagulant properties through direct effects on reducing platelet aggregation (Puddey *et al.*, 2003; Ruf, 2004). For example, a particular intervention of moderate wine consumption (Hansen *et al.*, 2005) showed an increase (11–16%) in fasting plasma high density lipoprotein cholesterol, with concurrent decrease (8–15%) in fasting fibrinogen, compared with water and red grape extract, although other indices of cardiovascular health did not change significantly, possibly because of a relatively short time frame (four weeks).

Although it appears that low to moderate alcohol consumption provides cardiovascular protection through a number of suggested mechanisms (Agarwal, 2002), including enhanced insulin sensitivity along with increase in blood high density lipoprotein cholesterol levels and improvements in atherosclerotic inflammatory

processes, conclusive characterization of cause and mechanisms is yet to be found (e.g. Freiberg and Samet, 2005). Low to moderate alcohol intake is associated with a decreased risk of developing type 2 diabetes (Koppes *et al.*, 2005; Djoussé *et al.*, 2007). Furthermore, moderate alcohol consumption (taken with food) has been shown to increase insulin sensitivity in established type 2 diabetics (Greenfield *et al.*, 2005) and in nondiabetics (van de Wiel, 2004). The mechanism of increased insulin sensitivity caused by moderate alcohol intake is not fully understood, but may involve a number of factors (Greenfield *et al.*, 2005). The anti-inflammatory effects of low to moderate alcohol consumption have been demonstrated (e.g. Collins *et al.*, 2009).

There is also some evidence from population based cohort and case control studies, conducted in several parts of the world and across genders and racial groups, that the moderate regular consumption of alcohol, particularly wine, is associated with a lower risk of developing dementia (Alzheimer's disease and vascular dementia) (Pinder, 2009).

It has been found that age related cognitive decline is lower in regular light drinkers and older drinkers with mild cognitive impairment (MCI) are less likely to progress to Alzheimer's disease than abstainers of similar age. Mechanisms for these possible neuroprotective effects are unknown at present, but the antioxidant and related properties of wine may be involved, although light consumption of wine appears to modify the neuropathology of Alzheimer's disease, especially with regard to the deposition of amyloid plaque.

The relative nutritional significance of different nutrients found in the multifarious alcoholic beverages available, and their possible beneficial synergisms, have been considered previously in the appropriate sections of this chapter. Although the effects of some of these nutrients are sufficient to be implicated in protective roles, others are not, and actual definitive biological effects have yet to be demonstrated (van de Wiel and de Lange, 2008). Many of the constituents of alcoholic beverages with antioxidant potential, such as some vitamins, minerals and phytochemicals (derivatives of plant metabolism; see Section 5.4.3, Chapter 5.8 and Section 5.11.2), may promote health in moderate consumers because they appear to collectively scavenge and neutralize the harmful highly reactive oxygen and nitrogen species, or free radicals (Puddey *et al.*, 2003; Suter, 2004). Although such free radicals occur endogenously during metabolic processes and exogenously with ingestion and assimilation of foods, they are also produced as the result of detoxification of alcohol itself (see Chapter 5.5). Free radicals damage key structural and functional components of tissues and cells, such as proteins and DNA, unless they can be effectively neutralized by sufficient antioxidant capacity. The 'French paradox,' of relatively good cardiovascular health, even in the face of less healthy dietary and lifestyle practices, has been generally attributed to consumption of red wine, as it contains relatively large amounts of antioxidants (German and Walzem, 2000; de Lorgeril *et al.*, 2002; Ferrières, 2004). However, white wines (Lamuela-Raventós and de la Torre-Boronat, 1999), beers (Denke, 2000; Kondo, 2004) and some spirits (Goldberg *et al.*, 1999) have also been implicated in such health benefits as they too may contain significant levels of phytochemical antioxidants, albeit of different origins and amounts to those of red wines. Nevertheless, there may be differential effects according to the particular beverage concerned and its relative constituents, perhaps with wine consumption associated with more favorable levels in serum of high density lipoprotein cholesterol and fibrinogen, and lower systolic blood pressure, than consumption of either beer or spirits (Ambler *et al.*, 2003). In contrast, the effects of low to moderate alcohol consumption on health may be independent of the type of alcoholic beverage, with equal reduced risks of myocardial infarction being ascribed to beer, spirits and wine (Cleophas, 1999; Schröder *et al.*, 2007), and reduced incidence of type 2 diabetes (Djoussé *et al.*, 2007). It may be that the apparently greater benefits of red wine are more attributable to other factors such as healthier lifestyles (Grønbaek, 2006).

Laboratory studies have been used to try to elucidate the purported mechanisms of protection offered by various components of alcoholic beverages. In one illustrative example, the extent of DNA damage was measured in human lymphocytes exposed to either oxidative stress, with hydrogen peroxide, or to γ-radiation in the presence of individual beverage components and in constituent mixtures typically found in alcoholic

beverages, and compared to controls. Interestingly, the greatest protection against both of these stress agents was afforded by a mixture typical of wine, with ethanol and phytochemicals (e.g. catechin and caffeic acid) in a possible synergistic combination that exceeded the protective effects expected by the sum of the individual constituents (Greenrod and Fenech, 2003).

5.6.4 Conclusion

It is clear that excessive alcohol consumption, whether acute or chronic, may have severe deleterious effects on every cell, tissue and organ of the body, with the extent of damage being essentially dose dependent, but such effects may be quite variable between individuals and communities because of genetic and phenotypic differences in susceptibility. Conversely, there is now quite a substantial body of speculation and scientific opinion, mainly from epidemiological evidence, suggesting that low to moderate consumption of alcoholic beverages on a regular basis, rather than less frequent binge drinking, may actually reduce the risks of all cause mortality, particularly from coronary heart disease, and there may be other health benefits potentially attributable to alcohol per se and to certain other constituents of alcoholic beverages. However, there is little indisputable evidence of alcohol beverage consumption being *directly* causal of health benefits. There is much lively debate in the literature concerning these issues; for a recent example, see Stockley (2009), and for a comprehensive review of moderate drinking, see Gunzerath *et al.* (2004).

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5.7

Carbohydrates in Alcoholic Beverages and Health

The first part of this chapter is focused on the calorific value of certain alcoholic beverages (particularly beers) in the context of relative ethanol and metabolizable carbohydrate content, how this relates to health and how these drinks are perceived by the general public. For a more general account of carbohydrate nutritional value of alcoholic beverages, the reader is referred to Section 5.3.3. The next sections describe the production of low and high carbohydrate beers, and the final part is dedicated to a discussion of health values of nonmetabolizable carbohydrates (again, particularly with reference to beers), such as β-glucans and other fibrous oligomers and polymers.

5.7.1 Metabolizable Carbohydrate

Metabolizable carbohydrates, such as starch (polymers), sucrose, glucose and other sugars, are those that are utilized in cell metabolic processes as a source of energy. In most countries, for most people, they are the biggest providers of dietary energy. Many people in western countries are classed as overweight, as defined by their body mass indexes (BMI = mass (kg)/height^2 (m^2)) being over 25. Additionally, a significant number of these can be classed as obese, with BMI 30. This situation has become more acute since the 1950s, with increased richness of diet and increased use of machinery, leading to a general lack of physical exercise. Overweight in general, and obesity in particular, have been linked to higher risk of disease and to shorter lifespan. Of particular interest here are links with increased risk of cardiovascular disease, cancer and diabetes. Although genetic factors may contribute to some people's high BMI values, it is probable that the greatest contributor to this situation is a greater input of energy (calories) over output of energy – that is, overeating, overdrinking and underexercising. Increased awareness of this situation, especially over the last three decades, has led to considerable interest in special diets, exercise regimes (especially 'working out' and jogging) and in special beverages, both alcoholic and nonalcoholic, and both high and low carbohydrate types. Much of this interest is focused on intake of carbohydrates, the cheapest form of food energy or calories.

A calorie (cal) is defined as the amount of heat energy needed to raise the temperature of 1 g of water by 1 °C. The energy value of foods is commonly expressed in units of kilocalories (kcal = 1000 cal = 1 Cal), and it is generally agreed that, apart from very young children and the elderly, most people need an input of 2500–5000 kcal per day, depending on (amongst other factors) the amount of daily exercise.

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

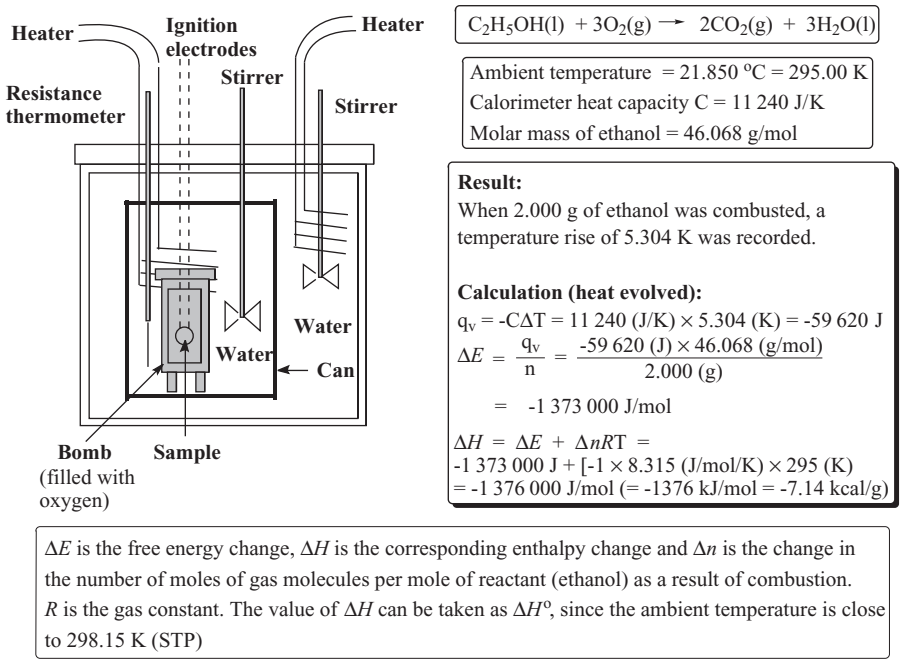

Figure 5.7.1 *Schematic diagram of a bomb calorimeter used to determine calorific value of substances and foodstuffs, via free energy and enthalpy (heat) of combustion. The calorimeter heat capacity is deduced by measuring the rise in temperature of the water as a result of applying a known voltage to the heater coil (of known resistance) for a specif ic time*

The total potential energy of foodstuffs (the 'calorific value') can be estimated by combusting the food in a steel bomb calorimeter (Siska, 2006) (Figure 5.7.1). Combustion releases heat, which is measured by the temperature rise of the bomb calorimeter, or more specifically of the water in which it is immersed. The heat released can be calculated as shown in Figure 5.7.1 and is expressed as kcal/mol or more usually as kcal/g, since most foods are complex mixtures of substances.

Although the measured calorific value of a particular substance (e.g. glucose or maltose) differs from those of other chemically related substances (e.g. fructose or sucrose) and will be higher than the *in vivo* calorific value of the substance (because of incomplete metabolic oxidation), useful rules of thumb for calorific values can be devised for estimating energy input on consumption of foodstuffs. These are (in kcal/g) 3.8 for carbohydrates, 4.0 for proteins and 9.0 for fats. To this, for alcoholic beverages, we can add 7.1 kcal/g for ethanol. Since most alcoholic beverages contain no fat and little protein (with the general exception of beer and other cereal based beverages), their calorific values can be related to ethanol and carbohydrate contents, with the former contributing about twice (w:w) as much as the latter.

The calorific value of an alcoholic drink can be estimated from Equation 5.7.1.

$$\text{Calorific value (CV)} = 7.1\text{E} + 3.8\text{C} + 4.0\text{P} \tag{5.7.1}$$

Here, CV is expressed as kcal/100 ml, and E, C and P are ethanol, carbohydrate and protein content, respectively, in g/100 ml. In many cases, the last term of Equation 5.7.1 will make a negligible contribution

to CV. An alternative calculation of CV is given by Equation 5.7.2.

$$CV = 6.9E + 4.0(RE - A) \tag{5.7.2}$$

This time, CV is expressed in kcal/100 g, and E, RE and A are ethanol, real extract (see Section 2.6.4) and ash, respectively, in % (w:w). Apart from Equations 5.7.1 and 5.7.2, CVs are often given as 'kcal per serving' in books and web pages giving dietary information for health conscious people. A serving is a typical portion of foodstuff (in mass or volume) that would be consumed as a unit, typically ~360 ml (~12 US fluid ounces) for beer, ~120 ml (4 US fluid ounces) for wine and ~45 ml (~1.5 US fluid ounces) for spirits and liqueurs. Typical calorific values, ethanol contents and carbohydrate contents are given for various alcoholic beverages in Table 5.7.1. Also shown here are CVs and carbohydrate contents for some nonalcoholic drinks, for comparison. Although the values in Table 5.7.1 are only typical or representative values, it can readily be seen that the drinks with the higher CVs are generally those with higher ethanol contents, and the drinks with the highest CVs of all are sweet, highly alcoholic drinks. It is also apparent from Table 5.7.1 that, contrary to widespread public belief, spirits and wine generally have higher CVs than beer or cider. If one takes the view that unburnt calorie intake equals fat, then wine is more fattening than beer.

Legislation exists, or is underway, in many countries to impose mandatory requirements on alcoholic beverage producers to include nutritional information with their products, along with health warnings and information on additives. This information is usually on a subsidiary label under the main label, or on the back of the bottle or can. In many countries, this is in line with general foodstuff labeling, especially of processed foods. The general public has long been aware that the only really sensible way to avoid weight

Table 5.7.1 *Calorific values, ethanol content and carbohydrate content of some alcoholic beverages*

Beverage	Typical carbohydrate content/% w:w	Typical ethanol content/% v:v	Calorific value/kcal per g
Barley wine	6.8	9.6	0.79
Lager	2.9	5.0	0.40
Pale Ale	3.4	5.6	0.56
Porter	4.6	5.7	0.56
Stout (sweet)	6.6	4.5	0.54
Stout (bitter)	2.8	4.0	0.35
Light beer	1.8	4.2	0.31
Low carb beer	0.7	4.2	0.26
Low alcohol beer	1.6	0.4	0.27
Cider	3.0	6.0	0.36
Sweet cider	5.0	6.0	0.56
Dry wine	0.03	12.0	0.80
Sweet wine	13.3	12.0	1.6
Spirits	–		2.3
Liqueurs	47		3.8
Cola drink	7.5	–	0.28
Diet cola drink	–	–	–

Source: Data from Bamforth (2005), USDA National Nutrition Database for Standard References, Release 20 and www.perfect.brewing.com. Data for popular soft drinks have been included for comparison.

gain or to lose weight is to strike the right balance between calorie intake and calorie usage (i.e. between eating and exercise). This, and recent labeling legislation, increasing amounts of information on the Internet and the growth of the health food industry has prompted many consumers to focus on the calorie content of their alcoholic beverages (see Section 1.3.10). Unfortunately, alcoholic drinks, and beer in particular, have suffered from being classed as 'high carb' by many influential diet regimes (Bamforth, 2005). Pressure is such that many breweries produce beers specifically labeled as 'low carb,' 'low C,' 'diet,' 'diät,' 'light' or 'lite,' thereby implying that normal beers are 'high carb.' Table 5.7.1 shows that beers in general have lower carbohydrate contents than many beverages (alcoholic or otherwise) and its average calorific value is lower than that for many beverages. Furthermore, the carbohydrate contents and CVs of all but the strongest and sweetest of alcoholic beverages compare reasonably with those for many nonalcoholic drinks (Bamforth, 2005). Finally, intake of carbohydrates per serving of alcoholic drinks compares favorably with intake per serving of nonalcoholic drinks and solid foodstuffs, such as bread, other cereal or cereal products, fruit and vegetables (Bamforth, 2005). For example, a 360 ml glass of pale ale contains around 12 g of carbohydrate, compared with 26 g in a serving of noodles or 29 g in a serving of grapes.

5.7.2 Low Carbohydrate Beers

Although it has been shown in the previous section (see Table 5.7.1) that ethanol, rather than carbohydrate, contributes most to the energy value of alcoholic beverages, there is still a significant demand for low carbohydrate beers. This is partly because of the continued general public misconception that carbohydrates are 'bad' and partly because of the need for alcoholic drinks with low sugar content for people with metabolic disorders, such as diabetes. Additionally, since low carbohydrate beers have about half the energy value of standard beers (Table 5.7.1), they are often heavily promoted by large brewing companies in order to attract more health conscious (usually younger) beer drinkers. As a compromise, some breweries produce a light or 'lite' beer, usually with an energy value somewhere between a regular beer and a low carbohydrate beer.

The production of low carbohydrate beers does not present the brewer with such a severe problem as low alcohol beers (Chapter 2.13). The secret is to provide the wort with enzymes that can cleave the branches from amylopectin (or branched α-glucans) to give linear starch (amylose) and linear oligosaccharides (Section 2.6.2). These can then be hydrolyzed by α- and β-amylases to lower sugars (predominantly maltose), giving a highly fermentable wort (i.e. one with low levels of branched α-glucans or dextrins, that are not fermented by normal brewing yeasts). There are several methods by which this can be achieved, as outlined in Figure 5.7.2.

Limit dextrinase (LD) is the barley enzyme that is able to hydrolyze α-glucan branches. However, in a normal mash, unlike α- and β-amylases, LD remains mostly bound to proteins (known as inhibitors) and hence its activity is rather low. Furthermore, LD activity in wort is strongly influenced by pH and the presence of reducing agents, and it seems that the enzyme is highly temperature sensitive, being essentially denatured at temperatures much above 60 °C. It appears that the presence of reducing agents stimulates cysteine dependent proteinases into cleaving the LD inhibitor protein linkages, thereby releasing free LD into the wort (Jones and Budde, 2003; Heisner and Bamforth, 2008). Furthermore, it has been demonstrated that a 3 h incubation period at pH 4 leads to a threefold increase in extraction of free (active) LD from malted barley at 24 °C, compared with extraction at the more usual mashing pH of 5 (Heisner and Bamforth, 2008). It should therefore be possible to produce highly attenuated beers by dint of pH adjusted, ramped temperature mashing. An initial low pH, low temperature period should produce enough LD activity to cleave many of the branches of amylopectin to give mainly linear starch and linear α-glucan molecules. Increase of pH and ramping of temperature to a rest at 60–65 °C should then allow α-amylase and β-amylase to hydrolyze these molecules to maltose (mostly), maltotriose and glucose.

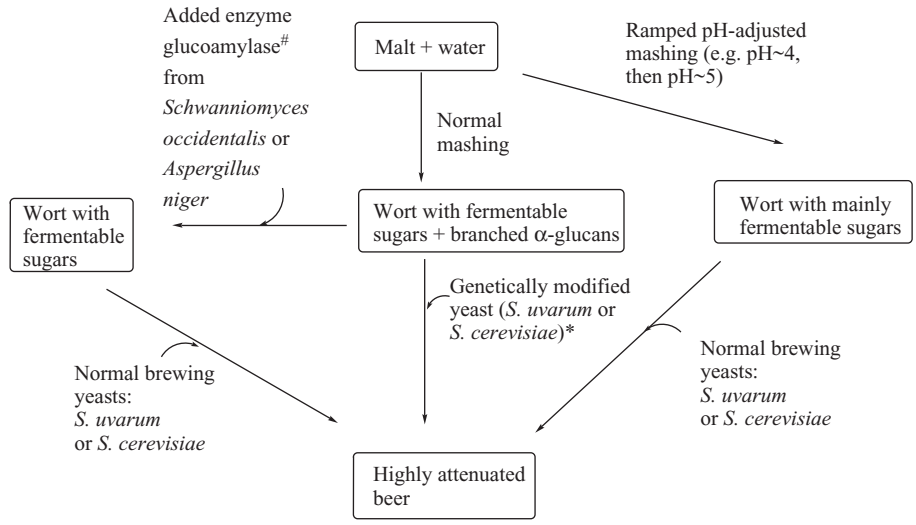

Figure 5.7.2 *Methods for the production of low carbohydrate beers. *With genes that express the α-glucoamylase of* Aspergillus niger *or* Schwanniomyces occidentalis. Saccharomyces diastaticus *will give a compromise result; its glucoamylase will degrade α-glucans, but not at the branches. #Pullulanase or limit dextrinase (LD)*

At the present time, most low carbohydrate beers are produced either by use of exogenous glucoamylase enzymes (usually from *Aspergillus niger* or *Schwanniomyces occidentalis*) in the mash or by fermenting conventional worts with genetically modified yeasts (Figure 5.7.2). Recombinant DNA techniques allow the incorporation of the glucoamylose genes from *Aspergillus niger* or *Schwanniomyces occidentalis* into the *S. uvarum* or *S. cerevisiae* brewing strain genomes. The resultant yeasts are usually genetically stable and exhibit normal fermentation patterns. An example (Hornsey, 1999) comes from the Brewing Research Foundation Institute (Nutfield, UK), who were pioneers in this field. Here, a lager yeast (NCYC 1342) with incorporated glucoamylase genes from *A. niger* was able to attenuate a 1044 °OG wort (80% malt; 20% maltose syrup) at 998° in 12 days, producing a beer with 6.2% ethanol (v:v) (diluted to 5.0% ABV prior to packaging). High attenuation brewing is also of interest to distillers of whisky (and whiskey), because of increased efficiency of distillation (Chapter 3.2).

Most of the large brewing companies produce at least one low carbohydrate beer, usually described as ‘light’ or ‘lite’ (‘low carb’ or ‘low C’ are other names), which refer to the low carbohydrate content rather than the color, although all the well known brands are pale, like Pilsner beers. Indeed, they are bottom fermented, but there the analogy with true Pilsner beers ends; low carbohydrate beers generally do not have the balance of flavor and delicacy associated with the better Pilsners.

5.7.3 High Carbohydrate Beers

The brewing of high carbohydrate beers, by the very nature of the brewing process, leads to a low alcohol product (called malt beer, Malzbier, malt beverage, etc.). The types and styles are discussed in Section 2.6.13. Because of their low ethanol (usually <2% ABV and often below 1% ABV) contents and high carbohydrate contents, high carbohydrate beers are of high nutritional value and as such have been prescribed for convalescent patients in the past. Similarly, some of the better known versions were originally brewed by seaport

breweries (or those close to the sea), in the nineteenth century, for sailors about to embark on a long voyage. Nowadays, the selling point of high carbohydrate beers is still nutritional value, but the target consumers are athletes, pregnant women and active, health conscious people in general. The high carbohydrate content is suitable for supplying energy for both short bursts of vigorous activity or more protracted, milder activity associated with athletic events or exercise, like working out or jogging. Sufficient exercise ensures that the carbohydrates are nearly all combusted, with little, if any being stored as glycogen or fat. Likewise, sufficient exercise will ensure that the low ethanol content consumed is not converted to fat. Also, exercise will help to maintain low blood serum low density lipoprotein cholesterol (LDL-C) levels, high high density lipoprotein cholesterol (HDL-C) levels, low cholesterol levels and low triglyceride ester levels, which are commensurate with low risk of cardiovascular disease (Sections 5.6.2 and 5.6.3). Furthermore, the very low amount of ethanol consumed in a normal serving will be in the beneficial category (Section 5.6.3), thus enhancing to some extent the benefit of exercise.

High carbohydrate beers can be brewed from worts that have been mashed at high enough temperatures to disable β-amylase (Section 2.6.3). This means that the worts contain relatively little maltose, maltotriose and glucose, the carbohydrates being mostly partially fermentable or unfermentable branched α-glucans. Some German high carbohydrate beers (Malzbier) are brewed from a standard, dark, low hopped wort of about 12 °Plato, but cooled to just above freezing point before the yeast is pitched. It then undergoes a very limited, slow fermentation, ultimately giving a beer with 0.5% ABV or less, thus qualifying it in German law as an alcohol free beer.

5.7.4 Nonmetabolizable Carbohydrate

The numerous enzymes and other substances of the human digestive system are unable to cleave covalent β-links in oligomeric and polymeric carbohydrates (see Section 2.6.2, Figure 2.6.5). Carbohydrates (typically cellulose or β-glucans), as well as other fibrous constituents of cereals, fruits and vegetables, such as lignins, polyphenols and proteins, that are not directly metabolized by the human digestive system are well known to be essential items of human diet, despite their lack of direct nutritional qualities. One main benefit of these fibrous materials is in the binding of waste in the gastrointestinal tract and its rapid passage through the large intestine. Another benefit is the stimulation of the immune system, particularly phagocytes such as macrophage cells. Linked with this is the fact that gut lactic acid bacteria thrive in the presence of fiber; they are able to metabolize some of these fibrous substrates, producing chemicals that may stimulate the immune system. Diets high in fiber are known to decrease the risk of several diseases, notably colon cancer, heart disease and diabetes. Refinement of food (e.g. dehusking of cereals and filtration or clarification of beverages) leads to products with lower fiber content. A combination of low fiber content, high refined carbohydrate content, high meat content and high refined sugar content in the diet, which is typical of many western countries, is thought to account, at least in part, to the higher incidences of colon cancer, heart disease and diabetes amongst westerners, as opposed to Asian or African peoples.

It is probable that polyphenols, which are naturally bound to fibrous carbohydrates and proteins, make a significant contribution to the health benefits associated with dietary fiber, since they are known to be readily fermented by gut microflora in the large intestine to simpler phenols that can be absorbed through the gut wall (Section 5.8.9). Indeed, the polyphenol content of the insoluble fibrous material of various beverages has been found to be in the range 1.4–50.7% (dry weight) and in the range 2.9–62.8% (dry weight) for soluble fiber (Goñi *et al.*, 2009).

The large intestine or lower gut is the largest of the immunological organs in humans: a well functioning intestine therefore protects the whole body against a wide range of external pathogens (bacteria, cancerous cells, dead cells or debris, foreign cells, etc.). The intestinal natural immune defence system is composed

mostly of phagocytes: macrophages, T- and B-lymphocytes, and dendritic cells. It appears that the presence of dietary fiber in the gut changes the relative quantities of phagocytes, influences signaling of T-cells and increases the activity, potency and recognition ability of macrophages, without normally overactivating them (Muscat *et al.*, 2007). These events are probably mediated by gut lactic acid bacteria (or their fermentation products of the fiber), which grow well after the consumption of fiber.

Intraperitoneal injection of yeast derived β-glucans were shown to increase leukocyte and lymphocyte cell numbers and the same time they increased the level of natural killer (NK) and lymphokine activated killer (LAK) activities of these cells in mice exposed to whole body X-ray irradiation (Gu *et al.*, 2005). Injected mice showed much greater resistance to tumor growth and secondary infections associated with irradiation.

Cereal derived β-glucans (from oats) in the diet have been shown to offset the risk of upper respiratory infection with exercise stress in mice (Davis *et al.*, 2004) and (from barley) to decrease total and non-HDL cholesterol in hypercholestemic Syrian golden hamsters (Wilson *et al.*, 2004). The latter effects were observed with both lower molecular weight and high molecular weight β-glucans, suggesting that β-glucans in general possess cholesterol lowering and antiatherogenic activities. These results from intervention experiments on animals are in broad agreement with epidemiological or meta-analytical observations on the incidence of disease related to dietary fiber intake in human populations. Human diet intervention studies on well controlled type 2 diabetes patients have revealed further beneficial effects of cereal derived β-glucans. A diet including β-glucans (from oats) and nonsucrose sweeteners led to even greater metabolic and anthropometric improvements than a successful diet based on the American Diabetes Association's nutrition recommendations (Reyna *et al.*, 2003).

The cell walls of cereals used in brewing contain arabinoxylans (pentosans) and β-glucans, mostly as medium to high molecular weight polymers. During malting to produce well modified barley malt, many of these polymers are degraded (hydrolyzed) by β-glucanase enzymes (Section 2.6.2) to give lower polymers and oligomers. These are still classed as β-glucans (or pentosans) since they still possess (mainly 1→4) β–linkages between monosaccharide units, which makes them indigestible to humans, and they are known more generally as soluble dietary fiber. Further degradation of these molecules occurs during low temperature mash in (Section 2.6.3).

In the production of more highly refined beers (the majority), particularly those where filtration is involved, it is in the brewer's interest to minimize the amount of high molecular weight β-glucans and pentosans. High concentrations of these polymers (especially arabinoxylans) in the beer increase its viscosity and decrease its filterability through membrane filters (Sadosky and Schwarz, 2002). However, despite these precautions, dimeric and trimeric β-glucans (cellobiose and cellotriose, respectively) as well as low molecular weight oligomers (e.g. cellohexose) and arabinoxylans of similar molecular weights survive in these beers (∼0.5–3 g/l) (Gromes *et al.*, 2000; Hughes and Baxter, 2001), thus giving even the more refined beers a valuable, if low fiber content. In the USA, the FDA daily recommended intake of dietary fiber is 25 g, which means that a liter of processed beer a day could supply around 10% of this requirement, thereby classifying the beer as a good nutrient source, according to this Authority.

At the other extreme, unfiltered and unrefined cereal based alcoholic drinks of Africa (bouza, burukuta, pombe, shakporo and others), Asia (makkoli and others) and South America (Section 2.7.2) must be relatively rich in both 1,4-β-glucans and arabinoxylans from cereal, and 1,3- and 1-6-β-glucans from yeast, including laminaribiose, laminaritriose, higher oligomers and polymers. These drinks are usually served 'green' (still fermenting) and as thin or thick porridges, depending on the style of the brewer or tradition of the area. Either way, the solids contents of the thinnest unfiltered drinks are probably around 5–10% and so must provide a significant measure of nutrition with respect to both digestible carbohydrates (α-glucans) and nondigestible (dietary fiber) carbohydrates (β-glucans and arabinoxylans). In addition, these drinks supply proteins, amino acids, minerals, vitamins and antioxidants, including polyphenols.

In between these two extremes, the unfiltered cask-conditioned beers and bottle-conditioned beers (both minority products) of Australasia, Europe and USA can contain up to ∼10 g/l of dietary fiber (Hughes and Baxter, 2001). Additionally, suspended yeast cells will supply a small quantity of 1,3- and 1,6-β-glucans. Interestingly, an earlier study of 15 commercial beers revealed arabinoxylans to be much more abundant than β-glucans, with concentrations ranging from ∼0.5 g/l for an American light beer to ∼4.2 g/l for a German wheat beer (Schwarz and Han, 1995).

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5.8
Antioxidant Value of Alcoholic Beverages

5.8.1 Overview

The following sections give a brief description of aerobic metabolism, oxygen in metabolic processes and reactive oxygen species, followed by an account of the nature and effectiveness of natural antioxidants and free radical scavenging substances in alcoholic beverages and their raw materials and precursors. The emphasis is on polyphenols, since the major part of the huge amount of dietary antioxidant research has been directed towards them and they are well known to be potent *in vitro* antioxidants and are widespread in alcoholic beverages and some of their raw materials and precursors.

It is now generally believed that oxygen was absent from the atmosphere at the time of the Earth's formation, about 4.8 billion years ago; the atmosphere was basically a reductive atmosphere. The first organisms were anaerobic, but photosynthetic organisms (such as cyanobacteria) began to evolve around 2 billion years ago. These produced oxygen as a photosynthetic product and it was another half billion years before the first aerobic organisms emerged. The photosynthetic organisms thrived and evolved further, and as the concentration of oxygen in the atmosphere increased, the aerobic species did likewise. Many anaerobic species did not adapt to the increasingly oxidative atmosphere and died out, as oxygen is toxic to anaerobic species. However, many anaerobic species survive today by inhabiting oxygen free environments, such as soil, but the fact is that these organisms thrive in the absence of oxygen, but die in its presence. So, it appears that there is some fundamental cellular toxicity associated with use of oxygen in respiration and that aerobic organisms have some in built defence mechanisms acting against this toxicity, which anaerobic species do not possess.

It is also known that the many aerobic organisms that exist today sometimes suffer from incomplete or faulty protection against oxygen toxicity and are subject to 'oxidative stress.' The stress is caused by the presence of reactive oxygen species (ROS) or reactive nitrogen species (RNS), many of which are free radicals. ROS are necessarily formed at the end of the respiratory electron transfer chain, which is itself linked to aerobic cell metabolism, especially the tricarboxylic acid cycle. ROS are also produced by biotransformation enzymes (e.g. cyclooxygenases (COX) and cytochrome P450 isoenzymes) during the elimination of exogenous substances (e.g. foreign cell debris, drugs, toxins) and endogenous compounds (e.g. unwanted steroid hormones).

Biotransformation enzymes are active during immunoresponse processes, such as the destruction of pathogens (e.g. bacteria, defective or cancerous cells) by macrophages. They are also involved in the degradation of cell debris following necrosis (unprogrammed cell death caused by infection, injury or lack of oxygen). Each of the above processes is characterized by local tissue inflammation, which sets off a number

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
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of biochemical responses, modulated by cell signaling molecules such as leukotrienes and prostaglandins. The biochemical responses (e.g. upgraded synthesis of C-reactive protein in the liver), along with high cytochrome activity, elevated concentrations of oxidized lipids due to ROS action, and other factors can be used as markers for inflammatory diseases, such as atherosclerosis. Reactive oxygen molecules that escape the defensive mechanisms cause cell damage in many ways, by oxidation and by the setting up of complex free radical chain mechanisms.

There is good evidence to suggest prolonged damage of this kind can lead to or contribute to the onset of a number of diseases, particularly cardiovascular disease and cancer. More generally, it is thought that chronic oxidative stress results in accelerated ageing and progressive breakdown of cellular function. Along with good lifestyle, which includes sufficient exercise, good diet (including especially fresh fruit, vegetables and certain beverages, including alcoholic varieties, but only in moderation) is thought to offer some protection against ROS, possibly through the presence of natural antioxidant molecules in the foodstuffs. The observed lower incidences of cardiovascular disease and certain cancers in populations who follow the above-mentioned lifestyles, is thought by many to be (at least in part) the result of the presence of relatively high levels of antioxidants in the diet, including those found in red wine and other alcoholic drinks.

5.8.2 Summary of Aerobic Cell Metabolic Processes

In the aerobic cell metabolism, food molecules like carbohydrates are oxidized essentially by the removal of hydrogen and electrons, which are ultimately combined with oxygen to form water: $2H^+ + 1/2O_2 + 2e^- \rightarrow H_2O$ ($\Delta G^\circ = -158.1$ kJ/mol).

This actually occurs via a series of complex interconnected redox reactions, often called the electron or hydrogen transfer chain, and leads to the ultimate reaction of combining four protons and four electrons with one oxygen molecule to produce two water molecules. This occurs right at the end of the chain and involves the redox protein complex known as cytochrome a, a_3 or cytochrome c oxidase. In between, there is a host of reactions, the overall effect of which is to transfer protons and electrons from the original food molecules to oxygen molecules. Thus, the electron chain process is intimately linked to the redox reactions of the oxidative degradation of food molecules, as illustrated by glycolysis (see Figure 2.2.2 in Chapter 2.2) and the tricarboxylic acid (TCA) cycle (Krebs or citric acid cycle) as outlined in Figure 5.8.1. Also intimately linked to all of this is the regeneration of ATP from ADP and P_i (inorganic phosphate). Many enzymes and other proteins play crucial parts in this entire set of events.

The TCA cycle contains several redox processes, as well as other reactions. The redox reactions involve dehydrogenase enzymes, where hydrogen transfer is performed by either NAD^+/NADH (mostly) or FAD/$FADH_2$ coenzyme systems. The reduced forms of these coenzymes are converted back to the oxidized forms by a series of interconnected redox processes involving dehydrogenases, ubiquinones, ferrodoxins and cytochromes. The last named occur at the very end of the redox process and catalyze the combination of oxygen and protons to produce water. Cytochromes contain iron ($Fe^{2+} \leftrightarrows Fe^{3+}$) in heme units and the cytochrome a, a_3 complex also contains copper. The entire redox chain is called the electron (or hydrogen) transport chain and occurs, along with the TCA cycle and ATP synthesis, in cell mitochondria. The important events that occur in the inner mitochondrial membrane are outlined in Figure 5.8.2.

5.8.3 Short Review of Oxygen in Cell Metabolic Processes

The redox agents that follow the ubiquinone redox system in the electron transport chain are the cytochrome proteins, whose family name is derived from the color changes that accompany their oxidation-reduction. They

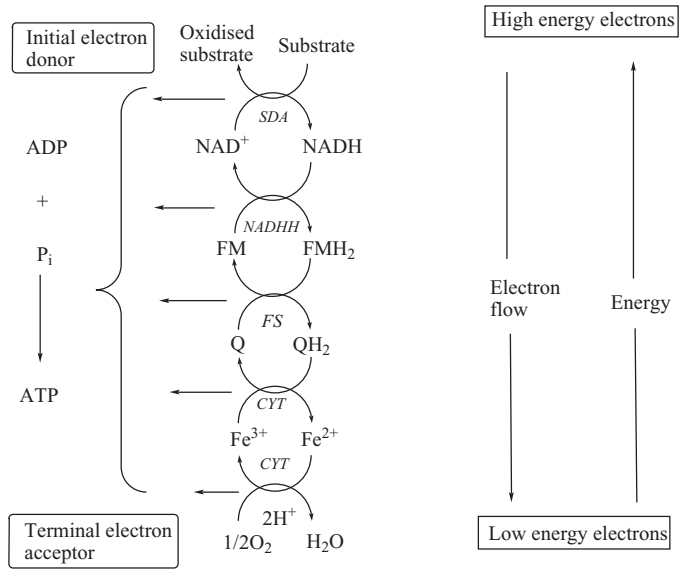


Figure 5.8.1 *The electron chain process. Simplified, showing principal redox cofactors. NAD+/NADH = nicotinamide adenine dinucleotide; FMN/FMNH2 = flavine mononucleotide; Q/QH2 = ubiquinone. The species in italics are enzymes: SDA = substrate dehydrogenase; NADHH = NAD dehydrogenase; FS = ferrodoxins; CYT = cytochromes*

are arranged in the order (after ubiquinone): cytochrome b, cytochrome c_1, cytochrome c and cytochrome a,a_3. It is the last protein (or rather, protein complex) that is responsible for transfer of electrons to molecular oxygen; it is the terminal electron donor of the electron transport chain. Cytochrome a,a_3 is often called cytochrome c oxidase. The redox centers of all the cytochromes are heme prosthetic groups (as in hemoglobin) and the redox reactions all inlvolve the interconversion $Fe^{II} \leftrightarrows Fe^{III}$. Hence, the cytochromes belong to the big family of heme proteins, which can be classified according to the structure of the heme group: heme a, as in

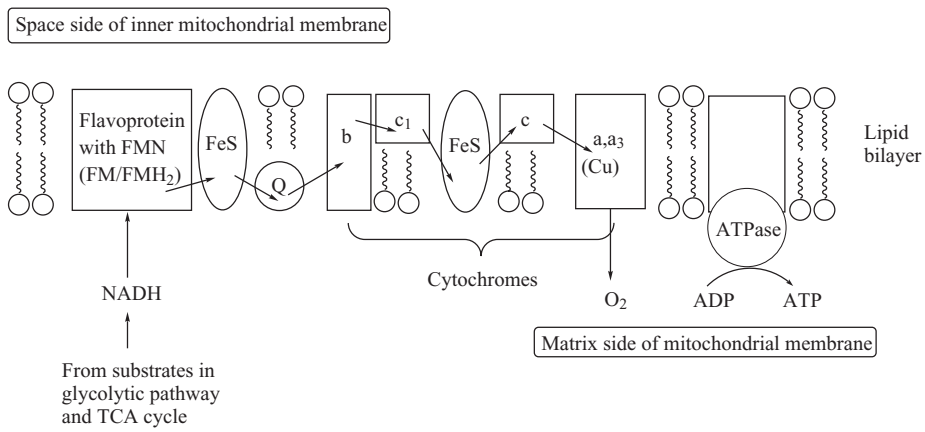


Figure 5.8.2 *Electron transport in inner mitochondrial membrane. Straight arrows represent electron flow. FeS = ferrodoxin (not all of them are shown)*

cytochrome a,a_3, heme b, as in cytochrome b, heme c as in cytochromes c, heme d, as in some catalases, etc. (see http://metallo.scripps.edu/PROMISE/). Other examples of heme proteins are myoglobin, hemoglobin, catalases and some peroxidases.

Cytochrome a,a_3 is the electron donor to oxygen molecules. It contains two heme redox centers (hence its name) and two $Cu^{I} \leftrightarrows Cu^{II}$ redox centers (one of which is a binuclear site) (see www.life.uiuc.edu/crofts/bioph354/cyt_ox.html for more details). At the binding site, heme a_3 and Cu_B act in conjunction to reduce O_2 to H_2O (Figure 5.8.2), coupled to the generation of a proton electrochemical gradient across the membranes in which protein is embedded: 4cyt c(red) + $8H^+$(in) → 4cyt c(ox) + $4H^+$(out) + $2H_2O$.

Cyt c (red) and cyt c (ox) represent the reduced and oxidized forms of the electron donor; H^+(in) and H^+(out) represent protons, which are pumped from the inner (matrix) side of the membrane toward the outer side.

5.8.4 Incomplete Reduction of Oxygen, the Formation of Reactive Oxygen Species and Cell Damage

The oxygen molecule itself, in its stable ground state, is not toxic. However, because of its biradical (triplet state) structure, it can act as an electron acceptor only in a specific fashion. In particular, it is probable that the four electrons donated to each O_2 molecule at the end of the electron transport chain are transferred in a stepwise manner, rather than simultaneously, as shown in Figure 5.8.3 (www.life.uiuc.edu/crofts/bioph354/cyt_ox.html).

All of the named species in Figure 5.8.3 are potent oxidizing agents that arise from the partial reduction of O_2 and all contain oxygen, hence they are known as reactive oxygen species (ROS). Moreover, hydrogen peroxide is produced during oxidations catalyzed by certain oxidase enzymes, such as monoamine oxidase and xanthine oxidase. Hydrogen peroxide, toxic in itself, can produce hydroxyl radicals via reactions of the type: $H_2O_2 + O_2^{-\bullet} \rightarrow OH^- + OH^{\bullet}$.

The hydroxyl radical is one of the strongest of all oxidizing agents and from a consideration of all the above factors, it can be seen that ROS pose a powerful threat to living cells because of the damage they can cause to biomolecules, especially to proteins and the lipid molecules of cell membranes, through oxidation and establishment of radical chain reactions (Figure 5.8.4).

The natural mechanisms that are available to cells for the removal of toxic reactive oxygen species fall into two categories: nonenzymic detoxification and enzymic detoxification. In nonenzymic detoxification, small molecules, such as glutathione, ascorbic acid (vitamin C), tocopherols (vitamins E), carotenes (and vitamin A), lycopenes and others are thought to scavenge ROS before they can cause damage to cellular tissues. The *in vivo* modes of action of most of these molecules are not known for certain, but some may react preferentially with one or more of the reactive oxygen species, whereas others (such as tocopherols) terminate

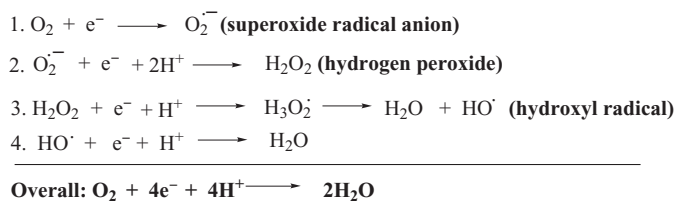


Figure 5.8.3 *Stepwise production of ROS from dioxygen, electrons and protons*

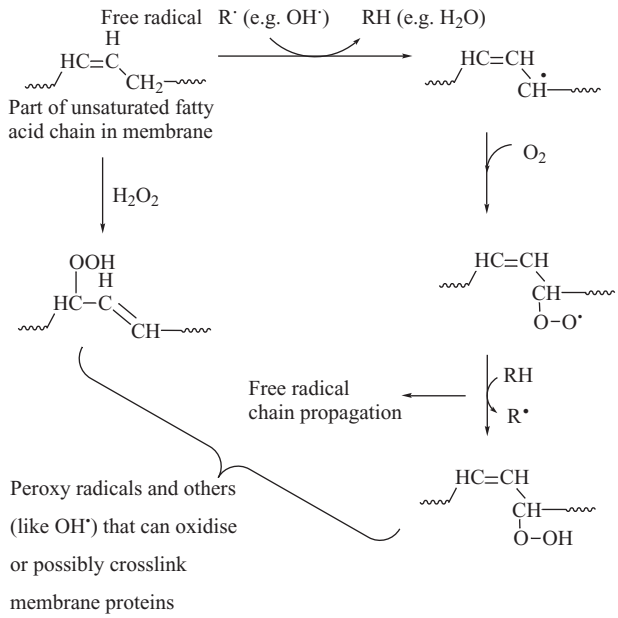


Figure 5.8.4 *Scheme for membrane lipid damage by ROS*

oxidative free radical chain reactions (as in lipid oxidation) or reconvert oxidized cellular constituents back to their naturally reduced forms. It has long been realized that a diet rich in fruit and vegetables can provide sufficient levels of certain of these antioxidants (especially vitamins A, C and E) and hence it is feasible that they help maximize the protection of cells from oxidative damage. Additionally, a host of other antioxidants exist in foods – again, especially in cereals, fruit and vegetables or foods derived from these, such as beers, juices, wines and extracts (e.g. tea). Many of these foodstuffs contain phenolic compounds and this aspect is considered in Section 5.8.6.

Cells possess a number of enzymes for removing ROS. For example, hydrogen peroxide can be reduced by catalase and peroxidase enzymes (Figure 5.8.5). DH_2 is a reduced organic compound, of which there are

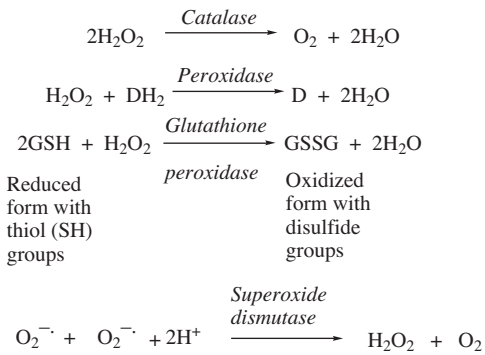


Figure 5.8.5 *Some cellular enzymic mechanisms for removal of ROS*

many, that behaves as a hydrogen donor. Glutathione is an example of such a compound and the enzyme glutathione peroxidase catalyzes the reaction shown in Figure 5.8.5. Superoxide, on the other hand, is disposed of by an enzyme known as superoxide dismutase (SOD). This catalyzes dismutation of $O_2^{-\bullet}$ (a dismutation is a reaction in which identical molecules have different fates) (Figure 5.8.5). The hydrogen peroxide product can then be reduced by enzymes or antioxidants, as described previously. Catalases, peroxidases and the cytochromes all belong to the heme family of proteins. Catalases, for example possess heme type b or d units (http://metallo.scripps.edu/PROMISE/), and are thought to catalyze the decomposition of hydrogen peroxide in the manner shown in Figure 5.8.5. Peroxidases also possess heme b units, although the Fe species is coordinated to a histidine residue (like heme a_3 of cytochrome a,a_3), rather than a tyrosine residue, as in catalases. The action of peroxidases is thought to be similar to that of catalases, except that the former also use an organic compound (e.g. glutathione – see Figure 5.8.5) for oxidation, in order to reduce H_2O_2.

Superoxide dismutase does not belong to the heme family of proteins; instead it is a more complex protein containing copper and zinc units, which are presumably used in the redox mechanism.

5.8.5 Natural Antioxidants in Food

Strictly, antioxidants are substances that interrupt the free radical chain mechanism promoting the oxidation of lipids (see Figure 5.8.4). Nowadays, upwardly spiraling interest in biological antioxidants has resulted in a much broader meaning for the term 'antioxidant.' It can be taken that any molecule that somehow works towards the minimization of oxidative cell damage is a biological antioxidant. This broader definition will be used in this section.

It has long been known from epidemiological studies that the inclusion of reasonable amounts of cereals, fish, fresh fruit and vegetables (or beverages derived from these) in the diet has a tendency to reduce the risk of certain diseases, particularly cardiovascular diseases (CVD) and a limited number of cancers. Likewise, epidemiological studies have demonstrated recently that intake of reasonable amounts of tea and wine, especially red wine, can have similar benefits, particularly with references to CVD. Since there is good evidence to suppose that cardiovascular diseases and cancer may be sponsored by oxidative cell damage in general and by oxidation of specific molecules, the beneficial effects from moderate intake of these foodstuffs have been linked, at least in part, to the presence of natural antioxidants (Table 5.8.1).

Whilst there is little doubt that the phenolic molecules listed in Table 5.8.1 are potent *in vitro* antioxidants (in the broadest sense), there is little firm evidence for their value as direct *in vivo* antioxidants. Indeed, particular health benefits attributed to certain diets may result from a combination of factors that depends on several components of the diet. Furthermore, diet molecules, such as wine polyphenols, may contribute to observed health benefits in several ways, most of which may not involve the molecule as a direct antioxidant at all (see Section 5.8.7).

At the present time, there is intense research activity in many aspects of this field, although there are still huge gaps and many uncertainties in our knowledge. Still relatively little is known about the bioavailablity, absorption and metabolism of antioxidants and even less is known about *in vivo* mechanisms of protection and mode of disease prevention. Also, there are differences in antioxidant assessment results between animal and human intervention studies and yet again between *in vitro* and *in vivo* studies. Additionally, *in vitro* testing methods have often employed phenol concentrations that are far in excess of those likely to be found *in vivo* (e.g. $\sim$1 μM in plasma) (see references in Cren-Olivé *et al.*, 2003; Halliwell, 2006).

Another problem is that certain antioxidants (such as ascorbic acid), in the presence of oxygen and Fe^{2+}, can actually behave as pro-oxidants and are able to generate hydrogen peroxide, which can promote numerous reactions, including hydroxylation of phenylalanine residues in proteins. Moreover, polyphenols have also been shown to generate hydrogen peroxide and other cytotoxic substances on contact with cell culture media

Table 5.8.1 *Some components of foodstuffs displaying* in vitro *antioxidant activity*

Antioxidant	Main dietary sources	Comments
Carotenes and vitamin A	Vegetables: carrots, broccoli and other green vegetables. Vitamin A from animal sources	Polyunsaturated lipid species: β-carotene is a precursor of vitamin A
Lycopenes	Tomatoes	Polyunsaturated lipid species
α-Tocopherol (vitamin E)	Green vegetables, seeds and cereals	A lipid phenolic compound with a long saturated side chain
Lecithin	Vegetables and animal sources	A phosphatidylcholine lipid found in cell membranes
Vitamin C (ascorbic acid)	Vegetables and fruits, especially capsicums (sweet peppers), tomatoes, brassicas, blackcurrants, kiwi fruit and citrus fruits	Water soluble and easily destroyed, especially in the presence of metal ions, like Fe^{2+}
Nonanthocyanin phenolic compounds	Vegetables and fruits, also tea and wine	Many different structures, from simple phenols and cinnamic acids to flavonoids and 3-flavanols – see Figures 5.8.6 and 5.8.7
Anthocyanins	Dark fruits, such as black grapes, mulberries, blueberries, also red wine	

(Long *et al.*, 2000), thereby putting the interpretations of several *in vitro* cell culture studies in considerable doubt; cancer cells possibly being killed by H_2O_2 rather than directly by polyphenols (Halliwell, 2006).

In general, it is much more difficult to assess the value of nonessential antioxidant components of diet (like polyphenols – see Section 5.4.3) than essential components (like vitamins). Their value should be considered as beneficial only with reference to defined circumstances. For example, polyphenols in red wine can be described as inhibitors of cardiovascular disease in healthy individuals on well balanced diets that include the moderate daily intake of red wine. This does not guarantee that the same polyphenols per se taken as intensive supplements will give the same benefits or will offset the effects of a less healthy diet or lifestyle, such as one with high fat and/or high alcohol intake. Furthermore, human intervention studies involving purified antioxidant chemical components of diet, as opposed to the whole source, have failed to give consistent results with respect to protection against heart disease and cancer (Stanner *et al.*, 2004; Frankel and German, 2006). Frankel and German (2006) have advised that such studies should be redesigned to resolve conflicting results of earlier intervention studies. In particular, using separate biomarkers to distinguish healthy from diseased individuals may be of help (Astley, 2003). Frankel and German (2006), and Halliwell (2006) have given very useful summaries on the antioxidant values of phenols and other substances.

5.8.6 Phenolic Substances in Alcoholic Beverages

Phenols are found in all higher plants; in leaves, stems, roots, flowers, seeds, skins, husks and fruit. They have great diversity of structure, but they must have at least one phenolic hydroxyl group (i.e. with OH covalently bonded to an aromatic carbon atom, usually a benzene ring carbon). Most possess several phenolic OH groups and consequently are often known as polyphenols. Many natural phenolic compounds exist as monomers

(composed of just one molecular unit), whereas others exist as oligomers or polymers, containing from two to a large number of repeating or similar molecular units. The latter are often known as tannins, although this term is sometimes used to describe plant phenols in general.

As a result of fermentation and other processes involved in the manufacture of alcoholic beverages, some phenols are inevitably present in the finished drink, although their levels vary enormously, according to the type of processing and aims of the processor. Thus, red wine is rich in phenols, rosé wine less so, cider, perry and white wine less still, with beer generally having the lowest phenolic content.

Phenolic compounds in alcoholic drinks are derived from three main sources:

- From materials used to craft the beverage (e.g. malt, hops, fruit, stalks, pips)
- From metabolic and other reactions that occur during processing, especially during alcoholic fermentation (yeast metabolism) and malolactic fermentation (bacterial metabolism)
- From additives (e.g. from contact with oak or chestnut casks or chips, or from added spices, herbs or pine resin).

The major classes of monomeric phenolic compounds found in alcoholic beverages are shown in Figure 5.8.6. Although flavonoid phenols tend to receive the greatest attention in the literature (especially with regard to antioxidant activity), the nonflavonoid phenols are probably just as widely distributed in alcoholic beverages. Certain classes of phenols, such as isoflavones, coumarins and chromones are not included in Figure 5.8.6 mainly because of their more limited occurrence in alcoholic drinks.

Figure 5.8.7 shows the major classes of polymeric phenols (tannins) found in alcoholic drinks. Of these, the procyanidins (such as B_2, B_3, and larger oligomers) are the most widespread, being found in beer, cider, perry and wine (including fruit wines). Table 5.8.2 lists typical or major phenolic types found in the most common fermented beverages, although it is worth remembering that many alcoholic beverages contain a large number of phenolic compounds, often at low levels.

Monomeric phenols are found in beverages as glycoside or ester derivatives (as well as underivatized forms – aglycones – in the case of flavan-3-ols), whereas procyanidin type polymers are present as aglycone forms. Glycosides include monosaccharides D-arabinose, D-glucose, L-rhamnose and D-xylose and disaccharides such as rutinose (see Section 2.8.1, Figure 2.8.2, Section 2.11.2, Figure 2.11.1 and Section 2.11.3, Figure 2.11.5), which are sometimes esterified (e.g. as acetyl, coumaroyl or galloyl derivatives; see Section 2.10.7, Figure 2.10.13). In the source materials, some phenols are bound to proteins, pectins, β-glucans or arabinoxylans of cell walls; this is especially the case for hydroxycinnamic acid type phenols (such as *p*-coumaric acid and ferulic acid) in barley and malted barley (Dvořáková *et al.*, 2008), and for procyanidins in cloudy apple juice (Barth *et al.*, 2007).

Polyphenols and lignins constitute an important part of dietary fiber, along with digestion resistant carbohydrates and proteins, the polyphenolic content sometimes exceeding 50% (dry weight) of insoluble dietary fiber and exceeding 60% of soluble dietary fiber in some beverages (Goñi *et al.*, 2009). The percentages of phenolic substances in the fiber in beer, cider and red wine were in the middle to higher part of the above ranges. It is believed that fermentation of some of these polyphenols in the large intestine by bacterial microflora contributes a significant amount to the health benefits associated with dietary fiber.

5.8.7 Some Chemical Characteristics of Phenolic Compounds

It is of interest to give a brief survey of selected *in vitro* reactions of phenols, as this may help in understanding their many *in vivo* reactions. The fact that many phenolic substances are relatively easily oxidized means that they are potent *in vitro* antioxidants, often being preferentially oxidized when in mixtures containing

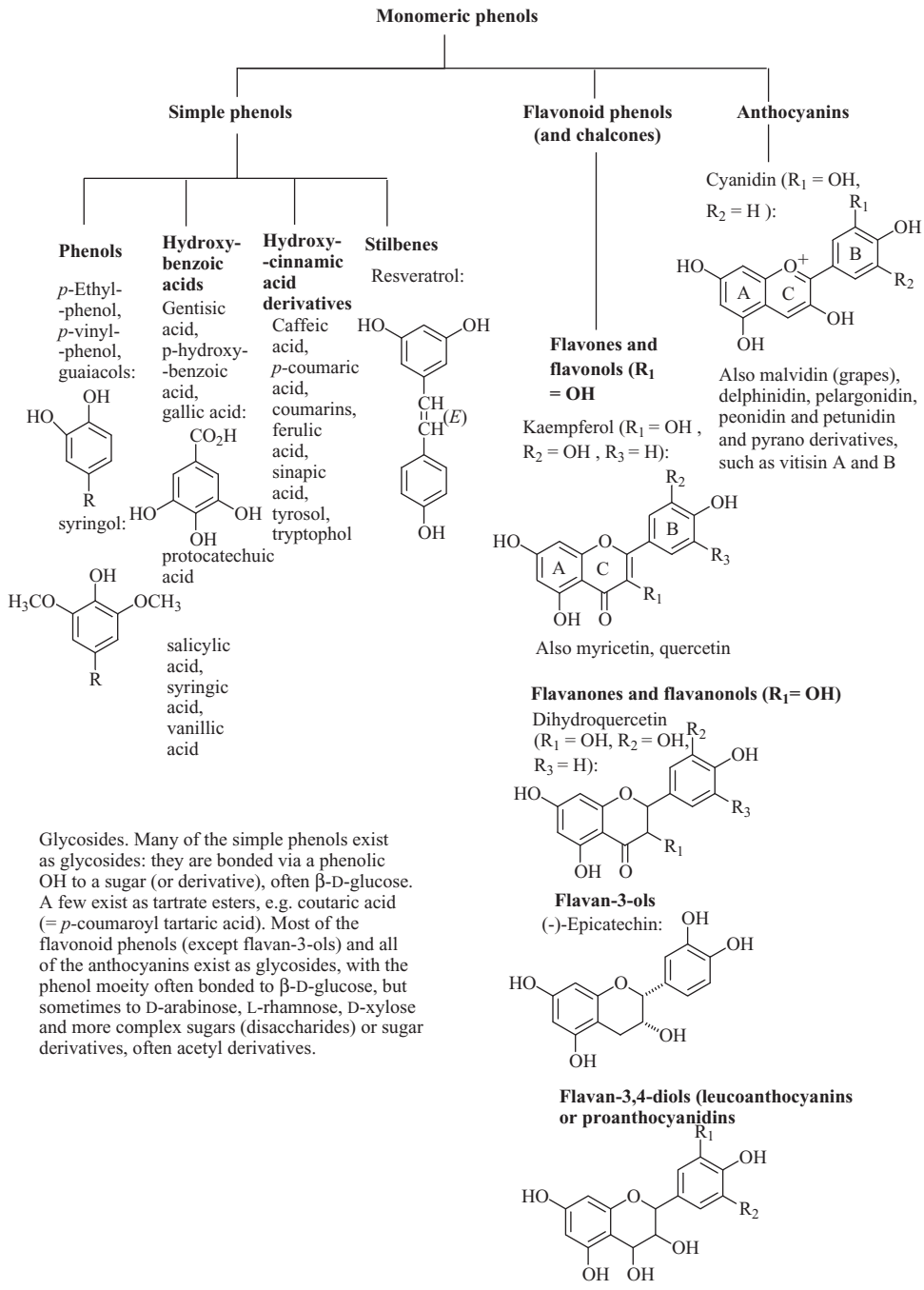
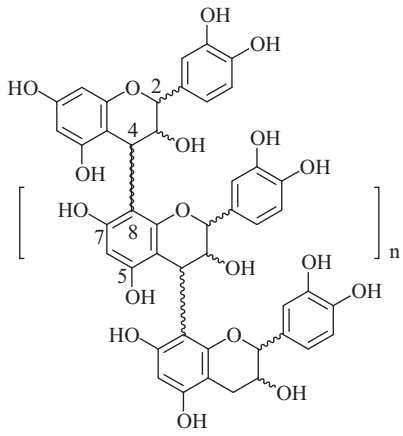


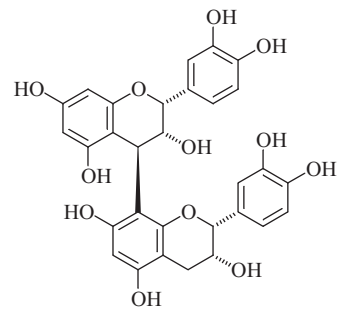
Figure 5.8.6 *Monomeric phenols in alcoholic beverages*



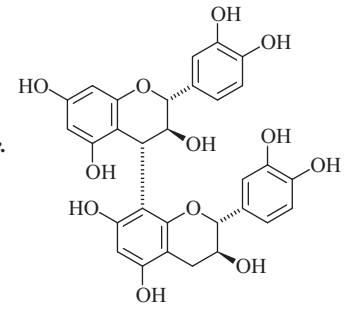
Procyanidin general structure, with type B (4-8) interflavan bonds

These are condensed catechic tannins, made from flavan-3-ol monomers. They are found in most fermented beverages, derived from the source.

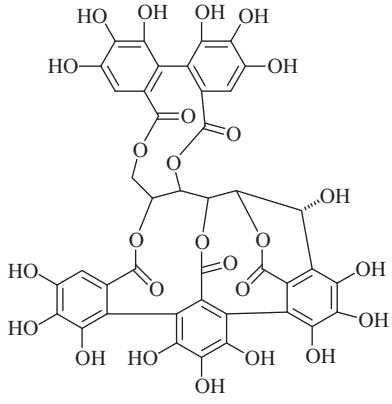
Type B procyanidins have just one interflavan bond between monomers, either C(4)-C(8) or C(4)-C(6) (not shown here).
Type A procyanidins have an ether bond between C(5) or C(7) on the lower unit and C(2) on the upper unit, as well as either a C(4)-C(8) or C(4)-C(6) bond.



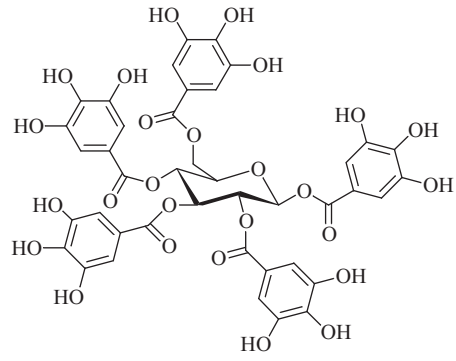
Procyanidin B2, based on (-)-epicatechin



Procyanidin B3, based on (+)-catechin



Castalagin, an ellagitannin from oak or chestnut.



Pentagalloylglucose

These are hydrolyzable tannins, based on sugar derivatives of phenolic acids, gallic acid, ellagic acid (hexahydroxydiphenic acid) nonahydroxytriphenic acid and others. They are derived mostly from woody materials.

Figure 5.8.7 *Some major polymeric phenols found in alcoholic beverages*

Table 5.8.2 *Major types of phenolic substances in alcoholic beverages*

Beverage	Representative monomeric phenol types	Polyphenol and tannin types	Typical total phenolic content (TPC)/g/l gallic acid equivalents (GAE)	Comments
Beer and other cereal based drinks	Cat, cin, fol, sp	Pro	<< 0.1	The brewing processes remove most phenols
Cider	Cat, cin, fol, sp	Pro	≤ 0.5	See Figures 2.8.2 and 2.8.8; Tables 2.8.3 and 2.8.4 in Section 2.8.2
Perry	Cat, cin, fdl, fol, sp	Pro	≤ 0.5*	See Table 2.8.2, Section 2.8.2
White wine (including fruit wine)	Ba, cat, cin, sp, fdl, fol	Pro	~0.3	Some phenols of type ba, sp may arise from wood ageing
Red and rosé wines (including fruit wines)	An, ba, cat, cin, fol, sp	Pro, ell†	≤ 3	See Figure 2.10.13, 2.10.7, Figure 2.11.5, 2.11.3. Some phenols of type ba, sp may arise from wood ageing

An = anthocyanin and nonpolymeric derivatives, such as vitisin A and B, ba = benzoic acid derivatives, cat = flavan-3-ols, cin = hydroxycinnamic acid derivatives, ell = ellagitannins, fdl = flavan-3,4-diols (leucoanthocyanins), fol = flavonols, sp = simple phenols, pro = proanthocyanidins and polymers based on flavonoid monomers. *for bittersweets; †in strawberry – see Figure 2.11.5, Section 2.11.3

other oxidizable substances. Simple phenols are oxidized by mild oxidizing agents (Figure 5.8.8), whilst 1,2- and 1,4-dihydric phenols are even more easily oxidized to 1,2- (or *o*-) and 1,4-(or *p*-)-benzoquinones (respectively). Many natural phenols possess catechol (1,2- or *o*-dihydroxy units) that can be chemically or enzymically oxidized to *o*-benzoquinone groups, as shown for (+)-catechin, a common component of alcoholic beverages (de Gaulejac *et al.*, 2001) (Figure 5.8.9). It is understood that *o*-benzoquinone groups can bind covalently to proteins, one of the factors that contribute to permanent hazes in maturing beer (Section 2.6.9). It will be emphasized later that a common general feature of polyphenols and tannins is their ability to bind proteins.

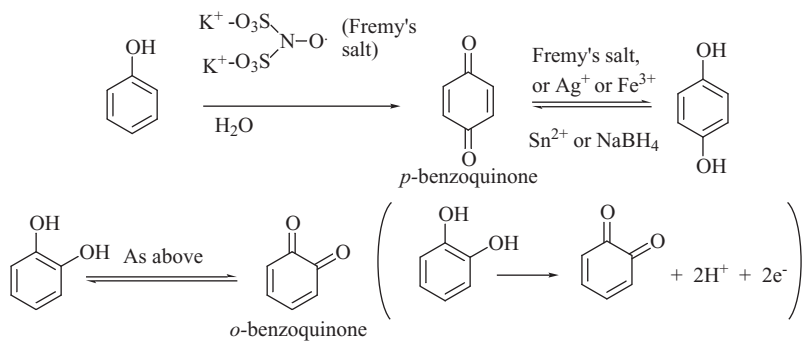


Figure 5.8.8 *Oxidation of phenols* in vitro

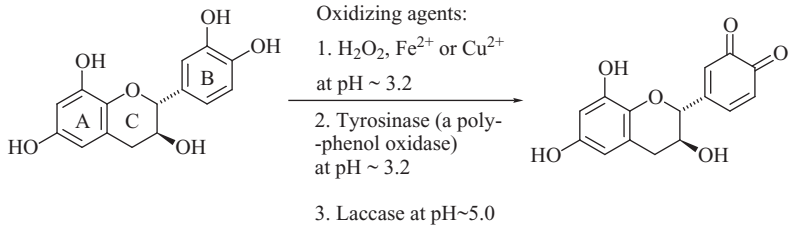


Figure 5.8.9 *Oxidation of B ring catechol unit of (+)-catechin*

It is believed that the first step in the oxidation of polyphenols is the formation of resonance stabilized phenoxyl radicals. Simple phenols, and especially conjugated phenols are able to form relatively stable phenoxyl radicals by hydroxyl hydrogen abstraction and are thus capable of disrupting free radical propagation in chain mechanisms such as those observed in the peroxidation of lipids (Figure 5.8.4). This phenomenon, shown by many conjugated and aromatic molecules, is known as radical scavenging. Phenoxyl radicals are resonance stabilized (a form of thermodynamic stabilization), but some with flanking bulky groups are also sterically (kinetically) stabilized. In a study of the antioxidant values of citrus flavanones, Di Majo *et al.* (2005) showed antioxidant power, as measured by scavenging competition for peroxy radicals with crocin, is dependent on both methylation at the catechol unit in ring B and on the nature of glycosation at position 7 in ring A. For the aglycones, the presence of C(4)-OCH_3 enhanced antioxidant activity somewhat, whereas with the 7-rutinosides, antioxidant ability was greater with an unmethylated catechol ring B unit. This catechol unit (methylated or not) appears to be important, but not essential for radical scavenging activity, since narengin, with only one ring B OH group, also shows antioxidant properties.

Phenolic substances, tannins in particular, are noted for their binding to proteins – indeed this quality is important in the tanning of leather materials, and contributes to influences on overall nutrition (Section 5.4.3). Hydrolyzable tannins especially contain large numbers of phenolic OH groups that can act as either hydrogen bond acceptors or hydrogen bond donors with proteins, besides having carbonyl oxygen and other oxygen atoms that may act as hydrogen bond acceptors. Castalagin (Figure 5.8.7) for example has 16 phenolic OH groups and 10 other oxygen atoms that could conceivably participate in hydrogen bonding. Given the right conditions, say a local mildly alkaline environment, phenols may be able to form ionic bonds with arginine and lysine residues of proteins. Additionally, lipophilic attractions between the phenol aromatic rings and the side chains of phenylalanine, tyrosine and other aromatic amino acid residues may also be possible.

Polyphenols should also be capable of hydrogen-bonded attractions with carbohydrate and nucleic acid molecules. Given the extent of the many weak bonding capabilities of phenols, it is likely that polyphenol–carbohydrate or polyphenol–protein interactions (e.g. at receptor sites) are important in some of the *in vivo* processes that result in the known health benefits that can occur when phenol containing alcoholic beverages are consumed in moderation.

The fact that polyphenols are generally good metal chelators is of interest because many oxidations are catalyzed by transition metal ions (Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, etc.), since these ions can readily exchange electrons, thus enabling them to initiate free radical chain reactions. Polyphenols, especially those with catechol units, by forming chelate complexes with metal ions such as Fe^{2+} and Fe^{3+} can render these ions less able to participate in the generation of free radicals, as initiators (Bujanda, 2000). This may be one of the mechanisms by which inhibition of the oxidation of membrane or lipoprotein lipids may occur in the presence of phenols.

Polyphenols and other antioxidants exhibit a wide range of nonantioxidant *in vitro* activities, some of which could contribute to *in vivo* disease protection by the presence of such constituents in the diet. For

example, some flavonoids and other phenols are inhibitors of oxidative or degradative enzymes, such as cyclooxygenases, matrix metalloproteinases, lipoxygenase and xanthine oxidase. *In vivo*, these effects may limit unsaturated fatty acid oxidation and protect against atherosclerosis, or limit the invasive properties of cancer cells and metatstasis. Some polyphenols (e.g. flavan-3-ols) block folate metabolism, thus possibly inhibiting cell proliferation. Likewise, catechins inhibit telomerase, a nucleoprotein whose activity has been linked to the 'immortality' of cancer cells. Several polyphenols are known to downregulate transcription factors such as NF-κ-B, or are active receptor binders or are known to be inhibitors of angiogenesis. Carotenes and selenium are known to stimulate immune response or to upregulate the production of protective enzymes (Halliwell and Gutteridge, 2006).

5.8.8 Health Benefits and Antioxidant Potency of Alcoholic Beverages, their Phenolic Extracts and their Individual Phenolic Components

The association of certain components of diet, commonly known as antioxidants, with reduced risk of certain diseases and pathological conditions (notably cardiovascular diseases, diabetes and certain cancers) is well known. Amongst those components, and sometimes prominent, are phenolic compounds. Red and rosé wines, including fruit wines, generally possess the highest phenolic contents, with white wine, beer and spirits being relatively poor sources, unless phenols have been added, as additive components, such as in liqueurs, bitters, cocktails, aperitives, vermouths and retsina.

The primary evidence comes from whole beverage and whole food epidemiological studies, which have been critically summarized (Bujanda, 2000; Stanner *et al.*, 2004; Meyskens and Szabo, 2005 and many references therein). As discussed in Section 5.6.3, low to moderate consumption of alcoholic beverages in general is associated with reduced risk of cardiovascular disease (in particular) and certain cancers, such as some of those of the gastrointestinal tract. Ethanol itself plays a role in reducing the risk of cardiovascular disease, but the consensus of opinion is that phenols also play an important part and that the two may be most effective together. It is possible that other components (e.g. β-glucans (Section 5.7.4), pentacyclic triterpenoids, Maillard reaction products and degraded chlorophylls (Section 5.8.10) play some part too.

Given such evidence, many animal intervention and human intervention studies (*in vivo*), as well as *in vitro* studies have been undertaken in recent years using either whole beverage samples, phenolic extracts or single/mixed specific phenolic components. Intervention studies are usually carried out by observing changes in risk factors or changes in the levels of disease biomarkers in a group of subjects that are randomized into subgroups ('arms') to take (injested or injected) beverages or placebo. In the case of humans, the risk factors can be physical ones such as heart rhythm or blood pressure, or concentrations in biological fluids of specific chemicals (say for cardiovascular disease), such as blood HDL-C, LDL-C, trigycerides and fibrinogen levels. Biomarkers (usually measured in plasma, serum or urine) can be specific ones for particular conditions (say atherosclerosis) such as homocysteine, C-reactive protein, interleukin-6, intercellular adhesion molecule-1, etc., or more general markers for oxidative stress, such as F_2-isoprostanes and 19- or 20-hydroxyeicosatetraenoic acid (19- or 20-HETE) (Section 5.8.9). The same argument applies to animal intervention studies, with the addition that there is the opportunity to study biomarkers in whole tissue, as well as biological fluids.

Human intervention studies have tended to show that moderate intake of alcoholic drinks (especially red wine) favorably alters the levels of certain cardiovascular disease risk factors in healthy individuals (Hansen *et al.*, 2005; Natella *et al.*, 2002; Vásquez-Agell *et al.*, 2007). Similar benefits were found on moderate red wine intake by subjects with type 2 diabetes mellitus (Marfella *et al.*, 2006) and by mildly hypocholesteremic subjects (Naissides *et al.*, 2006).

Intervention studies on rats have demonstrated that those fed with red wine had lower lipid peroxidation, greater reduced glutathione levels and higher antioxidant enzyme activity compared with alcohol-fed rats (Assunção *et al.*, 2007). Also, red wine was found to attenuate alcohol induced rise in blood pressure in Sprague–Dawley rats, although the effect was not mediated by the inhibition of cytochrome catalyzed hydroxylation of arachidonic acid (Section 5.8.9) (Cowpland *et al.*, 2006).

Thus, intervention studies tend to corroborate the association between moderate red wine consumption and lesser risk of cardiovascular disease. Moreover, individual polyphenols (catechin and quercetin) were found to improve F1-labeled blood clot lysis on injection into ApoE knockout mice (atherosclerosis model) to the extent that lysis was at the same level as in 'wild type' control mice (Booyse *et al.*, 2007). Furthermore, polymeric proanthocyanidins from grape pips were found to increase antioxidant defences in rat plasma (Koga *et al.*, 1999), whilst a combination of gallic acid, total catechins and total resveratrols showed similar effects in rabbits, whereas total anthocyanins contributed more to vasodilatory effects than to antioxidant effects (Burns *et al.*, 2000).

Thus, there is a body of *in vivo* evidence for the involvement of phenolic compounds, as such, in protection against cardiovascular disease, thought not necessarily via direct antioxidant pathways. This is discussed further in Section 5.8.9.

There is less clear direct *in vivo* evidence of protective effects of beverage polyphenols, as such, against gastrointestinal and other cancers; beneficial effects appear to depend on dose, biological redox status and biological environment (Bujanda, 2000). Recently, cloudy apple juice was found to be more effective than clear juice or phenolic extracts in the modulation of 1,2-dimethylhydrazine-induced colon cancers in rats (Barth *et al.*, 2007). This implies that the colloidal suspension of proteins, fatty acids, cell wall polysaccharides, pectins, polyphenols and a host of other compounds, has a greater protective effect than polyphenols alone. This has been interpreted as a synergistic action between pectins and procyanidin type polyphenols in particular, which is discussed in Section 5.8.9.

More recently, Zhang *et al.* (2009) have shown that dietary grape extract, taken as 5% of the diet and corresponding to daily consumption of 7 μg of resveratrol, significantly reduced the incidence and multiplicity of tumors in the small intestine of male Fischer rats. The tumors were induced by azoxy methane in the diet. Extrapolation of animal data suggested that a 100 ml glass of red wine per day might provide protection against small intestinal cancer development in humans. Interestingly, it was also shown that resveratrol (although at much higher levels than in the animal study) inhibited the *in vitro* growth of human colonic adenocarcinoma (Caco-2) cells, mainly by inhibition of cell proliferation, rather than by cell viability.

The *in vitro* antioxidant value (often known as total antioxidant activity or TAA) of juices, alcoholic beverages and extracts can be estimated by a number of chemical/spectroscopic methods. These methods are discussed in more detail in Section 4.4.3, but include the crocin bleaching method, the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) method, the ferric reducing ability of plasma (FRAP) assay, the ferricyanide reducing method, the oxygen radical absorbance capacity (ORAC) method and the trolox equivalent antioxidant capacity (TEAC) assay.

In general, there is a positive correlation between TAA and total phenolic content (TPC). Thus, red wines tend to have higher TAA values than white wines (Stasko *et al.*, 2002). The best correlation between TAA and phenolic concentration in red wine was for proanthocyanidin (polymer) fractions (Sánchez-Moreno *et al.*, 2003), partly explaining why older wines (with higher polymeric phenol contents) usually have higher TAA values than young wines (Larrauri *et al.*, 1999; Burns *et al.*, 2001). Radical scavenging power (often measured by the $DPPH^{\bullet}$ method) was likewise in the order red wine $>$ rosé wine $>$ white wine, and in white wines, hydroxycinnamic acid derivatives and flavonols were found to be largely responsible for radical scavenging ability, whereas flavan-3-ols were the main antioxidants (Psarra *et al.*, 2002). For red wines, reasonable correlations (R^2 up to 0.55) were found between TAA and catechin, gallic acid, myricetin and peonidin 3-glucoside concentrations (Di Majo *et al.*, 2008).

In nongrape based alcoholic beverages, high TAA values have been measured (by the Fe^{2+}/Fe^{3+}-thiocyanate method, Section 4.4.3) for Chinese yellow wine (or its concentrate), made from glutinous rice and wheat (Section 2.7.1) (Que *et al.*, 2006). The total antioxidant activity of 14 unmalted barley varieties has been determined by several methods, showing that total phenolic content of the barleys was well correlated with reducing power and radical scavenging power (by both ORAC and $DPPH^{\cdot}$ assays) (Zhao *et al.*, 2008).

Wines from black fruits, such as blueberry, have been shown to have similar TAA values to red wines (Sánchez-Moreno *et al.*, 2003). Similarly, dark fruits that are the basis of red fruit or country wines (e.g. blackberry, blackberry × raspberry hybrids and black cherry) were found to have similar TAA values to those of black wine grapes by the FRAP method (Pantelidis *et al.*, 2007). Likewise, North American huckleberries had high TAA values (ORAC assay), with *Vaccinium ovatum* being superior to *V. membranaceum* in this respect. The former were also higher in TPC and total anthocyanin content (Lee *et al.*, 2004).

There are a great many *in vitro* studies in the literature that were designed to test the polyphenol antioxidant hypothesis and to shed light on antioxidant and other mechanisms of interaction between polyphenols and biological systems. Much of this work involves the observation of physiological and biochemical changes in cells in response to contact with whole beverages, phenolic extracts or individual phenols. The main potential problem with results from these types of studies is the extrapolation to *in vivo* situations; cell responses *in vitro* may be quite different to *in vivo* responses. Also, the concentrations of phenolic compounds exposed to *in vitro* cells were often far greater than those ever experienced *in vivo* (Frankel and German, 2006). Furthermore, in experiments involving exposure of cancer cells to individual phenols or even phenol rich beverages, cell death (or other effects) may sometimes be attributed to the production of H_2O_2 from reactions of polyphenols with cell media (Long *et al.*, 2000).

Finally, although *in vitro* results generally support the antioxidant hypothesis, there is sometimes seemingly contradiction in detail (such as which class of phenols, like anthocyanins, flavonoids or proanthocyanidins, are most effective), which might be a result of differences in experimental conditions.

Earlier studies suggested that wine (especially red wine) and phenolic extracts were able to inhibit the (often Cu^{2+} catalyzed) *in vitro* oxidation of LDL and that the activity was collective and not due to a single phenolic component, although the best correlation between concentration and extent of inhibition of oxidation was for catechin (Frankel *et al.*, 1995; Teissedre *et al.*, 1995). Other studies under similar conditions using phenolic fractions from juice, wine and grape pips pointed to the greater antioxidant activity of procyanidin oligomers, flavan-3-ols and myricetin (Teissedre *et al.*, 1996). Wines made from mixtures of blackcurrants and crowberries or bilberries were effective in the inhibition of methyl linoleate peroxidation (Heinonen *et al.*, 1998). Wines were more effective than liquors, but there was no correlation between effectiveness of peroxidation inhibition and total phenolic content. In a study of the *in vitro* LDL oxidation in blood platelets, a red wine anthocyanin fraction, rather than whole wine, dealcoholized wine or phenolic acid/flavonol fractions was most effective at inhibiting LDL oxidation and platelet aggregation, two major events in the pathogenesis of atherosclerosis (Ghiselli *et al.*, 1998).

On the other hand, a study of *in vitro* inhibition of oxidative damage to catalase inactive human erythrocytes using red wine anthocyanin fractions demonstrated the polymeric subfraction to be the most effective (Tedesco *et al.*, 2001). Likewise, wine phenolic oligomers/polymers have been shown to be the best radical scavengers (de Gaulejac *et al.*, 1999; Rigo *et al.*, 2000). More recently, a blueberry extract of vinylpyranoanthocyanin-catechin oligomers (portisins, see Section 2.10.7, Figure 2.10.13) proved to be more effective than other phenolic extracts in reducing 2,2′-azobis(2-methylpropanamide).2HCl induced peroxidation of liposomal membrane lipids (Faria *et al.*, 2005). Also recently, both anthocyanin and procyanidin extracts of blackcurrant and boysenberry were able to protect human HL-60 promyelocytic cells and neuroblastoma cells against oxidative damage by hydrogen peroxide (Ghosh *et al.*, 2006).

Resveratrol from black grapes or red wine was shown to effectively counter apoptosis of human peripheral blood mononuclear cells caused by the oxidative activities of added 2-deoxy-D-ribose. However, inhibition of fatal damage was highly dependent on resveratrol concentration, maximum activity occurring at the 20 μM level (Losa *et al.*, 2001). Higher concentrations of this phenol actually showed pro-oxidative behavior, something that could apply to doses of polyphenols in general, though it should be borne in mind that there is little evidence that *in vivo* cells experience even 20 μM concentrations of phenolic substances. Thus, most *in vitro* evidence suggests that phenolic oligomers and polymers are generally more effective lipid oxidation inhibitors than other (monomeric) polyphenols, but there is *in vitro* evidence that all the important phenols found in alcoholic beverages have some effect against oxidative damage. Also, most data suggest that red wines are rather more effective *in vitro* inhibitors of oxidative cell damage than white wines, cider, perry or beer.

However, experiments have shown that white wine made with just 18 h skin contact (most white wine receives virtually no skin contact time) had a similar *in vitro* inhibition of LDL oxidation as a red wine, despite the fact that the total phenolic content of the white wine was still only 23% that of the red wine. This suggested that white grape skin substances (phenols and other compounds) are pro rata more powerful antioxidants than black grape/red wine compounds (Fuhrmann *et al.*, 2001).

Other *in vitro* studies on oxidative damage related to cardiovascular disease have taken rather different approaches. Extracellular addition of Chinese yellow wine to cultured rat vascular smooth muscle cells reduced homocysteine induced matrix metalloproteinase-2 (MMP-2) (Guo *et al.*, 2007). MMP-2 has been implicated in atherosclerotic plaque growth and instability (Section 5.8.9). In experiments inducing oxidative stress in myotube cultures by hypotonic shock, it was found that the presence of catechin and quercetin lowered the level of ROS and osmolyte release (of leukotrienes and taurine in particular), as well as inhibiting the oxidation of 2′,7′-dichlorodihydrofluorescein (Young *et al.*, 2004). However, trolox (Section 4.4.3) or α-tocopherol did not significantly influence leokotriene and taurine efflux, thus suggesting that the myotube culture responses to the presence of flavonoid phenols may not be confined to redox activity. Oxidative damage to DNA of *in vitro* cell cultures has been the focus of attention of some studies. Thus, monomeric and polymeric phenolic fractions from red wine were observed to inhibit chemically induced oxidative damage to herring sperm DNA (Lodovici *et al.*, 2001), whereas caffeic acid and catechin were shown to offer protection to human lymphocyte DNA against oxidative damage caused by either H_2O_2 or γ-rays (Greenrod and Fenech, 2003).

As a result of the observation from epidemiological studies that polyphenol rich diets (including low to moderate intake of some alcoholic beverages, especially red wine) may reduce the risk of certain cancers, there has been much research activity centered on the *in vitro* effect of polyphenols on cancer cells. Some earlier work in this area is summarized in Bujanda (2000) and in the references that follow.

Recent research has shown that whole berry black fruit extracts (Seeram *et al.*, 2006) and individual phenols (Ma *et al.*, 2007) have some marked effects on certain human cancer cell lines. Berry extracts at concentrations of 25–200 μg/ml were evaluated to inhibit the proliferation of human breast (MCF-7), colon (HT-29 and HCT 116), oral (KB and CAL-27) and prostrate (LNCaP) cancer cell lines (Seeram *et al.*, 2006). All the extracts exhibited inhibition of cell proliferation in all the cell lines, especially at the higher concentrations. Furthermore, black raspberry and strawberry extracts in particular exhibited high pro-apoptotic activity (∼threefold over untreated controls) against human HT-29 colon cancer cells. Earlier work has also shown berry extracts to have *in vitro* pro-apoptotic effects on human cancer cells (Ramos *et al*., 2005; Heo and Lee, 2005). Additionally, phenolic fractions or individual polyphenols have demonstrated inhibitory effects against various human cancer cell lines, such as red wine phenolic fractions against melanoma cell colonies on agar (Gómez-Cordovés *et al.*, 2001) and isorhamnetin (a flavonone glycoside) against esophageal squamous carcinoma cell line Eca-109 (Ma *et al.*, 2007).

5.8.9 Mechanisms of *In Vivo* Health Benefits of Dietary Phenols: Antioxidants or Otherwise?

The possible roles of phenols in food, wines and other beverages as *in vivo* antioxidants have been reviewed (de Beer *et al.*, 2002, de Lange and van de Wiel, 2004; Manach *et al.*, 2004; Tripoli *et al.*, 2007; Serrano *et al.*, 2009). Although there is substantial evidence that phenolic substances in alcoholic beverages, upon moderate consumption, contribute to health benefits, and polyphenols are well known to be potent *in vitro* antioxidants, there is little firm evidence that they act as direct antioxidants *in vivo*. Indeed, there is seemingly conflicting evidence in the literature regarding *in vivo* mechanisms – see for example Cowpland *et al.* (2006) and Ghiselli *et al.* (1999). More specifically, there is *in vivo* evidence that conflicts with *in vitro* evidence (Section 5.8.8) for the inhibition of lipid oxidation mechanism of phenolic antioxidant activity in, for example, preventing or delaying atherosclerosis (Hodgson and Puddey, 2005; Cowpland *et al.*, 2006). Part of the problem lies in the design of and interpretation of results of intervention (i.e. *in vivo*) studies and corresponding *in vitro* studies, as already discussed in Section 5.8.8 (Frankel and German, 2006). Another problem is the extreme complexity of the *in vivo* processes; relatively little is known about the bioavailability and metabolism of phenolic compounds. Also, there is a wide choice of biomarker molecules (in plasma, serum or urine) with which to correlate general protection from diseases (e.g. cardiovascular disease) or with particular aspects of a disease, such as the growth and instability of plaque in atherosclerosis.

As far as antioxidant activity is concerned, quite a few biomarkers of *in vivo* lipid oxidation or general oxidative stress have been used, including eicosanoids (such as isoprostanes), isofurans, isoketals (isolevuglandins), malondialdehyde and neuroprostanes. In recent years, much work has focused on isoprostanes in blood or urine as products of *in vivo* free radical (nonenzymic) peroxidation of lipids (especially arachidonic acid) (Montuchi *et al.*, 2004). The most studied isoprostanes are of the $F_{2\alpha}$ group of stereoisomers: they are isomeric with cylcooxygenase-1 (COX-1) derived prostaglandin $PGF_{2\alpha}$ (Smith, 2008) (Figure 5.8.10). Of these, probably the best known and most common stereoisomer is 8-isoprostaglandin- $F_{2\alpha}$ (8-*iso*-PG $F_{2\alpha}$

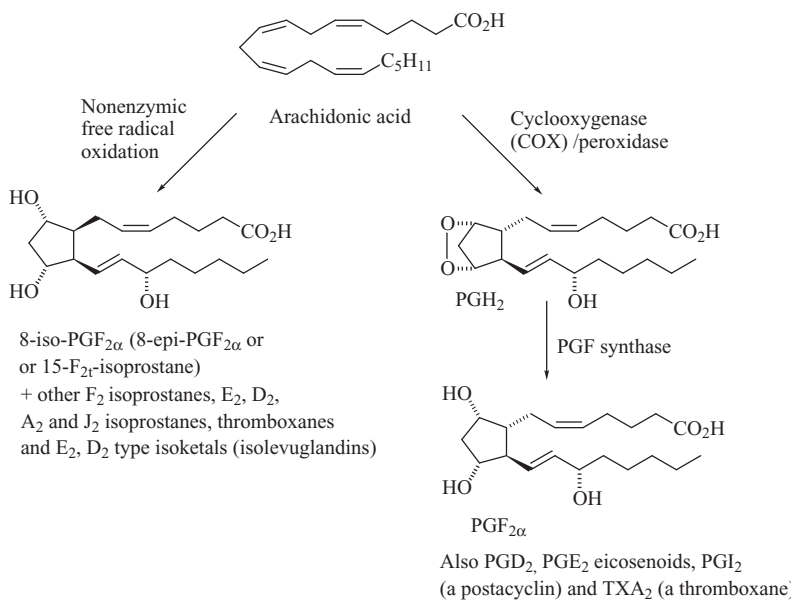


Figure 5.8.10 *Isoprostanes and prostaglandin $F_{2\alpha}$ from oxidation of arachidonic acid*

or 8-*epi*-PG $F_{2\alpha}$), sometimes known as 15-F_{2t}-isoprostane (Figure 5.8.10). Many other products of free radical oxidation of arachidonic acid are produced via the isoprostane pathway, including some with 3-hydroxycylopentanone rings (E_2- and D_2-isoP), cyclopent-2-enone rings (A_2- and J_2-isoP), γ-ketaldehydes (isoketals or isolevuglandins: iso-LGs, or E_2- and D_2-isoK) and thromboxanes (isoTxA_2 and isoTxB_2). The latter are also produced from arachidonic acid by COX enzymes (Smith, 2008).

Other cell membrane unsaturated fatty acids (e.g. α-linoleic acid, γ-linolenic acid and eicosapentaenoic acid, as well as docosahexaenic acid in the central nervous system) can also be oxidized by ROS via free radical chain mechanisms to give isoprostane type molecules. Hence there is theoretically an enormous number of isomeric and nonisomeric oxidation products of lipid oxidation. It is believed that the F2-isoprostanes are at first formed as phospholipid esters in membranes and are then hydrolyzed to free forms by phospholipases.

Apart from free-radical oxidations promoted by ROS, enzymes such as the COX isoenzymes (COX-1, COX-2 and COX-3) and cytochrome P450 isoenzymes are able to oxidize lipids to a range of products, such as prostaglandin $PGF_{2\alpha}$, thromboxanes and 20-hydroxyeicosatetraenoic acid (20-HETE). COX isoenzymes are widely distributed in body tissues. Additionally, myeloperoxidase (MPO), was observed to be excreted by phagocytes in wild type mice upon stimulation by pathogens. MPO is able to synthesize isolevuglandins (isoLGE_2 and iso[4]LGE_2) via the isoprostane pathway, beginning with the peroxidation of arachidonic acid. These isolevuglandins are able to form stable plasma protein adducts, which may be useful as a sensor of *in vivo* oxidative stress (Poliakov *et al.*, 2003).

It is now widely held that the observation of elevated concentrations of F_2-isoprostanes in biological fluids is a good (maybe the best, but not perfect) indicator of the extent of general oxidative stress. Furthermore, increases in F_2-isoprostane levels have been noted as early events in many pathological conditions, including asthma, cardiovascular disease, hepatic cirrhosis and Alzheimer's disease. Although not proven in every case, this suggests a causative role for oxidative stress in at least some of these diseases, especially cardiovascular disease (Voutilainen *et al.*, 1999; Montuschi *et al.*, 2004; Stocker and Keaney, 2004; Gross *et al.*, 2005).

In some human intervention studies with phenol rich beverages (red wine, dealcoholized red wine, grape juice and tea) reduced levels of oxidative damage biomarkers (such as F_2-isoprostanes) have been observed (Halliwell *et al.*, 2005). In other experiments, however, red wine polyphenols or other phenol rich beverages showed beneficial effects against atherosclerosis (by analysis of other risk factors), but did not influence lipid peroxidation (i.e. there was no significant effect on serum or urine F_2-isoprostane levels) (Halliwell *et al.*, 2005; Hodgson and Puddey, 2005; Hodgson *et al.*, 2002). It thus appears that phenolic substances in beverages may act against a variety of cardiovascular pathological conditions via a number of different mechanisms. The mechanisms and extent of beneficial health effects appear to depend on the type and range of phenolic substances present, and also on the medium in which the phenols are ingested. Additionally, there may be synergistic effects between phenolic substances and ethanol (Saucier and Waterhouse, 1999) and other beverage substances such as pectins (Barth *et al.*, 2007).

Two main general modes of *in vivo* action of beverage phenolic compounds, depending upon their fate in the gastrointestinal tract, need to be considered:

- *Postabsorption action*. Phenols are absorbed somewhere in the digestive system where some metabolic changes occur and the metabolites pass into the blood stream into tissues where health benefits occur before excretion.
- *Preabsorption action.* Phenols perform their beneficial reactions prior to absorption and excretion. This mode of action is thought to be most likely in the large intestine, where levels of phenolic substances can be comparatively high.

In the first mode of action, phenol metabolites have been found in human plasma, but usually in concentrations lower than 1 μM (Halliwell, 2006). For example, conjugated flavonoids, methylated gallic acid

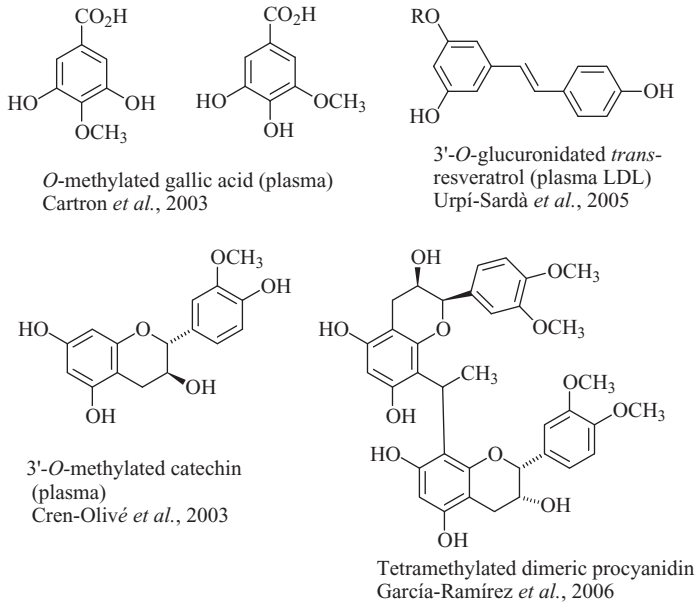
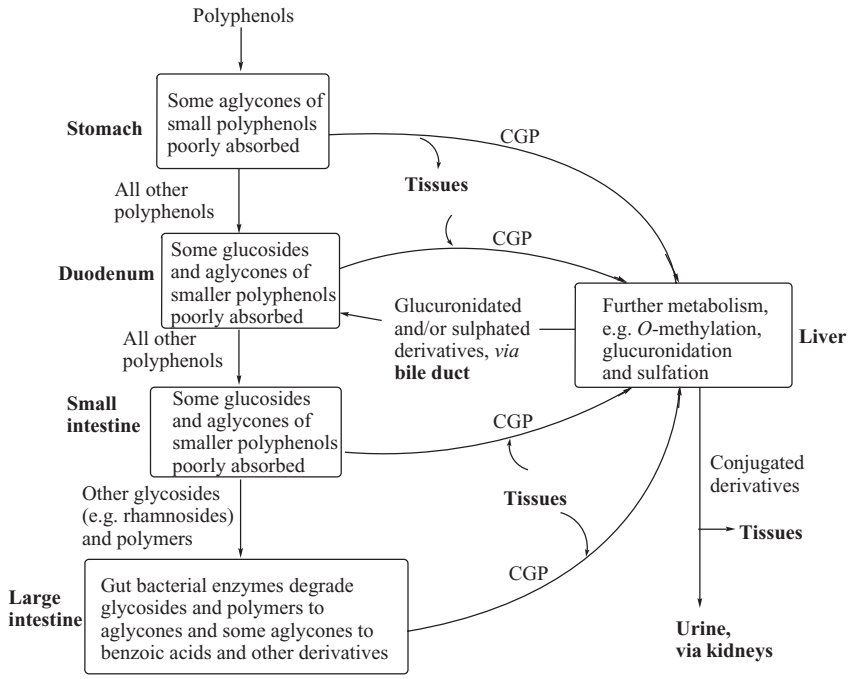


Figure 5.8.11 *Some polyphenol* in vivo *metabolites*

and various catechin metabolites (Figure 5.8.11) have been detected in blood. Catechin metabolites in the bloodstream may be of sufficient concentration to offer protection against LDL oxidation (Donovan *et al.*, 1999), giving some support to the LDL oxidation theory of atherosclerosis (Donovan *et al.*, 1999; Cren-Olivé *et al.*, 2003). However, many of these metabolites have been shown to be poor *in vitro* antioxidants compared with the aglycones or original beverage phenols, possibly because *O*-methylation (a common metabolic transformation) lowers radical scavenging ability. Phenolic compounds in general are not well absorbed, their bioavailability being dependent on dietary source (Manach *et al.*, 2005). Some scientists believe that the presence of ethanol enhances the bioavailability of phenols, which may explain why red wines (and red fruit wines) have often been found to offer somewhat greater health benefits than phenolic extracts or dealcoholized wine alone.

Figure 5.8.12 sketches the metabolic fate of dietary phenols. In general, polyphenols are poorly absorbed: for example, plasma concentrations of quercetin metabolites are no more than 0.1 μM following consumption of red wine samples containing 35 mg of quercetin each (Henning *et al.*, 2005; Manach *et al.*, 2005). The least well absorbed of all polyphenols are anthocyanins and proanthocyanadin polymers. Flavan-3-ols and flavonols are rather better absorbed, whilst the most effectively absorbed flavonoids are isoflavones, which are minor polyphenols in most alcoholic beverages. It seems that very little absorption of polyphenols occurs in the stomach and most are not degraded by the highly acidic conditions in the stomach, hence they pass on to the rest of the digestive system more or less intact.

Some absorption of flavonoid aglycones and glucosides, as well as free benzoic acid and hydroxycinnamic acid polyphenols occurs in the duodenum and small intestine. Glucosides are hydrolyzed in enterocytes by cytosolic β-glucosidases or by extracellular enzymes of the brush border membrane of the small intestine. At the same time, most of the resulting aglycones are glucuronidated by membrane bound glucuronosyl transferases and/or *O*-methylated by catechol *O*-methyl transferase. *In vitro* studies (Yi *et al.*, 2006) showed that anthocyanins can be transported through Caco-2 intestinal cell monolayers with a general efficiency of ~ 3–4% (1% for delphinium glucoside).



CGP = circulatory (plasma) conjugated polyphenol metabolites, usually *O*-glucuronides and *O*-methylated derivatives bound to albumin mostly, but also to other proteins, such as LDL

Figure 5.8.12 *Summary of metabolic fate of dietary polyphenols*

In general, the best absorbed here were glucosides and anthocyanidin rings with fewer OH groups. Cyanidin 3-glucoside of black raspberry (this is a common anthocyanin of black fruit wines) is absorbed through the human small intestine mostly as the intact glucoside, which appears as such in the plasma and urine, along with a small amount of *O*-methylated metabolite (Ichiyanagi *et al.*, 2007). Other cyanidin 3-glycosides (the 3-rutinoside and 3-xylosylrutinoside), like most polyphenols, are present in urine as metabolites, in this case as *O*-methylated derivatives at the 3′ position for the rutinoside and at both 3′ and 4′ positions for the xylosylrutinoside (Tian *et al.*, 2006). More recently, 18 phenolic acid metabilites acids were detected in the urine of human subjects 2–6 h after being fed bilberry-lingonberry purée, which contains anthocyanins and phenolic acids (Nurmi *et al.*, 2009). Several of these, including homovanillic, vanillic acids and hydroxylated phenylpropanoic acids, were judged to be metabolites of methylated anthocyanins, but their low concentrations in urine indicated that still a large part the metabolic products remained unknown.

Other flavonoid glycosides (e.g. arabinosides, rhamnosides and xylosides), carbohydrate bound hydroxycinnamic acid derivatives and proanthocyanidins, along with other unabsorbed polyphenols, must pass to the large intestine, where enzymes of the gut flora hydrolyze glycosides to aglycones and some flavonoid aglycones to hydroxyphenylalkanoic acids, hydroxybenzoic acids and phenols (Figure 5.8.13). These microbial metabolites are absorbed, whence they appear in the bloodstream as glucuronidated, *O*-methylated derivatives or as glycine-conjugated derivatives (Manach *et al.*, 2004). Recent studies have shown that plasma and urine levels of these metabolites are higher than those of tissular metabolites, suggesting that they may contribute significantly to the reduction of atherosclerosis risk factors and other health benefits associated with moderate wine consumption. Indeed, this may be the major mode of action regarding proanthocyanidins (some of the most abundant beverage polyphenols), since only the B_2 dimer has been found in human plasma

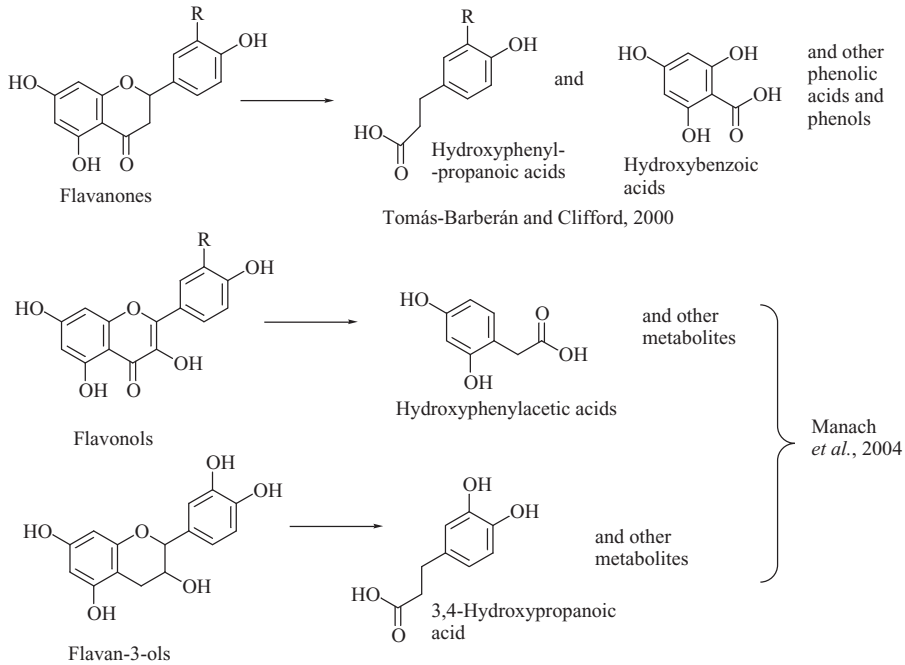


Figure 5.8.13 *Summary of metabolic fate of dietary polyphenols*

and even then, only at 0.04 μM concentration. There is also evidence that raspberry anthocyanins per se are only poorly absorbed before reaching the small intestine and substantial amounts pass to the large intestine, where degradation by microflora occurs (Borges *et al.*, 2007). Gut bacteria also hydrolyze large carbohydrate bound polyphenols, thus releasing the aglycones for further metabolism in the colon. This could explain why cloudy apple juice (and the same could apply to cloudy cider) was more effective than clear juice in modulating chemically induced colon cancers in rats (Barth *et al.*, 2006).

Polyphenol concentrations in the colon can reach several hundred μM, and even if the majority are neither metabolized nor absorbed, they may still play an important local role in the transformation of potential toxins and carcinogens, quite possibly acting as extracellular antioxidants. After all, the colon is particularly exposed to oxidizing agents, and most other dietary antioxidants, such as vitamins C and E are mostly absorbed before they reach the middle and lower colon.

Circulatory polyphenols (Figure 5.8.11) exist mostly as conjugated metabolites (*O*-methylated, *O*-glucuronidated and sulfated derivatives) that are extensively bound to plasma proteins, notably albumin, although red wine *trans*-resveratrol and the 3-*O*-glucoronides (Figure 5.8.11) of *cis*- and *trans*-resveratrol were found in human LDL particles after moderate red wine consumption (Urpí-Sardà *et al.*, 2005). This is consistent with the hypotheses that early events in atherosclerosis involve LDL lipid oxidation and that polyphenols behave as antioxidants in the prevention or delay of this condition. Recently, *trans*-resveratrol itself has been shown to bind strongly to bovine serum albumin (BSA, which has a high homology and a similarity in sequence and conformation with human serum albumin) in aqueous solution (i.e. *in vitro*) (Cao *et al.*, 2009). Fluorescence and IR spectroscopic results indicated the guest–host binding distance was less than 7 nm, suggesting that energy transfer from BSA to *trans*-resveratrol may occur via the formation of electrostatic attractions.

O-Methylation and *O*-glucuronidation can occur in a wide range of tissues, including enterocyte cells of the intestines, but sulfation occurs in the liver. Once in the blood stream, polyphenol metabolites are known

from radioactive labeling studies to be widely distributed in tissues, including those of the gastrointestinal tract, the brain, the liver and the endothelium (inner lining of blood vessels). The last named is probably one of the major target sites for flavonoid uptake, with concentrations of metabolites ranging from 0.03 to 3.0 μg of aglycone equivalents per g of tissue. There are relatively few reports of polyphenol metabolites in tissues, probably because of the difficulty in extraction of sufficient quantities of metabolites for analysis. However, a tetramethylated metabolite of a synthetic dimeric procyanidin, consisting of (–)-epicatechin units linked by an ethyl bridge (Figure 5.8.11), was isolated from the livers (as well as plasma) of Wistar rats after 2 h of feeding (García-Ramírez *et al.*, 2006). The procyanidin feeding rate was 200 mg/kg body weight, which led to a maximum of 15 μg of metabolite per gram of liver tissue. Conversely, there was no evidence of the presence of metabolites of higher synthetic procyanidin oligomers (trimers etc.) in either plasma or liver tissue. This suggested that natural procyanidin dimers (but not higher oligomers) are probably rapidly absorbed and metabolized and hence may be good agents of beneficial biological activity.

Some ingested *trans*-resveratrol is metabolized *in vivo* by hydroxylation (probably by various cytochrome P450 isoenzymes, such as CYPB1 or 1A2) at the 4′ position, giving piceatannol (Potter *et al.*, 2002, Piver *et al.*, 2004), a stilbene that shows even greater general *in vitro* antioxidant value than the resveratrols. This is of particular interest, because as already mentioned, polyphenol metabolites frequently exhibit reduced *in vitro* antioxidant activity compared with the original.

However, data is still scarce and the nature of the tissue metabolites may be different from that of plasma metabolites, due to specific uptake or specific intracellular metabolism. Nevertheless, it is possible that certain polyphenol metabolites (say, those of flavonoids) may be present in certain tissues (say, endothelial cells) in sufficient concentration to afford direct antioxidant protection (say, against atherosclerosis). As already discussed, there is little direct conclusive evidence of this, although there are many other ways that *in vivo* phenol metabolites may be of health benefit; these are discussed in the closing paragraphs of this section.

Very little is known of the kinetics of uptake and elimination of polyphenols in body tissues, but the major excretion route are known to be via bile or urine. It seems that larger more highly conjugated polyphenols are preferentially excreted in bile to the duodenum, where further metabolism and absorption can occur in the intestines, recirculating the metabolites via the liver. In this way, it is possible that metabolites of certain polyphenols could remain in the body for some considerable time ($>$7 h). Smaller, less conjugated polyphenols are preferentially excreted via urine. Recovery of polyphenol metabolites in urine tends to be moderate ($\sim$30% for epicatechin in cocoa), to low ($<$10% for wine catechin and anthocyanins), although the lowest experimental recoveries may be due to analytical difficulties or lack of knowledge of metabolites.

Again, there are few precise data regarding half lives of polyphenol metabolites in human plasma, but common phenols of alcoholic beverages, such as anthocyanins and flavan-3-ols generally have half lives of the order of 2–3 h, whereas isoflavones (less common in alcoholic beverages) and flavonols (such as quercetin) have half lives of about 4–8 h and 11–28 h, respectively. Since the major polyphenols in alcoholic drinks are anthocyanins, flavan-3-ols and proanthocyanidins, it seems unlikely that significant accumulation of plasma metabolites will occur in the body, except perhaps after rapidly repeated intake (binge drinking?), which will have negative health effects (Section 5.6.2). Thus it is likely that polyphenol metabolite concentrations fluctuate between repeated the distant intake of alcoholic beverages associated with regular moderate drinking.

There is growing evidence that polyphenols exert at least part (and possibly a major part) of their health benefits by influencing cell signaling pathways, (Williams *et al.*, 2004). Cells respond to stresses by increasing or decreasing the availability of certain proteins, by altering the expression of specific genes, using signal transduction pathways. These pathways regulate many cell processes, such as apoptosis (programmed cell death), growth and proliferation. Kinase enzymes are crucial for signal transduction pathways: they catalyze the phosphorylation (or dephosphorylation) of signal transduction proteins and eventually influence the activity of transcription factors (proteins that bind to specific response elements on DNA, promoting or inhibiting specific gene transcriptions).

There is good evidence to suggest that flavonoids such as catechin or quercetin (as well as ethanol) in alcoholic beverages reduce the risk of cardiovascular disease by increasing the expression of endothelium fibrinolytic proteins, such as t-PA (Booyse *et al.*, 2007). This increases the fibrinolysis or lysis of atherosclerotic fibrin clots, thus preventing build up and rupture of plaque that leads to thrombosis or stroke. In particular, it has been proposed that polyphenols (and ethanol) increase the expression of t-PA by activation of p38 MAPK signaling. Transcription of t-PA is up regulated via specific polyphenol and ethanol transcription factor binding sites in the t-PA promoter protein.

There is also evidence that certain polyphenols are able to inhibit directly the action of certain metabolic enzymes. For example, it is possible that this kind of action demonstrated by procyanidins may occur earlier in the sequence of events that can eventually lead to atherosclerosis and stroke; a sequence that is linked to obesity in many individuals. It is known that a diet high in procyanidins can minimize weight gain and prevent obesity, although on the negative side, this may be accompanied by less efficient digestion and a drop in nutrient uptake (Section 5.4.3). Simultaneous ingestion of apple polyphenol extract (rich in oligomeric procyanidins) and triglycerides fats significantly inhibited an increase in plasma triglyceride levels in both mice and humans (Sugiyama *et al.*, 2007). Likewise, the same group showed that procyanidins, especially the higher oligomers, inhibited the *in vitro* action of pancreatic lipase on 4-methylumbelliferyl oleate, thus suggesting apple polyphenol extract procyanidins inhibited triglyceride absorption by inhibiting the activity of pancreatic lipase in mice and humans.

In general, polyphenols may inhibit a variety of chronic diseases by selective inhibition of kinases (Williams *et al.*, 2004). For example, cell growth and proliferation are regulated by growth factor proteins that start signal transduction cascades by binding to specific receptors in cell membranes. Polyphenols may alter growth factor signal transduction by either blocking receptor growth factor binding or by inhibiting receptor phosphorylation (that is, blocking the action of certain kinases). Thus polyphenols may help prevent cancer by inhibiting proliferation, inducing apoptosis and preserving cell cycle regulation, by a number of specific cell signaling mechanisms. Additionally, some polyphenols are known to inhibit angiogenesis (development of new blood vessels) and matrix metalloproteinase (MMP) expression. New blood vessels are essential for the supply of oxygen and nutrients to growing tumors, and MMPs, overexpressed and excreted by tumor cells, break down extracellular tissues (collagen, gelatin and other fibers), allowing invasion of surrounding tissue by tumor cells. Hence polyphenols may prevent cancer by inhibiting the growth of cancerous masses and the spreading of cancer cells.

It was previously mentioned that *trans*-resveratrol is partially metabolized to piceatannol in humans. Density function theory (DFT) calculations suggest greater reactivity of piceatannol over *trans*-resveratrol towards hydroxyl and peroxyl radicals (Rossi *et al*., 2008), in agreement with observation of the greater *in vitro* antioxidant power of the metabolite. Similarly, molecular modeling showed that piceatannol is more able than *trans*-resveratrol to form a complex with tetrameric transthyretin (TTR), a cerebrospinal fluid protein that is thought to offer protection against Alzheimer's disease by binding to Aβ peptides and preventing them forming amyloid fibrils (Costa *et al.*, 2008). The stilbene polyphenols are known to bind (*in vivo*) to specific plasma membrane binding sites, which could increase the expression of protective TTR and they also bind to Aβ peptides *in vitro*, thus preventing fibril formation, although the molecular mechanisms of these interactions are unknown.

Polyphenols may prevent CVD by decreasing cell adhesion molecule expression, thus discouraging the incorporation of inflammatory monocytes from blood into the endothelium (Choi *et al*., 2004; Ludwig *et al.*, 2004; Stocker and Keaney, 2004), which is thought to be important in the early stages of CVD. They are also known to increase endothelial nitric oxide synthase (eNOS) activity (Anter *et al.*, 2004). Nitric oxide is required for arterial relaxation, which reduces the risk of arterial injury, inflammation and subsequent cardiovascular disease. Finally, polyphenols are known to inhibit platelet aggregation (Deana *et al.*, 2003; Bucki *et al.*, 2003).

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5.9
Additives in Alcoholic Beverages

5.9.1 Introduction

Additive are substances that are intentionally added in small amounts to a foodstuff during its manufacture. In general, additives play important roles in the preparation of alcoholic drinks and are often integral parts of processing aids that are essential for their production. Additives can be used at any time during manufacture, although for a particular additive, legislation will generally specify at what point(s) in the manufacturing process it must be used. Plant materials or plant products that are an essential part of the production of particular alcoholic beverages are not classed as additives. This includes hops, other herbs and spices that are used to flavor beer (Section 2.6.3), vermouth (Section 2.12.2), spirits (Sections 3.4.2 and 3.5.5) and liqueurs (Section 3.8.3). The health values of selected plant components (phytochemicals) are dealt with in Section 5.11.2.

There are many additives that are allowed in the production of alcoholic drinks and these perform many functions, as outlined in Table 5.9.1. Permitted additives that perform specific functions, or are part of specific processes, vary from country to country, and from beverage to beverage, so the information in Table 5.9.1 is meant as a general guide only. Although undoubtedly useful, many additives have negative health benefits if ingested at high concentrations. Largely for that reason, each country has its own regulations governing additives, and each sets its own legal maximum limits permitted in alcoholic beverages. However, bilateral and collateral agreements exist between different countries or groups of countries that seek to rationalize differences in production methods (including use and nature of additives) for ease of trading (see Section 5.9.5). Analysis is carefully performed at approved or government laboratories as a periodic check on certain additive levels, as well as certain residues and trace substances (Chapters 5.10 and 5.11).

Sections 5.9.1–5.9.4, while giving some details on the major additives and processes in which they are used, emphasize health aspects. A brief account on legislation and regulation regarding additives is given in Section 5.9.5.

5.9.2 Sulfur Dioxide and Other Preservatives

Sulfur dioxide in its various forms has been used as an antiseptic agent in wines for centuries. Originally obtained by burning sulfur candles, sulfur dioxide is nowadays added in the form of potassium or sodium metabisulfite crystals, or in larger operations as gas form cylinders (Section 2.5.2). It is also used as a

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

Table 5.9.1 *Some permitted additives used in the production of alcoholic beverages*

Additive or process	Examples	Additive or process	Examples
Sterilization/ preservative	Sulfur dioxide and sulfites; sorbic acid and sorbates; dimethyldicarbonate; N_2; CO_2	Yeast nutrition/ fermentation aids	Ammonium phosphates; autolyzed yeast; soy flour; thiamine hydrochloride; oxygen or compressed air
Clarification, fining and stabilization	Acacia (gum Arabic); albumen; bentonite (and other clays); caseinates; gelatin; isinglass (fish collagen); PVPP; silica gel; tannin. Enzyme stabilizers include: catalase; cellulase, glucose oxidase; lysozyme; pectinase; various proteinases; urease	Color, odor and tartrate stabilization	Activated charcoal; ascorbic acid; copper sulfate; ferrous sulfate; potassium bitartrate; potassium ferrocyanide
Acidification	Citric, malic and tartaric acids; calcium chloride, calcium sulfate; Burton salts	Colorant	Caramel; natural cochineal (vermouth)
Deacidification	Calcium carbonate; other carbonates or bicarbonates; neutral tartrates	Oak flavoring/ maturation aid	Oak chips

preservative in some ciders and beers, although in the latter case at much lower levels. Sulfur dioxide in alcoholic beverages exists as 'bound' and 'free' forms, the sum of the levels of both forms giving the total sulfur dioxide concentration. The bound forms of SO_2 are reaction products with carbonyl compounds (e.g. acetaldehyde, pyruvic acid, α-ketoglutaric acid), phenolic compounds (e.g. anthocyanins, hydroxycinnamic acid derivatives) and thiamine. The main forms of free sulfur dioxide are SO_2 hydrates, hydrogen sulfites (HSO_3^-) and sulfites (SO_3^{2-}) (Section 2.5.2 – see Figures 2.5.1 and 2.5.2). The first mentioned has the most potent antimicrobial activity, and in excess, probably has the greatest allergenic and irritant abilities.

The big majority of people show no physiological reactions to sulfur dioxide at the levels found in alcoholic beverages and hence this additive poses no health hazard in these cases. However, some individuals (estimated at ~3% of the population) are hypersensitive to sulfur dioxide and some of these suffer severe asthmatic reactions, even fatal ones (caused mainly by bronchoconstriction), at low levels of ingestion (Hughes and Baxter, 2001; Vally and Thompson, 2003). Less severe allergic reactions to sulfites in alcoholic beverages include nasal congestion, abdominal pain, dizziness, skin flush and nausea (Kline, 2005).

It is thought that these symptoms are mediated by IgE associated immune responses and by genetic defects in mitochondrial enzymes, such as rodanase. Gaseous SO_2 dissolves in the moisture of the respiratory system to produce H^+, HSO_3^- and SO_3^{2-}, which affect the smooth muscles and nerves involved in bronchoconstriction, as well as influencing sodium and potassium currents in neurons (Du and Meng, 2004a; 2004b). Lipid peroxidation of cell membranes also occurs, because of inhibition of natural antioxidant (superoxide dismutase, etc.) activity (Meng *et al.*, 2003). Once sulfur dioxide is absorbed into the blood stream it becomes associated with globulin proteins. It is metabolized in the liver to sulfate and is excreted as such in urine.

People who are sensitive to sulfur dioxide are advised to drink spirits, liqueurs, certain ciders (and perry) and certain beers. As far as wines are concerned, the safest are organic wines, which by definition have the lowest SO_2 levels (although in the USA, organic wines are allowed up to 100 ppm of total SO_2), some having only natural levels, of up to 40 ppm. Nonorganic wines can have 199–450 ppm of total sulfur dioxide present, depending on the style of wine (sweet white wines tend to have the highest levels, dry red wines the lowest)

and the philosophy of the winemakers. Cask-conditioned beers may have up to 50 ppm of total SO_2, but this will often arise from the suspension of isinglass that is used as finings, as it is preserved with sulfites.

Many other beers have much less than this level of total SO_2, and some are produced with no added sulfites. High concentrations of ethanol kill or prevent the growth of microorganisms as well as providing some protection against oxidation of flavor compounds. For this reason, spirits, liqueurs and fortified wines do not need added sulfites. Similarly, beverages with high CO_2 contents – sparkling wines, sparkling cider or perry and most beers – are protected from microbial spoilage, and can be made with minimal or no addition of SO_2. Also certain wines, such as fino sherry and other flor wines (Sections 2.10.3 and 2.10.4), and some ciders (and perry) (Section 2.8.5) are deliberately made without added sulfites in order to facilitate essential processes, such as growth of flor yeasts and the occurrence of malolactic fermentation, respectively.

FDA regulations in the USA require all wines (domestic or imported) that contain more than 10 ppm of total SO_2 state 'contains sulfites' on the label. Similar regulations apply to alcoholic beverages produced in the European Union, Australia, New Zealand, South Africa and other countries.

Estimates of the toxicity of SO_2 and sulfites have reported the oral LD_5o for rates at 2 g/kg body weight, using 5% aqueous solutions (Amerine and Ough, 1980a). The acetaldehyde–SO_2 complex (the 'bisulfite complex') was found to have an oral LD_5o for rats of 1.5 g/kg body weight, making it a little more toxic than acetaldehyde itself ($LD_5o = 1.9$ g/kg). Thus, ignoring allergenic effects, the evidence suggests that no toxic effects should occur on consumption of alcoholic beverage that have less than the maximum allowed levels of total SO_2. At levels very much higher than those encountered in the most highly sulfited beverages, sulfur dioxide is cytotoxic; in particular it impairs the proper functioning of alveolar macrophages and it inhibits the production of tumor necrosis factor and interleukin-1 β-cytokines (Knorst *et al.*, 1996). At even higher levels, SO_2 is genotoxic, causing chromosome breakage (Yi and Meng, 2003). Concentrations of SO_2 as high as these are only ever met in industrial accidents and are only of passing interest to the drinker of alcoholic beverages. The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have recommended human acceptable daily intake (ADI) of sulfites to be less than 0.7 mg/kg body weight; well below toxic levels.

Production of wines with no added sulfites must be carried out with care, in order to eliminate factors that spoil wine – especially microbial spoilage (Chapter 2.4) and browning (oxidation). Thorough precleaning of wine must and rapid heat treatment prior to fermentation in a pressure controlled tank at 20 °C resulted in lower acetaldehyde concentrations upon fermentation (Seckler *et al.*, 1992). Further storage on the yeast lees with regular stirring helped to maximize the effect of reductive power of yeast cells. If such a wine was sterile filtered and hot filled into bottles, then a measure of protection against microbial infection was obtained, and the wine was claimed to be of organoleptic merit (Seckler *et al.*, 1992).

As a compromise, provided the risk of microbial infection is minimized, the use of combined antioxidants ascorbic acid and sulfites may prevent browning in white wines (Bradshaw *et al.*, 2004). Oxidation of ascorbic acid in the wine leads to a loss of sulfur dioxide, thus compromising the latter's antimicrobial potential.

There are processes for removal of SO_2 from alcoholic beverages, but these tend to adversely affect the quality of the beverage (Lin and Georgiou, 2005). These include the use of ion exchangers, membrane reactors in conjunction with nondiffusible oxidizing agents, and the use of hydrogen peroxide. Wheatgrass (*Triticum aestivum*) chloroplasts have been shown to reduce the sulfite concentration of red and white wines by up to 95% in less than 3 h, without influencing the flavor of the beverage if removal of SO_2 is carried out just before consumption (Lin and Georgiou, 2005).

Sorbic acid – (2*E*),4(*E*)-hexadienoic acid – and its potassium salt (Section 2.5.3) have long been designated as GRAS substances ('generally recognized as safe'). They are widely used as antiseptic agents in European wine and carry approved 'E-numbers': E200 and E2002, respectively. The maximum allowed levels of sorbates in wine varies from country to country, but in the EU is 200 mg/l (200 ppm). An acceptable daily intake (ADI) of sorbates, expressed as sorbic acid, of 25 mg/kg of body mass has been suggested by the

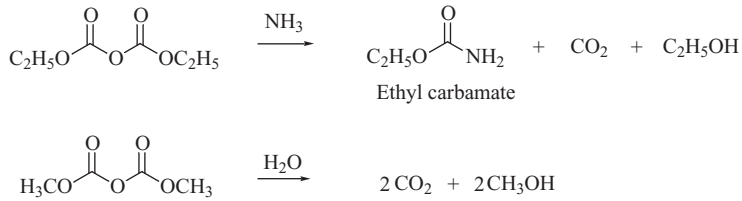

Figure 5.9.1 *Reactions of DEDC and DMDC in wine*

Joint Expert Committee on Food Additives (JECFA, 1973) of FAO/WHO. This value is far in excess of sorbate levels that are likely to be ingested in normal wine drinking.

Sorbates are used only at bottling time to inhibit the growth of yeast, especially in wines and other beverages that have significant residual sugar levels. They are ineffective against bacteria and hence are added with sulfites. This is of special importance since sorbates are metabolited by some lactic acid bacteria to the malodorous 2-ethoxyhexa-3,5-diene (Section 2.5.4). Sorbic acid is mostly metabolized to CO_2, but some is excreted unchanged in urine and a minor amount is metabolized to (*E*,*E*)-muconic acid (EEMA; 2*E*, 4*E*-hexadienedioic acid) and excreted in urine as such (Renner *et al.*, 1999). All toxicity experiments on animals proved negative at levels of feeding less than 1.5% of the diet (Amerine and Ough, 1980b). The oral LD_{50} for rats has been determined as 4.0–7.2 g/kg of body weight for potassium sorbate and 7.4–10.5 g/kg of body weight for sorbic acid.

The conjugated double bond system of sorbates is reactive to attack by nucleophiles, such as thiols, amines and nitrites, some examples of which (particularly amines) can be found in alcoholic beverages. In particular, many products of reaction between sorbates and amines have been found in other foodstuffs (Ferrand *et al.*, 2000) and it was feared that some of these may be mutagenic. A model system involving sorbic acid, sorbates and simple amines showed the formation of 2-pyridones at 50 °C (Ferrand *et al.*, 2000) – e.g. *N*-benzyl-6-methyl-3,6-dihydro-2-pyridone from sorbate and benzylamine. These compounds were shown to be neither mutagenic nor genotoxic, thus emphasizing the relative safety of sorbates as additives.

Pyrocarbonates, such as diethyl dicarbonate (DEDC) and dimethyl dicarbonate (DMDC), are powerful fungicides for many yeast species present in alcoholic beverages. They are generally more effective than SO_2, but unfortunately DEDC was found to produce very small amounts of ethyl carbamate, a known carcinogen, by reaction with ammonia (Figure 5.9.1) (Schlatter and Lutz, 1990). DMDC, on the other hand, has not been found to produce any carcinogenic hydrolysis products, although it does produce methanol (Figure 5.9.1), but not at toxicologically significant levels, when added to wine at levels of around 200 mg/l. DMDC can thus be used to supplement SO_2 in the stabilization of sweet botrytis affected wines, such as Sauternes (see Section 2.5.5) (Divol *et al.*, 2005). In this way, the maximum allowed total SO_2 level of 400mg/l can be avoided, thus decreasing the likelihood of 'sulfur stink' in young wines – a common criticism of botrytized wines.

Benzoic acid and benzoate salts (such as calcium or potassium benzoate) are used throughout the food industry as antimicrobial agents (preservatives) and flavoring agents. Use in wine has fallen in recent years in favor of sulfites and sorbates. Despite some concerns about allergenic reactions in some individuals, the JECFA of FAO/WHO advise that there are no safety concerns at normal levels of intake and recommend ADI of no more than 5 mg/kg of body weight. The EU codes for benzoate additives are E210–E213.

5.9.3 Fining Agents and Adjuncts

Like preservatives, clarification agents are very useful and find widespread use in the alcoholic beverage industry. Fining is followed by filtration in the production of many alcoholic drinks and hence there is little

chance of finding residual clarification agent in many beverages. Unfiltered drinks may have residual finings, but none of these have been found to present a health hazard. However, there is presently some public concern about the use of animal derived agents that may contain allergenic or pathogenic contaminants, or which may themselves be allergenic. In particular, isinglass has been (tentatively) listed as an allergen by the European Union, despite the fact that over decades of use in the UK and some other countries, no intolerance problems have been reported. In many countries, potential food allergens (Section 5.11.3) must be listed on food package labels (Section 5.9.5) and this is seen as a disadvantage by many producers of alcoholic beverages. Consequently, brewers and isinglass producers in the UK are investigating the potential allergenicity of isinglass in order to achieve a permanent exemption from the EU regarding labeling of (unfiltered) bottle- and cask-conditioned beers (Chlup *et al.*, 2006). To date (2008), there is no conclusive evidence that isinglass causes allergenic reactions (Baxter *et al.*, 2007). Nevertheless, in view of the relatively common incidence of allergy to fish proteins (Sagasuchi *et al.*, 2000; Hamada *et al.*, 2003) efforts have been directed to investigate the beer fining abilities of nonfish collagens (Walker *et al.*, 2007). It was found that avian collagen and pea pod extract were as effective as isinglass in laboratory trials, and in brewery trials there were few organoleptic differences between beers fined with the different agents. It is awaited with interest as to whether pea protein extract proves to be a good clarification agent on an industrial scale, as this would be an attractive option for vegetarians and fish protein sensitive people alike.

The cross linked insoluble polymer polyvinylpolypyrrolidone (PVPP) is useful in the removal of excess phenols from white wine (Section 2.9.4) and beer (Sections 2.6.3 and 2.6.9), often by incorporation into filtration materials such as kieselguhr. Because of its insolubility, only trace amounts of PVPP can be found in alcoholic beverages, although the more soluble PVP (a linear polymer) and the monomer 1-vinyl-2-pyrrolidone are often contaminants and may be found in drinks at rather higher levels. However, the World Health Organization (WHO) does not specify an acceptable daily intake (ADI) for PVPP indicating that it does not represent a hazard to health (http://www.inchem.org/documents/jecfa/jecmono/v18je01.htm). An ADI of up to 25 mg/kg body weight has been suggested by WHO (JECFA, 1973) for PVP, although this substance has been assessed, via oral dosed studies, mutagenicity studies and inhalation studies (on the monomer), to be safe for use in cosmetics (Anonymous, 1998). PVP was found to have an oral LD_{50} of over 100 g/kg body weight for both rats and guinea pigs. It is worth noting that orally administered PVP significantly reduced the incidence of bladder tumors in mice exposed to bracken fern (Anonymous, 1998). More recently, PVP was found to induce anaphylaxis in a boy's skin, but only when the skin was broken. In this case, basophils contacted PVP in the presence of serum to cause an allergenic reaction, otherwise PVP showed negative skin prick tests (Yoshida *et al.*, 2008).

Residues of other natural or nature based biological fining agents, such as albumen, carrageenan, caseinates, gelatin and gum arabic (acacia) are not considered to present health hazards to drinkers and the JECFA of FAO/WHO does not issue ADIs for these additives. Carrageenan, used as a kettle fining agent in beer production (Section 2.6.4), is the only one these agents to show toxicological properties, and hence it is not recommended for infants. Likewise inorganic fining agents based upon clay (bentonite and kaolin) and those based upon silica (silica hydrogels and kieselguhr) present no health hazard upon ingestion in small quantities. Chronic exposure to the fine dust, however, does pose some health risks to workers in the companies that produce them. The EU additive codes for bentonite, kaolin and silica are E558, E559 and E551, respectively.

5.9.4 Other Additives

The only generally allowed colorants for alcoholic beverages are caramel (E20) (for beers, ciders and certain wines, such as vermouth, and certain spirits) and natural cochineal (for some vermouths and aperitive wines) for which there have been no reported health risks. Other colorants are illegal, but in the past, elderberry juice or azo dyes may have been added to some wines.

Potassium ferrocyanide is established as an additive for removal of excessive amounts of copper and iron salts in (white) wines in the European Union and countries that have trade agreement with the EU (Australia, New Zealand and South Africa). For years it was not allowed in the USA, the fear being that prolonged contact with wine may produce cyanides, although this has never been established. Its oral rat LD_{50} is 6400 mg/kg body weight and JECFA of FAO/WHO gives an upper limit of 0.025 mg/kg body weight as the ADI for humans. Its EC approved code is E536.

Copper sulfate can be used in the European Union on wines that have excessive levels of malodorous hydrogen sulfide (H_2S) or mercaptans (thiols). The copper ions combine with sulfide species to give solid products that can be removed by racking and filtration. Nevertheless, Cu^{2+} levels must be carefully monitored and should not exceed 1 mg/l in the final product, although it is generally estimated that about 0.5 mg/l of Cu^{2+} is adequate for removed of H_2S and thiols in normal cases, so that residual levels of Cu^{2+} should be much lower than 1 mg/l. In any case, higher levels of Cu^{2+} can induce hazes and may need to be lowered by addition of potassium ferrocyanide. Copper sulfate is used in the EU, whereas copper citrate on bentonite can be used in Argentina, Chile, South Africa and Switzerland, and either copper citrate alone or on bentonite can be used in Australia and New Zealand. Copper citrate is less soluble in wine than copper sulfate and hence any excess will be removed on decanting/fining/filtration, along with copper sulfide, leaving lower levels of residual Cu^{2+}.

Copper is an essential trace element; it is a cofactor in many enzymes that participate in cell metabolism (see Sections 5.8.2 and 5.10.3). Acute ingestion of Cu^{2+} at high doses can lead to nausea and diarrhea, and at higher levels, coma and death can occur. The ionic form (i.e. Cu^{2+}) is considered to be more toxic than bound forms of copper, as it is often found in foodstuffs, including alcoholic beverages. Chronic ingestion of Cu^{2+} has led to liver failure in an extreme case (30 mg/day) and studies with mice indicate possible damage to the reproductive system. There is no evidence that copper sulfate is mutagenic (bacterial assays) and there is no evidence of copper salts being carcinogenic, although it does appear to inhibit the absorption of certain nutrients. A joint FAO/IAEA/WHO expert consultation committee established an upper limit to the safe intake of copper for adults of 0.2 mg/kg of body weight (JECFA, 1973). Its EC additive code is E405.

Artificial sweeteners are compounds that impart a sweet taste when added to foodstuffs. In the EU, Council Directive 94/35/EC (EC, 1994) (and various amendments) (e.g. EC, 2004) deal specifically with foodstuff sweeteners. There are many authorized sweeteners: acesulfame-K, alitame, aspartame, aspartame-acesulfame salt, cyclamic acid, dulcin, neotame, neohesperidine dihydrochalcone, saccharin, sucralose and thaumatin, all of which are synthetic, except neohesperidine dihydrochalcone (semi-synthetic) and thaumatin (natural) (Wasik *et al.*, 2007). Of these, all but alitame, dulcin and neotame are authorized for use in the EU. They are intensely sweet and are required to be added to foodstuffs in small quantities only, hence they have no nutritional value. This makes them valuable in the production of soft drinks, bottled or canned fruit, and yoghurt for diabetics or people avoiding a high carbohydrate diet. Within the EU, it is not legal to use intense sweeteners in beer and wine, but cider and perry in the UK and certain ciders in France are allowed to be sweetened in this way (Section 2.8.6)

Propylene glycol alginate (PGA) is used as a foam stabilizer in many beers, particularly highly refined, brewery-conditioned beers brewed from worts with low malted barley content, or beers brewed by high gravity methods (Section 2.6.8). It can be added to beer late in the process, usually at levels of around 50 mg/l. Labeling legislation in many countries now requires PGA to be listed as an additive, thus compromising the perceived wholesomeness of the product.

Other foam stabilizers used in the production of certain beers include hydrolyzed albumen (essentially a mixture of peptides) and hydrogenated hop *iso*-l-acids: tetra-and hexahydro*iso*-l-acids in particular. The latter are less polar (more hydrophobic) than the *iso*-α-acids themselves and hence make a more pronounced contribution to foam stability, even at levels of 3–5 mg/l. They are much more bitter than *iso*-α-acids but are unobstrusive when added to beer (normally late in the processing) at this concentration level.

Both *in vitro* and *in vivo* studies indicate that partial hydrolysis of PGA occurs in the gastrointestinal tract; the alginate moeity is not absorbed, whereas the propylene glycol moiety is rapidly absorbed and metabolized to lactic acid and pyruvic acid. Acute and chronic toxicity tests on ingested PGA in dogs, guinea pigs, humans and rabbits were all negative, and PGA showed no carcinogenic, mutagenic and genotoxic effects. The Joint Expert Committee on Food Additives (FAO/WHO) (JECFA, 1973), on the basis of extensive data from toxicological experiments have suggested an acceptable daily intake (ADI) for man of up to 25 mg/kg of body weight. The Committee also noted that 5% PGA in the diet of rats (≡ 2500 mg/kg body weight) had no toxicological effect and that no LD_{5O} data exists. Its EC additive code is E405.

5.9.5 Regulations and Legislation Relating to Additives

Legislation on food additives is based upon positive lists that vary from country to country. In each country, only explicitly authorized additives may be used. Also, most food additives may be used only in specified limited quantities in certain foodstuffs. Food additives for which no quantitative limits have been established must be used according to good manufacturing practice, which essentially means that only enough additive needed to achieve the desired technological effect should be used. All new additives (or modified versions of established additives) must have their usefulness well demonstrated and must pass rigorous and thorough scientific evaluation tests before approval is obtained for use. Within the greater context of food additives there exist the additives that are used in the manufacture of alcoholic beverages.

Statutory authorities exist in each country in order to protect that nation's health and safety by maintaining a supply of safe food, including alcoholic beverages. These authorities work in liaison or consultation with government ministries and representative organizations of the alcoholic beverage industry. They (via an expert group or committee within the authority) are responsible for developing, modifying and reviewing practices and procedures within the industry. This includes developing codes of conduct covering composition, contamination, labeling and fixing of maximum residue limits.

In the European Union (EU), legislation on food additives is governed by the European Council (EC) through its Scientific Committee on Food (SCF), which is advised by and works in conjunction with a number of authoritative and professional bodies. These include the European Food Safety Authority (EFSA), the Office International de la Vigne et du Vin (OIV), the European Brewing Convention (EBC) and the Institute of Brewers and Distillers. Legislation on additives is governed by Council Directive 89/107/EEC, which is amended from time to time, as the need arises. Within these directives, there are papers that refer to specific types of additives, such as SO_2, isinglass, artificial sweeteners, etc.

In the USA, the Food and Drugs Administration (FDA) is responsible for regulation and legislation relating to additives and processing associated with alcoholic beverages and labeling is regulated by the Alcohol and Tobacco Tax Bureau (TTB, formerly the Bureau of Alcohol, Tobacco and Firearms or BATF). This body may work in conjunction with the Association of Official Analytical Chemists (AOAC) and the American Society of Brewing Chemists (ASBC), the Wine Institute and other organizations.

In Australia and New Zealand, the Food Standards Australia and New Zealand (FSANZ) is the body that approves standards or variations in accordance with the general policy of the Australia and New Zealand Food Regulation Ministerial Council. FSANZ liaises with alcoholic drinks industry representative bodies, such as the Winemakers' Federation of Australia.

Also, at the international level there is the Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization (FAO) and World Health Organization (WHO). This committee surveys existing toxicological and other data on food additives and publishes reports of the findings. It offers recommendations relating to additives, particularly regarding acceptable daily intake (ADI). Australia, New Zealand and South Africa have wine trade agreements with the European Union. Such trade agreements allow for differences in

permitted wine additives. For example, wines made in the EU in 2005 (say) using gum arabic (acacia) as a fining agent, were permitted for sale in Australia, even though gum arabic was not at that time on the FSANZ list of permitted additives. Conversely, Australian wines that have been treated with copper citrate can be sold in the EU, where copper sulfate, not citrate, is the allowed agent for removal of H_2S and thiols.

Any application to these statutory authorities to amend one of its articles (or standards, according to FSANZ) must include a sound rationale for the proposed changes, along with relevant data, including those relating to toxicology and residues. A relevant committee of the authority will take these into account in a draft assessment that also includes its own risk assessment using the best scientific studies available. A draft report with regulatory response will be prepared and submitted to the authority board. The draft report will then be amended as necessary and after resubmission, the board will accept or reject the final report. All these events occur with public consultation (comments, extra information, etc.) and with the appropriate government ministries. An outline of this process is shown in Figure 5.9.2, which, although relating specifically to Australia and New Zealand (FSANZ), is broadly applicable to all countries. The actual details of such processes depend on the urgency and complexity of the matter under investigation. A recent example of this process, again involving FSANZ, can be seen in the final assessment report for application A562 (2007) by the Winemakers' Federation of Australia to allow the use of copper citrate without a bentonite base (copper citrate/bentonite was already a permitted additive) (http:/www.foodstandards.gov.au/standardsdevelopment/). The request was

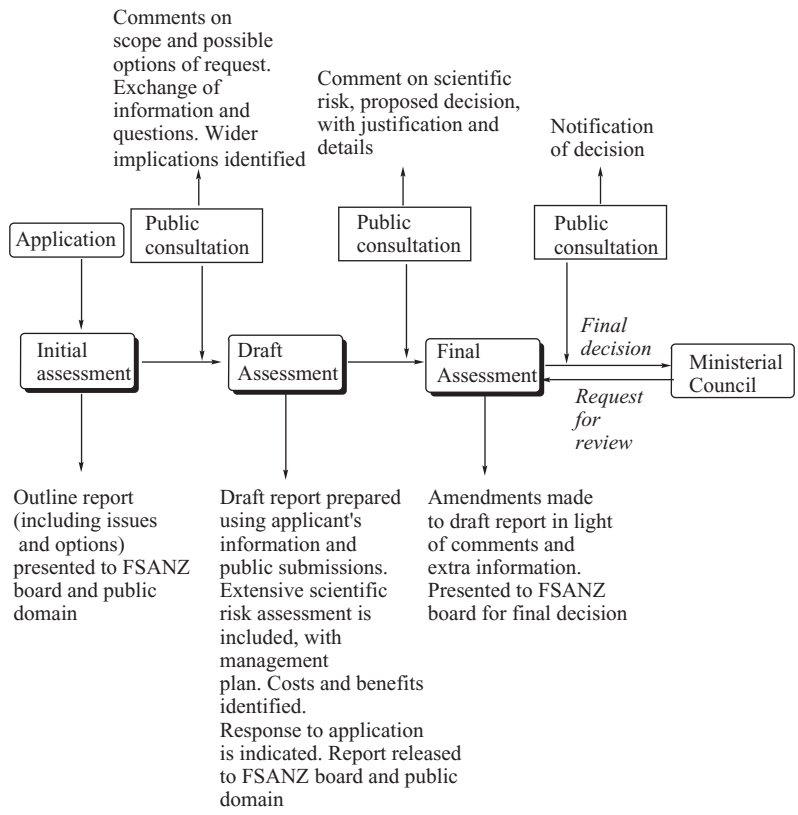

Figure 5.9.2 *Different stages in dealing with application for new or amended process in alcoholic beverage production in Australia and New Zealand (FSANZ)*

Table 5.9.2 *Legal limits for selected additives in alcoholic beverages*

Country	Beverage	Total SO_2 (mg/l)*	Total sorbate (mg/l)	Total Cu^{2+} (mg/l)*	Total $K_4Fe(CN)_6$ (mg/l)
Australia/ New Zealand	Wine (rs > 35 g/l)	400	200 (for all)	1 (for all)	
	Wine (rs < 35 g/l)	250			
	Country wines and mead (rs > 5 g/l)	300			
	Wine (rs < 5 g/l)	200			
	Mixed alcoholic drinks	250			
	Beer	25			
EU	Red wine (rs < 5 g/l)	160	200 (for all)	1 (for all)	1
	White wine (rs < 5 g/l)	210			
	Red wine (rs > 5 g/l)	210			
	White wine (rs > 5 g/l)	260			
	Various other wines (including Auslese)	300 350			
	Sweet white wines	400			
	Beer	200			
	Cider/perry	20–50†			
South Africa	Red wine (rs < 5 g/l)	150		1 (for all)	0§
	Natural wine (rs < 5 g/l)	160			
	Natural wine (rs > 5 g/l)	200			
	Noble late harvest wine	300			
USA	Wine	350 (100 for organic wine)	300	0.5	1

rs = residual sugar; *Includes contribution from natural or non-additive sources; †50 mg/l for cask-conditioned beer; §Allowed, but there must be no residue.

approved, but only after implementation of the procedures shown in Figure 5.9.2. Fixing maximum residue limits (including additives) is an important aspect of the work of statutory authorities such as SCF, USFDA and FSANZ. Table 5.9.2 shows maximum permitted levels of additives in various alcoholic beverages, fixed by various statutory authorities.

Temporary changes to legal requirements can be requested and approved in a short space of time, provided that sufficient grounds for the temporary change are demonstrated by the applicant. An example of this is the application to the European Union to amend Commission Regulation No. 1622/2000 that lays down the maximum allowed total SO_2 residue in EU wines. The application was made by the French Government to increase by a maximum of 40 mg/l, the total SO_2 content of wines from Alsace with less than 300 mg/l, as a result of very unfavorable weather conditions during the harvest (EC, 2007).

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5.10
Residues in Alcoholic Beverages

5.10.1 Introduction

For the purposes of this book, the term residues refers to low levels of substances that have found their way into alcoholic drinks by routes other than deliberate addition to the beverage. These residues fall into three major categories: pesticides (Section 5.10.2), inorganic materials (Section 5.10.3) and industrial organic contaminants (Section 5.10.4).

Residual substances that exist in alcoholic beverages through the use of additives during the manufacturing process to improve the product are dealt with in Chapter 5.9.

Pesticide residues – fungicides and insecticides – arise from spraying of crop plants (cereals, fruit trees, grapevines and hop bines) to prevent insect infestation or fungal disease. Additionally, herbicides, sprayed onto land surrounding the crop plants to eliminate or minimize competition from weeds, are included in this category. Inorganic residues, on the other hand, originate from plant uptake from the soil and from contact (deliberate or accidental) between plant and contamination source. They can also be acquired during the manufacturing process, by leeching out of equipment. Similarly, industrial organic contaminants can find their way into an alcoholic beverage via contamination of the crop or production materials, or during the production process.

The following sections review the more common residues found in alcoholic beverages, with emphasis on the health risks they pose to consumers. Included here are brief accounts of legal restrictions on the mineral (metal and nonmetal species) content of alcoholic beverages and on the mineral content of water used for brewing or dilution purposes (e.g. of spirits) (Section 5.10.3). Regulation of processes, in order to limit the levels of inorganic contaminants in the finished product, is also discussed briefly (Section 5.10.3).

5.10.2 Pesticides

A wide range of pesticides is used on the raw materials used to make alcoholic beverages. These are used to control fungal infection of plants or seeds (fungicides), weed growth (herbicides) and insect infestation (insecticides). Pesticides are of great economic importance since, all other things being equal, they maximize the yield of healthy crops of cereals, fruit and hops (etc.) by minimizing weed competition for moisture and soil nutrients, by preventing attack by insects and by protecting the plants from fungal infection. On the negative side, some pesticides are known to upset the local ecological balance by killing beneficial insects

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

(such as bees), earthworms and aquatic life. Additionally, residues persist to certain degrees in foodstuffs and this has been the cause of much public concern over the years, despite the fact that many residues in alcoholic drinks are of exceedingly low levels. Pesticide spraying programs are designed to ensure maximum crop benefit, whilst minimizing residue concentrations in the finished product. Thus, concentrations of pesticide (g/l/ha), spraying intervals (e.g. six weeks) and the time interval between application of the last spray prior to harvest are all laid down by the pesticide manufactures and are overseen by various governmental agencies (e.g. DEFRA in the UK, European Commission in the EU, USFDA in the USA and FSANZ in Australia and New Zealand).

Extensive work has been carried out regarding the toxicological properties of all pesticides. As a result of this work, the various governmental statutory bodies have defined maximum residue limits (MRLs) relating to the raw materials that are used to make alcoholic beverages. Furthermore, FAO/WHO have suggested acceptable daily intake (ADI) for some pesticides (Amerine and Ough, 1980). Maximum residue limits have been applied to the raw materials (in mg/kg or μg/g), rather than beverages, because it is known that pesticides are often significantly degraded or removed during processing (fermentation, fining, filtration, etc.) (Navarro *et al.*, 1999; Szerletics *et al.*, 1999; Ying and Williams, 1999; Angioni *et al.*, 2005; Navarro *et al.*, 2007).

Toxicological studies often involve administration of large doses (usually by ingestion, sometimes by skin contact or by inhalation) to rats, mice or other animals and under those conditions, with respect to those particular animals, some pesticides have been shown to have specific chronic toxicities. Some have been found to be carcinogenic or mutagenic, but more have been found to be endocrine disruptors.

As a result of these findings, and also because of other considerations, some insecticides (e.g. aldrin, DDT, dieldrin, heptachlor and lindane) and some herbicides (e.g. Atrazine, paraquat and simazine) are banned for agricultural use in many (or most) countries. Other pesticides are not used in some countries, but allowed in most others (e.g. the fungicide captan is not in use in the USA, and the insecticide carbaryl is not allowed in the UK).

Acute toxicity (LD_{50}) for pesticides has been determined by experiments on a wide range of animals, including mice, rabbits and rats. Some, like diquat and paraquat have very high acute toxicities (low values of rat oral LD_{50}), but most have low (and some exceedingly low) toxicities (very high values of rat oral LD_{50}). Indeed, the acute toxicities of many pesticides, especially fungicides, are lower than many common chemicals, including natural products and those used in the production of alcoholic beverages. Nevertheless, the various statutory bodies involved in agriculture and food production have imposed maximum residue levels (MRLs) for all pesticides that are allowed for agricultural use; levels that fall far below toxic levels or acceptable daily intake levels. These are reviewed at intervals, as additional toxicological information comes to light. It is emphasized that toxic residues arising from fungal infection of plants untreated with fungicides are in general far more dangerous than the fungicides or their degradation products. These toxic residues include aflatoxins (from *Aspergillus flavus*; infection of maize in subtropical climates), trichothecenes (from *Fusarium* spp. infection of cereals in temperate climates) and others discussed in Section 5.11.4. Tables 5.10.1 and 5.10.2 display some prominent (and once prominent) pesticides used on crops used in the production of alcoholic beverages, along with their MRLs, LD_{50} and other data. Figures 5.10.1 and 5.10.2 show some of their structures, and the chemical classes to which they belong. It can be seen from Tables 5.10.1 and 5.10.2, that the acute toxicities (given by LD_{50}) of herbicides and insecticides are frequently higher (sometimes much higher) than those of fungicides. MRLs are often higher for grapes and other crops destined to produce alcoholic beverages than those for crops for eating (e.g. table grapes) (for general information regarding Europe see http://europa.eu.int/, the European Union 'on-line') or for drinking water (Section 5.10.5 – see Table 5.10.6). The reason for this appears to be that lesser amounts of alcoholic beverages are likely to be consumed compared with fresh produce, juices and drinking water. Furthermore, it is expected that some residue will be removed during the production of alcoholic beverages as a result of biochemical, chemical and physical processes.

Table 5.10.1 *Some herbicides and insecticides used on agricultural crops for alcoholic beverage production*

Herbicide	Example of trade name	CAS registry number	MRL* mg/kg	LD_{50} mg/kgbw (oral, rat)	Comments
Atrazine		1912-24-9	0.05 (USA)	3090	In general use. Banned in the EU
Bispyribac--sodium	Nominee (Kumiai)	125401-92-5	0.01 (cereal)	2635	Used on rice fields
Diquat	Aquacide	2764-72-9 85-00-7 (as $2Br^-$)	0.05	120	Dipyridinium type
Fenoxaprop-p-ethyl	Acclaim-Super (Aventis)	71283-64-6	0.05 (cereal)	>5000	New phenoxy type. Used on wild oats in rye, wheat, etc.
Glyphosate	Roundup (Monsanto)	1071-83-6	0.1	5600	In general use
Paraquat	Gramoxone	1910-42-5	0.02 (USA)	57	Banned in the EU
Metsulfuron-methyl	Escort (Dupont)	74223-64-6	0.05	>5000	New, used in barley and wheat fields. Triazine type
Nicosulfuron	Accent (Dupont)	111991-09-4	0.05	>5000	New sulphonamide type used in wheat fields
Simazine		122-34-9	0.1 (USA)	3100	Triazine type. Banned in the EU
Terbuthylazine	Clip (Sipcam)	5915-14-3	0.1	1590	Triazine type
Insecticides†					
Acetamiprid	Assail (Aventis)	135410-20-7	0.01	146	Neonicotinoid type for fruit trees
Aldricarb	Temik (Bayer)	116-06-3	0.02	7 (solid)	Carbamate type, used on citrus trees
Azadirachtin	Align, Azatin	11141-17-6	1 (grape)	3540	Natural nortetraisoprenoid from neem tree seeds
Carbaryl	Sevin	63-25-3	0.05	250	Also an acaricide. Banned in the UK
Dichlorvos	Nuvam (Amvac)	62-73-7	0.01	61	Phosphinate type used on outdoor fruit trees. Highly toxic to bees
Etofenprox	Trebon (Mistui)	80844-07-1	5	42880	Used on fruit trees
Imidaclorid	Admire (Bayer)	138261-41-3	1 (vines) 3 (hops)	450	Used on pome fruit, stone fruit, vines, hops and cereals. Affects bees
Malathion		121-75-5	5	5400	Organophosphorus type, also an acaricide
Pirimicarb	Aphox	23103-98-2	1	142	For fruit trees and cereals. Also aphicide
Pyrethrins	Alleviate	e.g. Allethrin 584-79-2	1	685	In general use
Thiamethoxan	Adage, Cruiser (Syngenta)	153719-23-4	0.5 (grape)	1563	Neonicotinoid type. Used on fruit (Adage) and on barley seed for storage (Cruiser)

*EU, unless stated otherwise; †Insecticides such as aldrin, DDT, dieldrin, heptaclor and lindane are not included in this table as they are banned in many countries.

Table 5.10.2 *Some fungicides used on agricultural crops for alcoholic beverage production*

Fungicide	Example of trade name	CAS registry number	MRL mg/kg	LD_{50} mg/kgbw (oral, rat)	Comments
Azoxystrobin	Heritage (Zeneca)	131860-33-8	2	>5000	Used on vines against powdery and downy mildew
Captan	Orthocide		0.02	>9000	General use, not in the USA
Carbendazim	Benlate	10605-21-7	0.5	>15000	General, combats downy and powdery mildew
Chlorothalonil	Bravo	1897-45-6	3	>5000	General use
Copper (II)	Bordeaux mixture		50	30	Used on vines against downy mildew
Dimethomorph	Festival (BASF)	110488-70-5 (*E*-isomer)	3	3900	Active against downy mildew on grapevines
Dithiocarbamates	Maneb, Mancozeb, Zineb etc.	12427-38-2 (Maneb) 12122-67-7 (Zineb)	5 (for all)	4100 (Maneb), 7600 (Zineb)	Active against downy mildew
Dinocap	Karathane	39300-45-3	1	980	Usually a mixture of homologues
Difenoconazole	Bardos, Score (Syngenta)	119446-68-3	0.5	1453	Used on cereal crops
Folpet	Acryptan	133-07-3	0.1	2189	Used on fruit trees and for cereal seed storage. Not used in the USA
Hexaconazole	Anvil, Planate (Syngenta)	79983-71-4	0.1	2189	Used to mildew and scabs on fruit trees, and vines (Anvil). Also on cereals (Plante)
Iprodione	Rovral (Rhone Poulenc)	36734-18-7	10	>2000	Widely used in the EU on vines (*vs.* grey mold). Also cereal seed treatment
Kresoxym-methyl	Stroby (BASF)	143390-89-0	1 (general) 0.2 (apples/ pears)	>2000	Used mainly on apple and pear trees
Penconazole	Topas (Syngenta)	66246-88-6	0.2	2125	Used on fruit, grape and hop plants
Propiconazole	Banner	60207-90-1	0.05	1517	Wide spectrum, used on fruit and cereal plants

(*Continued*)

Table 5.10.2 (Continued)

Fungicide	Example of trade name	CAS registry number	MRL mg/kg	LD_{50} mg/kgbw (oral, rat)	Comments
Pyrimethanil	Scala (Aventis)	53112-28-0	5	>4000	Used on vines (*vs.* grey mold) and apples (*vs.* leaf scab)
Procymidone	Sumisclex	32809-16-8	5	>10,000	Active against grey mold (*Botrytis cinerea*)
Sulphur		7704-34-9	50	5000	Widely used against powdery mildew
Tetraconazole	Domark 40ME	112281-77-3	0.1	1030	Useful against powdery mildew on vines
Thiophenate-methyl	Alert (Nagarjuna)	23564-05-8	3	7500	Wide spectrum, used on fruit trees and sugar cane
Thiram	Fernacol	137-26-8	3	1800	For fruit trees and cereal seed storage
Trifloxystrobin	Compass (Bayer)	141517-21-7	5	5000	
Vinclozolin	Ronilan (BASF)	50471-44-8	5	>15 000	Widely used on vines (*vs.* grey mold). Endocrine disruptor
Zoxamide	Zoxium 80W (Rohm and Haas)	156052-68-5	5	>5000	For downy mildew on vines. Also used on potatoes

Many fungicides, belonging to different structural families, act by interfering with the sterol synthesis pathway used by pathogenic fungi, thus preventing the construction of cell membranes. A common class of fungicides used on grapes and other fruits (some are authorized for use on cereal crops) are the triazoles: difenconazole, hexaconazole, penconazole, propiconazole, tebuconazole, tetraconazole and others. Their acute toxicities are often higher (i.e. they have comparatively low LD_{50} values – see Table 5.10.2) than other fungicides, although they have lower MRLs because they can be applied to crops at much lower rates (e.g. ~60 g/ha, as opposed to ~250 g/ha) (Juan-Garcia *et al.*, 2004).

A more recent family of fungicides is based on strobilurin (a natural product obtained from *Strobilurus tenacellus* and other muhrooms) and these are known as strobins. They all have the β-methoxyacrylate structural moiety and include azoxystrobin, dimoxystrobin, kresoxin-methyl and trifloxystrobin.

Other fungicide families include (with examples) anilides (carboxin), benzimidazoles (carbendazim, thiophenate-methyl), dicarboximides (captan, folpet, iprodione, procymidone, vinclozolin) dithiocarbamates (maneb, zineb), pyridines (pyrimethanil), pyrimidines (fenarimol), and sulfonamides (Euparen, tolylfluanid). To these can be added the still widely used copper (II) (as Bordeaux mixture) and sulfur, both of which can be used on crops that are used to make organic beverages.

Herbicides and insecticides are generally more toxic than fungicides and several have unfortunate environmental side effects. For example, dichlorvos is highly toxic to bees. Earlier, more toxic herbicides and insecticides that persist in the environment have largely given way to less toxic, less persistent (more easily

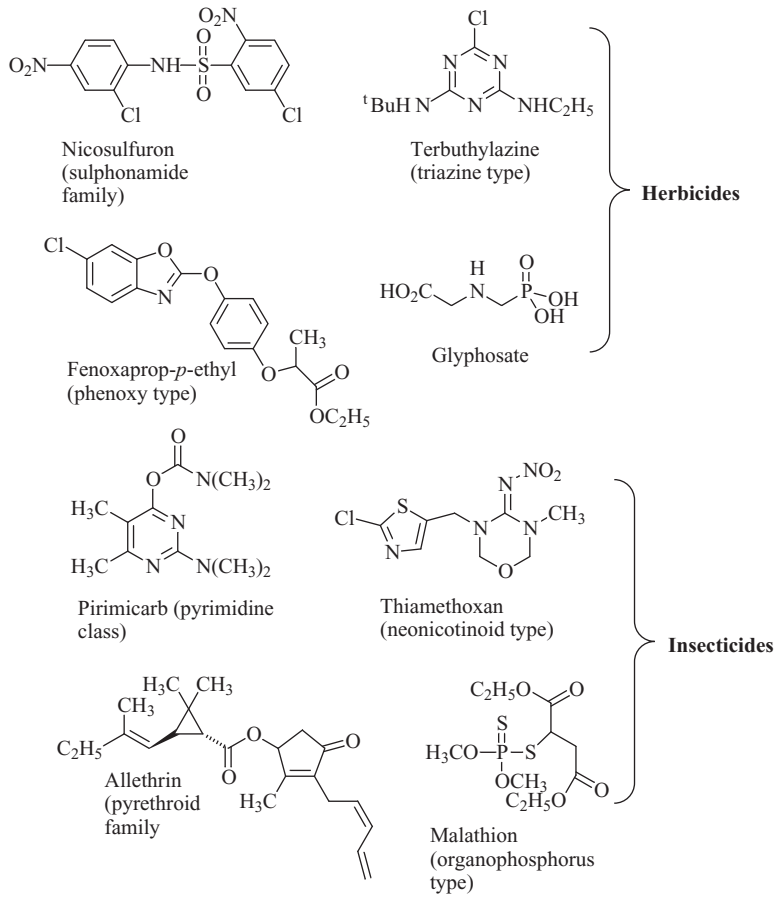

Figure 5.10.1 *Some herbicide and insecticide structures*

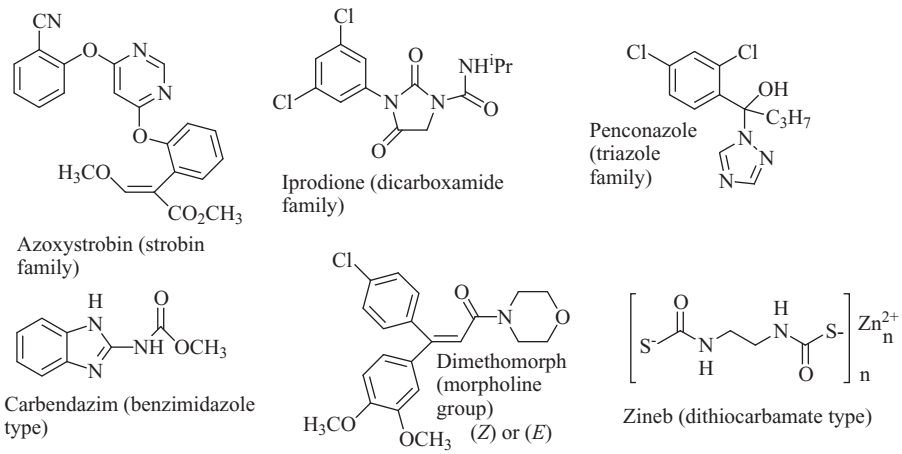

Figure 5.10.2 *Some fungicide structures*

degradable) types. Thus aldrin, DDT, dieldrin and others (insecticides) are banned in most countries, and atrazine and simazine (herbicides) are banned in the EU. Relatively few organophosphorus insecticides are now authorized, because of their high toxicities (cholinesterase inhibitors). More widely used insecticides nowadays are azadirachtin (a natural tetranorisoprenoid), pyrethrins, neonicotinoid types such as acetamiprid, imidacloprid and thiamethoxam, and pyrimidine types (e.g. pirimicarb). Prominent herbicides (used mostly on land growing cereal crops) include nicosulfuron, metsulfuron-methyl (sulfonamide types), glyphosphate, bispyribac-sodium and terbuthylazine (triazine type).

5.10.3 Metal and Nonmetal Residues

There has been much interest in the past few decades on the presence of heavy metal contaminants in alcoholic drinks, particularly Cd^{2+}, Cu^{2+}, Pb^{2+}, Mn^{3+} and Zn^{2+}. Because of the negative health consequences associated with excessive intake of certain compounds of these metals, their mere presence in alcoholic beverages can harm the reputation and commercial value of those beverages. However, in most cases, a significant proportion of the heavy metal content is derived from the soil, taken up by the plants from which the beverage is prepared and thus is difficult or impossible to avoid. These will be taken up along with a host of other metal cations (e.g. Ca^{2+}, Fe^{2+}, Mg^{2+}, K^+ and Na^+) as well as anions (Br^-, Cl^-, phosphates and sulfates), depending on the geological character of the local land and the prevailing climate. Additionally, metal and nonmetal residues can result from environmental pollution, adulteration and contamination by process equipment or methods.

The presence of certain inorganic residues in alcoholic beverages has both benefits (e.g. removal of malodorous substances and the provision of essential minerals) and drawbacks (e.g. spoilage, especially in the formation of hazes, sensory problems and health risks). This section seeks to review the range of inorganic residues in alcoholic beverages, their origins and their effects upon human health.

The inorganic components of alcoholic beverages have diverse origins; they can find their way into beverages at various stage in the production sequence, starting with the raw materials, through the various processes to storage and including adulteration (Pohl, 2007a). These possibilities are outlined in Figure 5.10.3, which also shows parts of the production sequence where metal ions in particular may be lost from the beverage. The plants from which beverages are derived obtain a wide variety of minerals from the soil, which is influenced by the use of fertilizers (K^+, NH_4^+, phosphates, Cl^-, NO_3^- and others, plus contaminants) and metal containing fungicides (5.10.2) (e.g. Cu^{2+}, Mn^{2+} and Zn^{2+} from Bordeaux mixture, maneb and zineb, respectively). Environmental pollution comes from both natural (e.g. NaCl and other minerals from sea spray on coastal crops) and industrial origin sources (e.g. Pb and Cd from factory exhaust or mining waste; F^- from ceramics factories).

Most of the Cu^{2+} and Mg^{2+} in beer are derived from the raw materials, mostly cereals. Some of the copper in wine is derived from Bordeaux mixture (Section 5.9.2), if it is used (but see next paragraph), whereas wines from coastal vineyards are richer in sodium content (Pohl, 2007a). Minerals in water or any additive or adjunct materials used in the brewing process will contribute to the mineral content of the finished product. Most of the Ca^{2+}, CO_3^{2-}, Cl^- and SO_4^{2-} content of beers comes from the brewing water.

During fermentation, the concentrations of many metal ions diminish, because of precipation (e.g. Ca^{2+} and heavy metal cations) and chemical reaction (e.g. Cu^{2+} forms insoluble CuS with hydrogen sulfide). Postfermentation processes, however, can lead to increases or decreases in mineral content, depending on the process. Distillation causes some minerals to be left behind in the residue, but some may be carried over in the distillate and copper ions are extracted from copper stills, which are popular in the production of many spirits, including malt whisky (Sections 3.1.2 and 3.2.2). The copper content of whisky and other spirits is thus derived mostly from the still material (Adam *et al.*, 2002), and is greater for malt whiskies (distilled

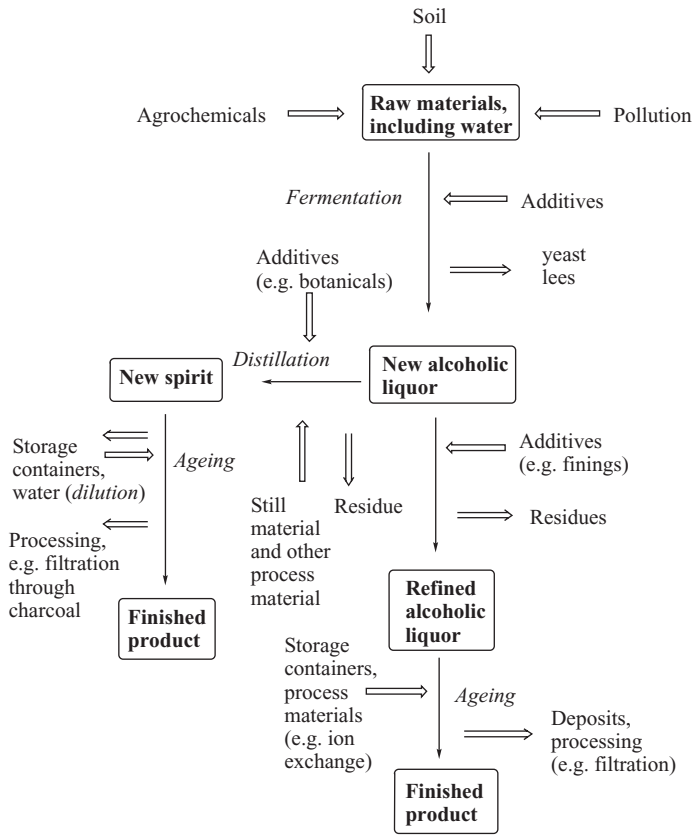

Figure 5.10.3 *Input (closed arrow) and output (open arrow) of metal and nonmetal ions during the production of alcoholic beverages*

from copper pot stills) than grain whiskies (distilled from stainless steel patent stills: continuous or Coffey stills). On the other hand, metal ions in the original fermented mash or wine are largely left behind in the residue upon distillation, explaining why higher levels of Ni^{2+} were found in beers and wines rather than in the spirits whisky and brandy (Dugo *et al.*, 2004).

The use of additives can make a significant contribution to the mineral content of alcoholic beverages. Fining agents (bentonite, isinglass, kieselguhr, silica gel, etc.) can remove metal ions bonded to proteins or adsorbed on yeast cells, but at the same time can lead to an increase of Na^+, Ca^{2+} and Al^{3+} levels in wine (Pohl, 2007b). Addition of copper sulfate or copper citrate to wine (to remove H_2S and thiols; Section 5.9.4) is the biggest contributor of the low levels of Cu^{2+} found in wines. Additionally, increased Na^+ or K^+ content of wines can come from the use of bentonite (Section 2.9.4), sulfites and sorbates (Chapter 2.5) and Ca^{2+} can come from added $CaSO_4$ (as gypsum) as in Sherry production (Section 2.10.2) or from added $CaCO_3$ (deacidification of must; see Section 2.9.1). Likewise, elevated levels of Na^+ and Cl^- can arise from the use of common salt as a clarifying agent (Amerine and Ough, 1980) and added herbs to wines or spirits can be a source of heavy metal cations (Moutatsou *et al.*, 2003).

Process equipment is often a key source of metal ions in the finished products. Copper stills provide most of the coppers content of spirits such as cachaça, rum, tequila and malt whisky (Adam *et al.*, 2002) and

relatively high levels of heavy metals in wastes from the distillation of ouzo (Section 3.5.6) are derived from the bronze pot stills (Moutsatsou *et al.*, 2003). Likewise, contact of wine with equipment materials (casks, pipes, tanks, etc.) is a major or significant source of Al (from concrete or steel), Cd, Cr (from steel), Cu (from copper pipes), Fe (from concrete or steel) and Zn (Pohl, 2007a).

The mineral content of alcoholic beverages can change as a result of certain practices that occur during ageing and in the packaging operations prior to distribution and sale. For example, the use of ion exchange resins can lower the concentrations of heavy metal ions, and K^+, but tends to increase the level of Na^+ (Amerine and Ough, 1980). The copper content of whisky decreases whilst aging in wood, as a result of Cu^{2+} binding to phenolic ligands in the wood (Adam *et al.*, 2002). Conversely, the levels of many ions (Ca^{2+}, Cl^-, NO_3^-, Pb^{2+}, SO_4^{2-} and Zn^{2+}) in Marsala wines tended to increase with increased length of cask maturation (Dugo *et al.*, 2005). At least one study on the Pb content of wines from different vintages has revealed a reverse correlation with vintage year (i.e. older wines tended to have higher Pb levels) (Ajtony *et al.*, 2008). This presumably reflects the lesser use in recent years of lead and its compounds for industrial purposes, leading to less pollution. Higher sulfate levels in wines can be traced to oxidation of some of the sulfite used to protect the wine throughout the maturation process

Many spirits are diluted with water to the required alcoholic strength before bottling (e.g. see Sections 3.2.1 and 3.5.3). Nowadays, purified or distilled water is used, after careful analysis of mineral content, although rainwater or mountain stream water is often used to dilute sugar cane spirits (Section 3.5.3). Water for spirits dilution can still be a source of Ca^{2+}, Mg^{2+}, Na^+, Cl^-, SO_4^{2-} and other ions. Many alcoholic beverages, especially beer, are offered for sale in cans, where higher levels of Cu^{2+} and Zn^{2+} are often apparent compared with glass bottled equivalents (Mayer *et al.*, 2003). On the other hand, the Ni^{2+} contents of bottled and canned beers have been found to be comparable (Dugo *et al.*, 2004). For many years, nearly all wine bottles were sealed with corks and many were 'finished' with lead capsules for attractive presentation and as an extra seal. After many years of storage, these wines may show rather elevated Pb^{2+} levels (Mena *et al.*, 1997). Nowadays, although screw cap or plastic seals for wine bottles are common, a great many wines are still bottled with cork seals. In the latter, however, lead capsules have largely given way to thinner aluminium or plastic capsules.

The mineral ion contents of alcoholic beverages are monitored regularly and carefully (see Sections 4.4.4, 4.5.1 and 4.5.2 for methods of analysis) and there are specified maximum allowed limits. Hence, although all mineral ions are toxic above certain concentrations, their actual concentrations in alcoholic beverages are very far below these levels. Typical selected metal ion contents for various alcoholic drinks are shown in Table 5.10.3, along with some recommended and legally allowed limits. The only (very rare) exceptions to the norm for alcoholic beverages are when either adulteration or contamination via use of inappropriate equipment or processes has occurred. Thus corrosion of copper distillation equipment has led to higher Cu^{2+} levels in tequila (Section 3.5.5) (Carreon *et al.*, 2001) and storage of vodka in poor quality steel or alloy vessels is known to introduce metals into the beverages, though not necessarily at toxic levels (Ibanez *et al.*, 2008).

It can be seen from Table 5.10.3 that spirits in general have lower levels of mineral salts than undistilled beverages, as might be expected from the distillation process. This is despite the fact that metal ions can be leached out of the still or storage material.

Except in the rather rare cases of contamination or adulteration, the drinker of alcoholic beverages, like the drinker of nonalcoholic beverages, can expect no negative health effects from the concentrations of cations and anions in those drinks. Indeed, moderate consumption of alcoholic beverages may provide a significant proportion of the nutritional requirements of some essential metals, notably Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Ni and Zn (Pohl 2007a). Additionally, there is good evidence to suggest that some metal cations in alcoholic beverages, that are known to be harmful in higher concentrations (e.g. Cu^{2+}, Fe^{2+}, Pb^{2+} and Zn^{2+}), are present as complexes rather than 'free' ions. Some of these complexes may decrease the bioavailability

Table 5.10.3 *Metal and nonmetal ion content of some alcoholic beverages (in mg/l)*

	Ca^{2+}	Cu^{2+}	$Fe^{2+/3+}$	Pb^{2+}	Mg^{2+}	K^{+}	Na^{+}	Zn^{2+}	Br^{-}	SO_4^{2-}	PO_4^{3-}	Cl^{-}
Beer		0.028–0.048 [0.05]	0.25–25	ND–0.245 (0.025)				0.1–68*				
Brandy	ND–14.8	2.0–14.6 (5)	ND–2.30	ND–0.224	2.0			3.0				
Gin	1.0	0.5	ND	ND–0.035	0.5							
Rum	4.0	0.5	1.0	ND–0.070	2.0			3.0				
Scotch whisky	0.5–4	0.1–1.7	0.02–28	<0.005	0.02–4		2–24	0.02–20				
Vodka	3.0	0.5	ND		0.5			0.5				
Wine	0.017–14.3	ND–7.62 (1.0)	ND–23.7	ND–1.125 (0.2)	7.8–718	265–3056	ND–310 (393)	ND–8.9 (5.0)	ND–0.42 [1.0]	ND–4390 [3000] (5000)	ND–900	ND–370 [500] (1000)

*Most values are much lower than the maximum quoted here.

Source: Data from Amerine and Ough (1980), Dugo *et al.* (2005), Ibanez *et al.* (2008) (and references therein) and Mayer *et al.* (2003). Figures in parentheses are EU maximum allowed limits, those in square brackets are suggested maximum limits.

Table 5.10.4 *Some methods for the removal of excess metal ions from alcoholic beverages*

Method	Cations removed	Comments
Ion exchange	Cu^{2+}, Pb^{2+} and others	Used on wine and spirits, such as Tequila (Ibanez *et al.*, 2008). Can lead to high levels of Na^{+} or K^{+}
Addition of potassium ferrocyanide	Cu^{2+}, $Fe^{2+/3+}$ and others	Allowed in the EU (for wine) but not in the USA. Level of Cu^{2+} in wine must be lower than that of added Fe^{II}
Addition of chelating insoluble polymers	Cu^{2+} and $Fe^{2+/3+}$	Detering *et al.*, 1991. A commercial version is Divergan (BASF), used in matrix filter sheets (1 g/l) (Ibanez *et al.*, 2008)
Addition $MgCO_3$ or $CaCO_3$	Cu^{2+}	Can be used to decrease Cu^{2+} content of rum (Almeida-Neves *et al.*, 2007)
Electrolysis and electroless cementation	Cu^{2+}	Cementation is on Fe. Used for decreasing Cu^{2+} content of tequila (Ibanez *et al.*, 2008)

of the metal ions in the gastrointestinal tract. It appears that lead and zinc ions preferentially complex with polyphenols (Green *et al.*, 1997), Cu^{2+} binds to the thiol groups of proteins and to OH grapes of carbohydrates, whilst Fe^{3+} forms complexes with hop bittering constituents (Wiese and Schwedt, 1997). *In vitro* digestion studies on Cu^{2+} and Pb^{2+} in port wines have suggested that *ca.* 50% and 10–22% of Cu and Pb respectively were bioavailable under intestinal digestion conditions (Azenha and Vasconcelos, 2000). The Pb^{2+} ions in particular, were converted to a largely insoluble form under these conditions.

There are many methods for the removal of metal cations from alcoholic beverages, should the need arise (e.g. accidental contamination leading to levels of metal in excess of the maximum allowed limit). The more widely used of these methods are summarized in Table 5.10.4. Not all of these methods are allowed in all countries, and alternative methods can be found in Ibanez *et al.* (2008). It should be noted that a certain concentration of a certain cation or anion may be beneficial to the organoleptic quality of the beverage. For example, Cu^{2+} present in wine or spirits combines with sulfur derivatives (e.g. H_2S produced by certain *S. cerevisiae* strains) to reduce sulfury odors (Amerine and Ough, 1980, Reaich, 1998). It is also involved in the formation of desirable congeners during the distillation of whisky (Reaich, 1998), sugar cane spirits (Section 3.5.3) and tequila (Section 3.5.5).

5.10.4 Industrial Contaminants

This section is concerned with the minor organic contaminants in alcoholic drinks that arise from pollution caused by general industrial activities or from use of specific equipment or materials. Potentially, there are many such contaminants. Table 5.10.5 lists the more prominent ones, along with their sources, the ways in which they can find their way into drinks, the most likely drinks to contain them, and ways of avoiding or removing the contaminants. It will be noted that all the substances entered in Table 5.10.5 are lipophilic; that is, they are fat soluble, with only limited solubility in water or aqueous alcoholic beverages – especially beer and cider with low ethanol contents. Also, crops from which alcoholic beverages are produced (cereals, grapes, fruit, etc.) are generally of low lipid content, although fruit skins contain terpenoid and other lipophilic substances. Nevertheless, because of the extreme toxicity of many of the substances in Table 5.10.5, crops, alcoholic beverages and water used in the production of drinks are periodically monitored to ensure that concentrations of these substances fall below statutory limits, or recommended international limits.

Table 5.10.5 *Some industrial contaminants sometimes found in alcoholic beverages*

Contaminant	Sources	Route to alcoholic beverage	Most likely beverages to contain contaminants	Methods of prevention or removal of contaminants
Benzene	Degradation of aromatic compounds, e.g. benzoates. From CO_2 supplies	Most likely through carbonation processes	Carbonated drinks: processed beer, cider and aerated wine	Monitor of CO_2 supply. Pass through activated charcoal
Polycyclic aromatic hydrocarbons (PAHs)	Incomplete combustion of carbon sources, including fossil fuels	Probably via contamination of raw materials, including water	Those produced from crops grown near pollution sources	Monitor of water quality. Use of activated charcoal beds
Dioxins and polychlorinated biphenyls (PCBs)	Combustion of materials containing both carbon and chlorine	Probably via contamination of raw materials, including water	Those produced from crops grown near pollution sources	Monitor of water quality. Use of activated charcoal beds
Trihalomethanes and chlorinated hydrocarbons	General industrial activities involving solvents. chlorination sterilization of water	Most likely via polluted water or through use of chlorinated water	Diluted beverages: spirits and beers brewed from high gravity mash	Monitor of water quality. Use of activated charcoal beds
Chlorophenols	Chlorination of water, bleaching of cork, use of chlorinated compounds in pesticide sprays	Water, cork stoppers, casks and vats	Wine (cork stoppers)	Monitor of water quality. Use of activated charcoal beds
Organotin compounds	Catalysts and stabilizers for PVC production	Most likely by storage in plastic containers. Possibly via pollution of raw materials	Cider, wine and brandy	Use of nonplastic containers
Phthalates	Plasticizers	Most likely by storage in plastic containers	Spirits	Use of nonplastic containers
Vinyl chloride and styrene	Monomers used to make plastic	Most likely by storage in plastic containers: PVC or fiberglass	Cider, wine	As above: PVC not widely used, but fiberglass is still in use

Benzene is classified as a Group 1 carcinogen by the WHO International Agency for Research on Cancer (IARC) (WHO, IARC 1987). The World Health Organisation (WHO) has suggested a guideline limit of 10 μg/l of benzene in drinking water (WHO, 2001), although many statutory bodies regulating water quality set much lower limits than this, often 1 μg/l (see Table 5.10.6 and Bamforth, 2002). Benzene can be produced by the degradation of aromatic compounds in foodstuffs during processing, such as cooking and irradiation sterilization (Fabietti *et al.*, 2001). In particular, benzene is formed in soft drinks that contain both benzoates and ascorbate preservatives, in the presence of transition metals (Gardner and Lawrence, 1993). However, this is unlikely to be the source of the benzene content of alcoholic drinks, since benzoates and ascorbates are not widely used in the alcoholic beverage industry. A much more likely source of benzene contamination here in bottled liquified carbon dioxide used for carbonation of processed beers (Wu *et al.*, 2006) and other drinks.

Investigation of the benzene content of 84 beers found that only six had levels above 1 μg/l (the detection limit) and all had levels below 10 μg/l, the WHO recommended limit (Wu *et al.*, 2006). Processed beers of six breweries in the UK were found to have detectable benzene levels that were traced to the use of contaminated liquid CO_2, but even here levels were lower than 10 μg/l (Long, 2003). Breweries now operate quality control programs on CO_2 supplies, in which the internationally suggested upper limit for benzene is 0.02 ppm (v:v).

Polycyclic aromatic hydrocarbons (PAHs), dioxins and polychlorinated biphenyls (PCBs) are produced in small amounts during the combustion of organic materials, all of which contain carbon and some also contain chlorine. In particular, common sources of PAHs are automobile and factory exhausts, although catalytic converters and filters can minimize pollution of crops and groundwater by fallout from these sources. Likewise, incineration of waste materials can produce PAHs, PCBs and dioxins if carried out below a certain temperature. These highly toxic compounds (many are known carcinogens) are removed from drinking water (and that used for alcoholic beverage production) by activated charcoal filtration. Typical maximum limits in drinking water for benz(a)pyrene and other PAHs are 0.010 μg/l and 0.10 μg/l, respectively (Table 5.10.6).

Trihalomethanes and chlorinated hydrocarbons in general are common environmental pollutants, being widely used in commerce and industry, particularly as solvents and cleaning fluids. Although of limited solubility in water, they are volatile, they can pollute a wide area of land and groundwater and tend to persist in the environment. Furthermore, chlorination of untreated water can produce trihalomethanes and other polyhalogenated organic compounds by reaction of chlorine with carbonyl compounds, carboxylic acids and other substance in the water. Trihalomethanes – chloroform ($CHCl_3$), bromoform ($CHBr_3$), dibromochloromethane ($CHBr_2Cl$) and bromodichloromethene ($CHBrCl_2$) – are classed as probable carcinogens. The main route into alcoholic beverages in via water used in the manufacturing process – brewing liquor and blending liquor used to dilute high strength beers (e.g. from high gravity brewing; see Section 2.6.8), ciders (e.g. factory cider (Section 2.8.6)) and spirits (see Sections 3.2.2 and 3.5.3, for example). The boiling stage of brewing (Section 2.6.3) dispels most of these volatile contaminants, but otherwise contamination will occur if these substances are present in the water (Baxter, 1999).

Regular checks on the treated water supply will ensure that chlorinated hydrocarbons are present below national regulation limits, such as those set in the UK for tetra- and trichloroethenes (10 μg/l) and trihalomethanes (100 μg/l) (Table 5.10.6; see also Bamforth, 2002). The average and maximum trithalomethane content determined in a study on 107 beers (27 of which were Chinese) were 1.2 mg/l and 5.2 mg/l, respectively (Wu *et al.*, 2006). In comparison, the range of trihalomethane levels in water from 20 beer brewing sites in China was 2.7–46.9 μg/l, with water from one site having 79.3 μg/l. These figures illustrate the typically lower level of contamination of alcoholic beverages, as opposed to water.

Haloanisoles and halophenols, especially 2,4,6-trichloroanisole (TCA) 2,3,4,6-tetrachloroanisole (TeCA), pentachloroanisole (PCA) and 2,4,6-tribromoamisole (PLA), are sometimes present in wine at low levels, too

Table 5.10.6 *Safety and quality parameters for water used in brewing and the production of alcoholic beverages*

Parameter	Maximum concentration or value	Units
Microbiological parameters		
Coliform bacteria	0	Number/100 ml
Clostridium perfringens	0	Number/100 ml
E. coli	0	Number/100 ml
Enterococci	0	Number/100 ml
Chemical parameters		
Acrylamide	0.10	μg/l
Antimony	5.0	μg/l
Arsenic	10	μg/l
Benzene	1.0	μg/l
Benz(a)pyrene	0.010	μg/l
Bromate	10	μg/l
Cadmium	5.0	μg/l
Chromium	50	μg/l
Cyanide	50	μg/l
1,2-Dichloroethene	3.0	μg/l
Epichlorhydrin	0.10	μg/l
Lead	25*	μg/l
Mercury	1.0	μg/l
Nickel		μg/l
Pesticides (individual substances)	0.030–0.10	μg/l
Pesticides (total)	0.50	μg/l
Polycyclic aromatic hydrocarbons (total)	0.10	μg/l
Selenium	10	μg/l
Tetra- and trichloroethene	10	μg/l
Tetrachloromethane	3	μg/l
Trihalomethanes (total)	100	μg/l
Vinyl chloride	0.50	μg/l
Quality Parameters		
Ammonium	0.50	mg/l
Aluminium	0.2	mg/l
Boron	1.0	mg/l
Chloride	250	mg/l
Copper	2.0	mg/l
Fluoride	1.5	mg/l
Iron	0.20	mg/l
Manganese	0.050	mg/l
Nitrate	50	mg/l
Nitrite	0.5	mg/l
Sodium	200	mg/l
Sulphate	250	mg/l

*10 mg/l from 25 December 2013.
Source: Based on The Water Supply (Water Quality) Regulations 2000; UK Statutary Instrument No. 3184, 2000.

low to be a serious health hazard. They are mentioned here because even at concentrations of a few μg/l their odor threshold values (OTVs) are such as that their presence gives rise to an off odor known as 'cork-taint.' It is believed that fungi present in cork metabolize halophenols by methylating them. The phenols, in turn, may be derived from the chlorination of cork lignin molecules by the use of chlorinated solutions to bleach the cork, although there are many other possible sources involving contact between winery equipment, materials (including grapes) and chlorine compounds (Pizarro *et al.*, 2007).

Organotin compounds are widely used in agriculture and industry: mono- and dibutyl tin (MBT and DBT) are common catalysts and stabilizers in the production of certain polymeric materials, notably poly(vinyl chloride) (PVC). Some organotin compounds (e.g. tributyl- and triphenyltin) may get into alcoholic beverages as pesticide residues, but it appears that a major route is via storage in plastic (including PVC) containers, as far as wine and brandy are concerned (Heroult *et al.*, 2008). Indeed, high organotin levels in some Canadian wines were attributed to storage in PVC containers (Forsyth *et al.*, 1992). More recent analyses of wines have found concentrations of MBT and DBT between 0.1 and 0.5 μg/l (Liu and Jiang, 2002; Azenha and Vasconselos, 2002), where the main source of these contaminants was probably high density poly(ethylene) containers used at an early stage in the vinification process (Azenha and Vasconselos, 2002). International and national bodies who deal with food safety and regulations (see Section 5.9.5) are working to define recommended or statutory maximum limits for organotin compounds in food, so at the time of writing (2008), no limits have been set.

PVC containers are not used for the storage of alcoholic beverages in most countries, the preferred media being wood, stainless steel, concrete, fiberglass and glass. The most widely used plastic material is poly(ethylene) for certain beers, ciders and wines, but not spirits. Poly(ethylene terephthalate) (PET) is used to make bottles and carboys, and Tetrapak™ containers for wine, beers and cider are made from neoprene. Any plastic material destined for (short- or long-term) contact with alcoholic beverages should be of food grade, where plasticizers, stabilizers and other adjuncts have not been used in the manufacture of the material. PVC is an especially undesirable material for the storage of alcoholic drinks, because of the possibility of monomer (vinyl chloride), plasticizers (often phthalate esters) and stabilizers (such as organotin compounds) leeching into the beverage. Vinyl chloride is a carcinogen and some phthalate esters are endocrine disruptors; they are estrogenic (antiandrogenic) and, for example, can alter testes function in pubertal male rats (Gray and Ostby, 2000). Organotin compounds are discussed earlier.

Phthalate esters are common environmental pollutants because of their widespread use as plasticizers. In fact, their presence in the laboratory environment (in the air, on glassware, etc.) can lead to false determinations (Fankhauser-Noti and Grob, 2007). The potential for phthalate ester contamination of alcoholic beverages is high, because of numerous situations during processing where beverage-plastic contact can occur. Despite this, there are few literature reports on phthalate ester residues in alcoholic drinks. Two recent studies on wine found at least one phthalate in a quantifiable amount in all 62 wines analyzed and 10 wines contained quantifiable amounts of others (Del Carlo *et al.*, 2008; Carillo *et al.*, 2008). Dibutyl phthalate (DBP) and di(2-ethylhexyl) phthalate (DEHP) were the two most common contaminants, although dimethyl and diethyl phthalate (DMP and DEP) were also quite common. In one study, total phthalate concentrations were between 2.7 and 15 μg/l and these were no significant differences observed for glass or plastic container (carton or bag in box) nor stopper material (cork, agglomerated cock or synthetic) (Carillo *et al.*, 2008). From this study, the origin of the major part of the residue was deduced to be likely from earlier in the production process: grape transport containers, pneumatic press material, pump material, pump lines and additives, for example.

At the time of writing (2008), there are no statutory limits with regard to phthalate ester levels in wine or other beverages, although the Environment Protection Agency suggests a limit of 6 μg/l in water for DEHP.

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5.11

Other Trace Substances in Alcoholic Beverages

5.11.1 Introduction

This section considers trace components of certain alcoholic beverages that are neither additives (as defined in Section 5.9.1) nor residues (as defined in Section 5.10.1). They include phytochemicals (Section 5.11.2), nitrogenous allergenic substances (Section 5.11.3), fungal metabolites (Section 5.11.4) and compounds that are formed naturally during some part of the processing of alcoholic drinks (Section 5.11.5). As in Chapter 5.10, health aspects are discussed in terms of toxicity studies on certain trace components, health risks for those who consume alcoholic beverages that contain these components and the regulations and legislation in force to ensure that minimal levels exist in beverages. Also considered here are phytochemicals (Section 5.11.2) that are known to have potent therapeutic value or beneficial *in vitro* activity. These are discussed in relation to their presence in either alcoholic beverages or the raw materials that are used to make them. Certain phytochemicals that are known to have toxic properties at high doses are also discussed.

5.11.2 Phytochemicals

The term phytochemicals refer here to components of alcoholic beverages originating from the plant materials used to make the beverages, as opposed to fermentation metabolites from microbiological sources. There are so many such components (usually at low or even trace levels) that it is not possible to give here a detailed account of their likely influences on health; indeed in many cases this has never been determined. Also, many different kinds of phytochemicals have been discussed with regard to a variety of alcoholic beverages (Part 2) and distilled drinks (Part 3) and earlier in Part 4 (e.g. Section 5.4.3 and Chapter 5.8). Instead, a few interesting phytochemicals have been selected for discussion here.

Consideration begins with some compounds present in ripening fruits that have not yet received much attention: chlorophyll degradation products. Chlorophyll a and b break down in ripening fruit, giving yellow nonfluorescent chlorophyll catabolites (NCCs). (Figure 5.11.1) in both skins and flesh. These NCCs (unlike the chlorophylls themselves, which are phototoxic), although only minor components, have been shown to be potent antioxidants, diminishing the rate and extent of *in vitro* linoleic acid hydroperoxidation

Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass
© 2011 John Wiley & Sons, Ltd. ISBN: 978-0-470-51202-9

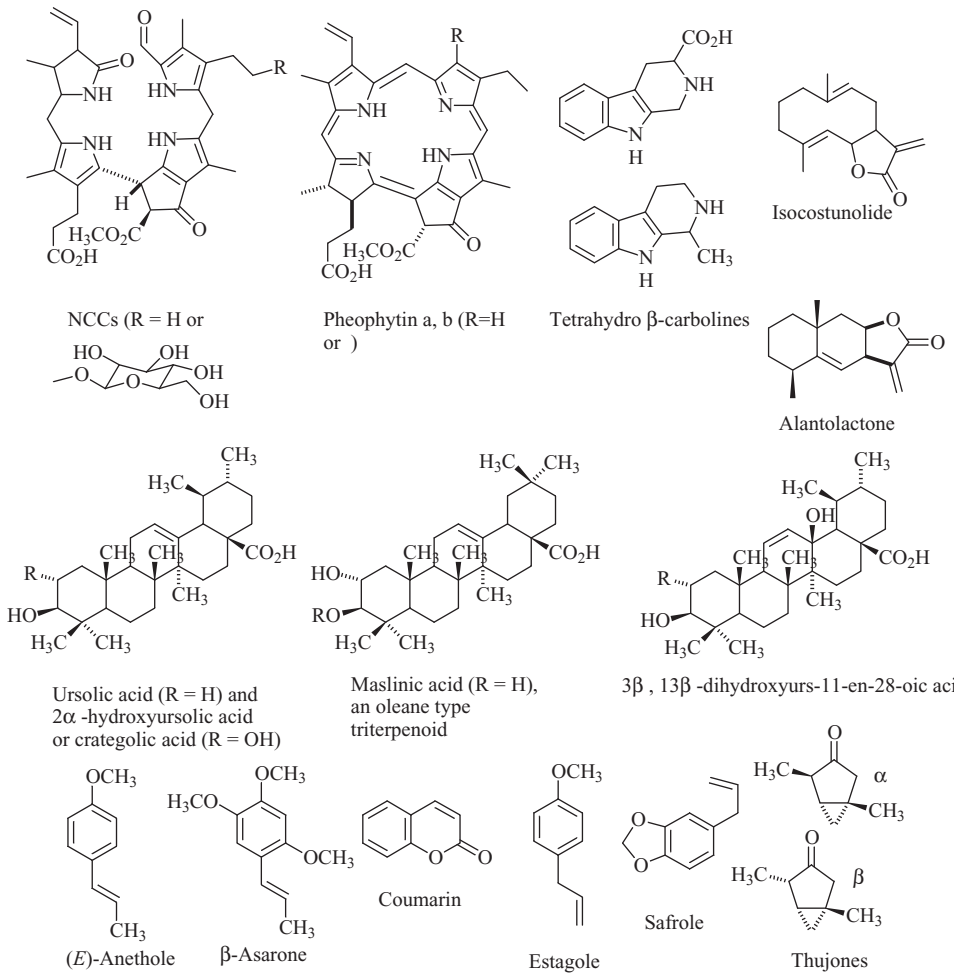

Figure 5.11.1 *A selection of phytochemicals from raw materials of alcoholic beverages*

(Müller *et al.*, 2007). Antioxidant activity, like that of bilirubin, is endowed by the tetrapyrrole structural moiety. NCCs have been found in the skins and flesh of ripe apples and pears, and from the discussion in the next paragraph it is not inconceivable that some may survive into finished cider and perry. Other chlorophyll degradation products, such as pheophytins a and b (but not NCCs) have been found in black grapes of the major port varieties (Section 2.10.7) (Mendes Pinto *et al*., 2005). Although further degradation occurs during the winemaking and ageing processes, some pheophytins survive and have been found in wine.

Carotenoids, common plant products, are well known as antioxidants and they are the precursors of important flavor compounds norisoprenoids, such as (*E*)-β-damascenone and β-ionone. Many carotenoids also survive the winemaking process and together it is likely that these compounds make a significant contribution to the antioxidant power of the wine. These compounds, as well as polyphenols, may contribute to health benefits occurring on light-moderate consumption of cider, perry or wine. See Section 5.4.3 for a discussion of nutritional value of carotenoids.

Another interesting group of compounds found in fruit such as grapes and cherries are the indole amines, melatonin and tetrahydrocarbolines, biosynthesized via the shikimate pathway, through tryptophan and serotonin (Figure 5.11.1). Melatonin has only recently been found in grapes (Iriti *et al.*, 2003), whereas the tetrahydrocarbolines were found in grape juice and wine some time ago, and are known to be antioxidants and radical scavengers (Herraiz and Galisteo, 2003). They are also reversible monoamine oxidase (MAO) inhibitors and potent *in vitro* anticancer agents (Chen *et al.*, 2004).

Isoprenoids, including terpenoids, carotenoids and norisoprenoids are ubiquitous and important constituents of nondistilled alcoholic beverages, as well as flavored distilled drinks. Some norisoprenoids, such as (*E*)-β-damascenone, also make important contributions to the aroma and flavor of certain distilled beverages, such as Bourbon whiskey (Section 3.3.4). Monoterpenoids are known to be synthesized in plastids in the skins of fruits (e.g. grape pericarp, apple skins) (Hardie *et al.*, 1996). The waxy skins of fruit such as apples are a comparatively rich source of terpenoids, including the pentacydic triterpenoids, especially derivatives of maslinic acid, oleanic acid (Figure 5.11.1) and ursolic acid (see Figures 2.12.4) (He and Liu, 2007). Ursolic acid is the most abundant triterpenoid in apple skins (~1.6 g/kg of skins). Many of these triterpenoids showed antiproliferative activity and cytotoxic activity against *in vitro* human HepG-2 liver cancer cells, MCF-7 breast cancer cells and Caro-2 colon cancer cells, with 2α-hydroxyursolic acid being generally the most potent compound. It is possible that *in vivo* these compounds act in a synergistic way, since apple phytochemical extracts were found to inhibit mammary cancer in a rat model in a dose dependent manner (Liu *et al.*, 2005).

Similarly sesquiterpene lactones are found in many plants, particularly the roots of *Inula helenium* (Horse Tail). Roots of this species are used (often as a concentrate) in certain herb liqueurs (Section 3.9.3) in order to add a mild camphor-like flavor and mild bitterness to the beverage. Sesquiterpene lactones such as alantolactone, isoalantalactone, costunolide and isocostunolide, known constituents of *I. helenium*, have been shown to possess *in vitro* anti-inflammatory properties, *in vivo* antibacterial properties and *in vitro* activity against human cancer cell lines. The eudesmane-like alantolactone (Figure 5.11.1) and isoalantalactone show either cytotoxic or antiproliferative activity (see references in Stojakowska *et al.*, 2006), while isocostunolide (Figure 5.11.1) induces *in vitro* apoptosis in human melanoma cells via a mitochondria dependent pathway (Chen *et al.*, 2007). Many plants containing these substances (including *I. helenium*) have long been used in folk and home medicine to remedy a variety of ills with some success (indeed the original purpose of liqueurs was medicinal – see Section 3.9.1), but there is nothing in the literature relating to *in vivo* anticancer activity.

The use of herbs and spices in alcoholic beverages introduces a multitude of phytochemicals into the drink, mostly at low levels (even trace levels for many), but occasionally at comparatively high concentrations. Beverages with added herbs and spices (or their oils and essences) include beer (Section 2.6.13), vermouth (Section 2.12.2), apéritifs (Section 2.12.3), retsina (Section 2.12.3), Maiwein (Section 2.12.3), some spirits (e.g. gin, Section 3.4.2) and some liqueurs (Section 3.9.3). While it is not possible to discuss in detail the health implications of such an enormous number of compounds, some of the better known or previously highlighted components of certain herbs and spices are considered here: (*E*)-anethole, β-asarone, coumarin, estragole, safrole, thujone and xanthohumol (Figure 5.11.1).

(*E*)-Anethole is a major constituent of anis and star anis, used to flavor a number of drinks, including vermouth (Section 2.12.2), anis-flavored spirits (such as arak, ouzo and pastis; Section 3.5.6) and certain liqueurs, such as anisette (Section 3.9.3). In large doses, this compound is an irritant, causing spasmolytic activity. Its LD_{50} is 2090 mg/kg body weight, although the maximum level that causes no toxicological effects in rats is 0.5% of the diet (250 mg/kg body weight). The FAO/WHO Joint Expert Committee on Food Additives (JECFA) have fixed an acceptable daily intake (ADI) of 0–2 mg/kg body weight and sees no safety concern at current levels of intake when used as a flavoring agent (JECFA, 2001a).

β-Asarone, estragole and saffrole, structural relatives of (*E*)-anethole (Figure 5.11.1), are of considerably greater health concern. β-Asarone (the (*Z*) or *cis* isomer) is a major component of certain varieties of *Acorus*

calamus Linn. (Sweet Flag); it constitutes ~75% of oil of calamus derived from the dried rhizomes of the Indian tetraploid variety, but only ~5% of the corresponding oils from the Kashmir (hexaploid) or European (diploid) varieties. It has antibacterial properties (including *in vitro* antitubercular action). The compound exhibits *in vitro* inhibition of monoamine oxidases and in higher doses causes sedative/tranquilizing action on animals (10–100 mg/kg body weight). Its acute toxicity (oral rat LD_{50}) is 1010 mg/kg body weight, whereas that of European oil of calamus (containing *ca*. 5% β-asarone, as well as many other constituents) is 3497 mg/kg body weight. Although low levels of β-asarone are nonmutagenic to various strains of *Salmonella hyphimurium* (via the Ames test), long-term oral administration to rats of high doses (1–2% of diet) caused liver and intestinal cancers. On this basis, use of the pure compound as a food additive is banned in most countries and JECFA of FAO/WHO have not allocated an ADI for this compound (JECFA, 2001b). In the European Union, the presence of β-asarone in alcoholic beverages (as a component of natural flavorings) is allowed up to 1.0 mg/kg (EC, 2008). Similarly, the Working Party on Herbal Medicinal Products of the European Agency for the Evaluation of Medicinal Products (EMEA) have recommended a temporary acceptable daily intake of β-asarone of 2 μg/kg bodyweight (EMEA, 2003).

Estragole, a constitutional isomer of (*E*)-anethole, is present in a wide range of herbs and spices: notably anis, basil, fennel and tarragon, all of which are used as flavorings in certain alcoholic beverages, particularly vermouth (see Figure 2.12.3, Section 2.12.2). It is a suspected carcinogen and genotoxic agent, as administration of large doses has been found to cause liver tumors in rats. Whilst appreciating that a number of plant materials used in the production of certain alcoholic beverages contain estragole, the Scientific Committee on Food of the European Commission (of the EU) have indicated that a safe exposure limit could not be determined (EC, 2002a). Estragole and (*E*)-anethole have also been found to be very minor constituents of certain grape musts (Caven-Quantrill and Buglass, 2006).

Safrole (EC 2002b) is a major constituent of the root bark or fruit of the sassafras plant and it is also present in basil, cinnamon and nutmeg. Like estragole, large oral doses have been shown to cause liver cancers in rats, the carcinogenic potency being low (TD_{50} = 441 mg/kg body weight/day, where TD_{50} is the dose required to produce tumors in 50% of the animals). Its acute toxicity is typically low: the rat oral LD_{50} being 1950 mg/kg body weight. Pure safrole per se is banned in the USA by USFDA as a food additive, and JECFA of FAO/WHO have not allocated an ADI for the compound (JECFA, 2001c).

Coumarin is a component of many plant materials, including bison grass, cassia bark (cinnamon), lovage, vanilla grass and woodruff (*Artemisia pontica*), some of which, especially cassia and woodruff are used to flavor alcoholic drinks such as May wine (Maiwein) and certain vodkas (subrowka). Coumarin has a moderate chronic toxicity to the liver and kidneys of rats, but not baboons. Its acute rat oral toxicity is comparatively high: LD_{50} = 290 mg/kg body weight. Coumarin per se is banned as a food additive in the USA, where it is listed by USFDA as 'a substance generally prohibited for direct addition or use in human food,' but natural additives that contain coumarin are allowed in alcoholic beverages. Likewise, in the countries of the European Union, pure coumarin may not be added to foodstuffs, only plant materials that contain it. At present, the 1988 EC ruling of maximum levels of 2 mg/kg of foodstuff (10 mg/kg of certain foodstuffs and alcoholic beverages) is still in force (EC, 1988). These levels were adopted from those in the *Codex Alimentarius* (1985). Recently, the European Food Safety Authority (EFSA, 2008) recommended a total daily intake (TDI) of no more than 0.1 mg/kg body weight to the European Council (EC) of the European Union. There were concerns about its genotoxicity in the 1980s, but more recent research has suggested that coumarin is not genotoxic (Lake, 1999).

Thujone, or more correctly α- and β-thujone (Figure 5.11.1), are terpenoids that once again can be found in a wide range of plant materials, but particularly in the leaves and stems of various *Artemisia* species, such as wormwood (*A. pontica*), mugwort (*A. vulgaris*) and common wormwood (*A. absinthium*). The two isomers are also found in green anise (*Pimpinella anisum* L.), Florence fennel (*Foeniculum vulgare* Mill.),

hyssop (*Hyssopus officinalis* L.) and lemon balm (*Melissa officinalis* L.). Many of these herbs are used to flavor certain alcoholic beverages, such as vermouth, arak and absinthe. Regarding the last named drink α-thujone was thought for decades to be the active ingredient responsible for this drink's notoriety between 1805 and 1915, when it was banned by the French wartime government. Absinthe was a green distilled drink, flavored with oil of wormwood, in which α- and β-thujone are present (Figure 5.11.1). It was popular and frequent overindulgence often led to various physical and psychological disorders, so that the drink became increasingly associated with fits and hallucinations, which contributed to a condition known as 'absinthism.' The supposed high thujone content of 'preban' (i.e. pre-1915) absinthe was for many years thought to be the cause of absinthism.

Recently, this supposition, based upon oft quoted data of unclear origin, has been found to be false (Lachenmeier *et al.*, 2008). Preban, as well as modern absinthe were both determined by precise and accurate methods to have an average thujone content of *ca.* 25 mg/l – about 10% of the claimed figure of 260 mg/l in preban absinthe. It therefore appears that the notoriety of preban absinthe is more likely to have been caused by ethanol and other components of the rough spirit that was used to make the beverage, combined with frequent overindulgence. Lachenmeier *et al.* (2009) substantiated their above claim by demonstrating the total stability of thujone (as well as fenchone and pinocamphone) in modern absinthe stored in green glass bottles (like preban absinthe) upon exposure to UV radiation over an extended period of time. Thus criticisms questioning their results, concerning decomposition of thujone in preban absinthe over the years (the last ones were produced in 1915), were refuted. It seems like old myths die hard.

Nevertheless, thujones (particularly the β-diastereoisomer) are neurotoxins for a number of animals, including insects, rats and worms. The mouse oral LD_{50} is 45 mg/kg body weight, but the presence of ethanol was found to protect some mice from otherwise lethal doses. There is one reported human poisoning by α-thujone in wormwood oil (Weisbord *et al.*, 1997). It was hypothesized in the 1970s that α-thujone activates the CB_1 cannabinoid receptors in the brain (del Castillo *et al.*, 1975), but this has been refuted by experimental evidence (Meschler and Howlett, 1999). Instead, it was found that α-thujone acts on the GABA receptors in the brain, thus modulating the GABA gated chloride channel (Höld *et al.*, 2000). Like other monoterpenoids, α-thujone is rapid acting and is easily detoxified (e.g. to hydroxy α-thujones, in the brain). In the EU, the maximum allowed levels of thujones in alcoholic beverages are 5 mg/kg for beverages of <25% ABV, 10 mg/kg for drinks of >25% ABV and 35 mg/kg for 'bitters,' such as Angostura bitters, that are used to make cocktails.

Hop bittering agents include α-acids (humulones) (Section 2.6.3) and xanthohumol. During the boiling stage of beer production humulones are isomerized to the more strongly bitter *iso*humulones (see Chapter 2.6, Figure 3.6.13) and xanthohumol is isomerized to *iso*xanthohumol, although the latter isomerization appears to be inhibited during boiling of stout worts, which have black or roast malt content (Biendl and Walker, 2004). Over the years, considerable evidence has accumulated concerning health benefits resulting from ingestion of these substances in hop products and of their isomerized equivalents in beer (Biendl and Walker, 2004, Biendl, 2007). These compounds have been shown to be estrogenic, anti-inflammatory and to have *in vitro* antimutagenic and carcinostatic properties. Thus, they may offer protection against osteoporosis (decreased bone mineral density), cardiovascular disease and cancer. Epidemiological studies have suggested a correlation between moderate consumption of alcoholic beverages and high bone mineral density and lower risk of cardiovascular disease (Section 5.6.3), so these phytochemicals could well make important contributions. Animal studies have so far confirmed their role as preventers of osteoporosis and 'metabolic syndrome' (one of whose ultimate results is cardiovascular disease) and more recently strongly hopped (freeze dried) beer were found to be more active than unhopped (freeze dried) beer in the prevention of intestinal cancers in rats (Nozawa *et al.*, 2005; Biendl, 2007).

Table 5.11.1 *Major natural nitrogenous allergenic substances in alcoholic beverages*

Allergenic compounds	Alcoholic beverage type	Typical allergic reactions	Comments
Biogenic amines: histamine, tyramine and others	Wine, especially red wine having undergone MLF	Migraine headache, hypertension.	In severe cases anaphylactic shock occurs. Ethanol enhances effect of allergens
Gliadin proteins (prolamins), notably glutens and hordeins	Beer, especially wheat beer	Coeliac disease: irritation of cells lining the stomach	Beers brewed from buckwheat, maize and rice are low in prolamins

5.11.3 Nitrogenous Allergenic Substances

In relation to foodstuffs and alcoholic beverages in particular, an allergenic substance is a component of a beverage that causes disturbing physiological reactions in certain individuals upon consumption of the beverage. The physiological ('allergic') reactions include headaches, hypertension and digestive disorders, and can be very serious for susceptible individuals. Allergenic substances include additives such as sulfites, benzoates and other preservatives (Section 5.10.2), and certain synthetic colorants (Section 5.10.4), although some of these additives are rarely or never used in alcoholic beverage production. Considered in more detail here are natural allergenic substances that are found in certain alcoholic drinks. The two major classes are biogenic amines and proteins, both nitrogenous organic derivatives of amino acids (Table 5.11.1). Biogenic amines are present in many alcoholic beverages in wide ranges of concentration, but most data available relates to beer and wine. The most biologically active (and hence most studied) amines are histamine, 2-phenylethylamine and tyramine, but agmatine, cadaverine, putrescine, spermidine and spermine are also found in alcoholic drinks (Figure 5.11.2) (Önal, 2007). They are produced by normal metabolic processes during yeast alcoholic fermentation and especially during the malolactic fermentation of wines. The monoamines are produced by decarboxylation of the corresponding α-amino acids (histidine-histamine; tyrosine-tyramine, etc.; see Figure 5.11.2), whereas the polyamines (spermidine and spermine) are formal by *de novo* synthesis from arginine, via putrescine (Figure 5.11.2) (Önal, 2007). The polyamines are involved in fruit growth and development, whereas other amines are involved in DNA, RNA and protein synthesis, where their physiological functions include cell membrane stabilization and cell proliferation regulation (Bardócz, 1995). Hence they can be found in fruit as well as wine (Esti *et al.*, 1998). Likewise histamine and other biogenic amines are formed in cereals during the malting process and hence are constituents of wort, as well as beer (Gasarasi *et al.*, 2003).

In wine production, the concentrations of biogenic amines are related to many factors, including grape variety, location and winemaking methods (especially with regard to the conduction of MLF) (Landete *et al.*, 2005; Hernández-Orte *et al.*, 2008). For example, red wine from Cabernet Sauvignon grapes had lower levels of histamine and tyramine than red Tempranillo wine, made in the same region (Huesca, Spain) under the same conditions (Hernández-Orte *et al.,* 2008). Red wines from Tempranillo grapes grown in different regions were found to contain 8.2, 2.4, and 4.5 mg/l of histamine, according to whether the wines originated in La Rioja, Utiel-Requena or Tarragona, respectively. Similarly, Tempranillo wine from these three regions had 1.9, 2.3 and 1.8 mg/l of tyramine, respectively (Landete *et al.*, 2005). Red wine samples produced in Sicily from indigenous varieties (e.g. Nero d'Avola, Aglianico), French varieties (Cabernet Sauvignon, Merlot, Pinot Noir) and one Spanish variety (Tempranillo) showed a wide variation in biogenic amine concentrations (Dugo *et al.*, 2006). In this case, the wines with highest histamine levels were made from strains of Pinot

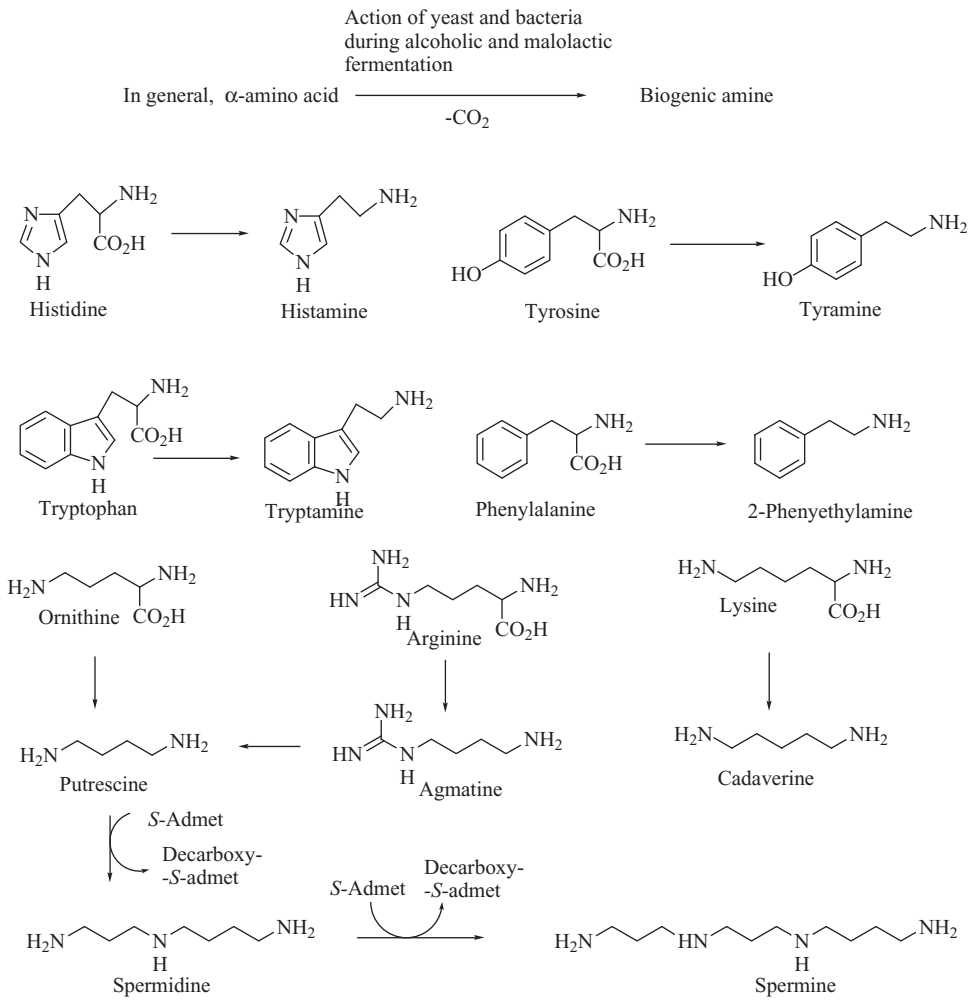

Figure 5.11.2 *Biosynthesis of biogenic amines.* S-*Admet* = S-*adenosylmethionine; decarboxy*-S-*admet* = *decarboxylated* S-*adenosylmethionine*

Noir, whilst Cabernet Sauvignon, Merlot and Tempranillo wines had no measurable histamine. In Turkish red wines (30 samples from different wine growing regions and different vine varieties), levels of histamine, 2-phenylethylamine, tryptamine and tyramine were 0–0.292 mg/l; 0–0.365 mg/l, 0–1.965 mg/l and 1.09–7.94 mg/l, respectively. It can be seen from the foregoing discussion that most of the factors that influence levels of biogenic amines in red wines, such as location, grape variety and certain winemaking techniques, cannot be modified as they are often laid down by law or are required for the production of quality wine. It does appear, however, that inoculation with strains of *Oenococcus oeni* to perform MLF leads to wines of lower biogenic amine levels than wines that have undergone MLF by indigenous bacteria, such as *Lactobacillus plantarum* (Hernández-Orte *et al.*, 2008; Landete *et al.*, 2005). The MLF bacterium species *O. oeni* exhibited lower amino acid decarboxylase (especially histidine carboxylase) activity than other MLF species, such as *L. hilgardii* and *Pediococcus parvulus* (Landete *et al.*, 2005).

In the production of beer, it has been demonstrated that typical beer worts already posses significant histamine and tyramine concentrations, which increase somewhat as a result of fermentation and standard processing (Gorinstein *et al.*, 1999). Histamine content of lager beers from Brazil, Israel and Mexico were between 3.02 and 3.23 mg/l, whilst tyramine levels were 3.61–7.4 mg/l.

Although severe allergic reactions to foods naturally rich in biogenic amines (some fish, meat, chocolate and fermented foods such as cheese and sauerkraut, as well as beer and wine) have been ascribed to the presence of those amines, there are contradictory conclusions in the literature. Histamine administered orally, by inhalation, or by subcutaneous or intravenous injection causes headaches and histamine free diets were found to reduce the incidence of headache (Steinbrecher and Jarisch, 2005). However, a meta-study of diet histamine and allergic reactions concluded that there was no correlation between oral ingestion of biogenic amines and food intolerance (Jansen *et al.*, 2003). Also, plasma histamine levels were observed to rise on consumption of wine, irrespective of the biogenic amine content of the wines (Kanny *et al.*, 2001). Nonetheless, histamine has been shown to be a vasodilator, whilst tyramine, tryptamine and 2-phenyletuylamine are vasoconstrictors, probably because they cause the release of noradrenahin and norephedrine. Histamine is released from mast cells as part of an IgE antibody dependent reaction, whereupon it is involved in inflammatory reactions. Cadaverine, putrescine and the polyamines appear to be nontoxic, although they may intensify the adverse effects of histamine, 2-phenylethylamine and tyramine, by interfering with the enzymes that metabolize them and by favoring their absorption in the intestine (Badócz, 1995).

Detoxification of amines occurs in the gut via the action of monoamine oxidases (MAOs) and diamine oxidases (DAOs). The amines are oxidized to physiologically less active degradation products. It is thought that the detoxification enzymes are unable to cope with a high intake of biogenic amines, which thus results in observed allergic reactions. Also, certain individuals are unable to metabolize even low levels of biogenic amines and so these compounds in general pose a serious threat to these individuals. The low amine oxidase activity of these individuals may arise from genetic predisposition, gastrointestinal disease or the presence of amine oxidase inhibitors, such as MAO inhibitor antidepressant drugs. In this respect, since ethanol is a mild MAO inhibitor, it is believed that ethanol may interact synergistically with biogenic amines in causing allergic reactions to alcoholic beverage in some individuals (Önal, 2008).

An alternative proposal, put forward by Kanny *et al.* (2001), was that biogenic amines in wine are not by themselves responsible for allergenic reactions. Instead, this group suggested that some other wine component, probably acetaldehyde, acts as an allergenic substance in itself and also as a plasma histamine releaser.

Gluten is the name given to the storage proteins of cereals; it is composed of albumins, globulins, prolamins and glutenins. Of these proteins, it is the prolamins that are of greatest interest here, because of their ability to cause severe allergic reactions in susceptible individuals. The prolamins are relatively rich in the α-amino acids proline, glutamine, asparagine and arginine, especially the first two. Prolamins from wheat are known as gliadins, and other cereals have related proteins: hordeins (barley), zeins (maize) avenins (oats) and secalins (rye). Gliadins are doughnut shaped glycoproteins that can be subdivided into three types: α, γ and ω. The α-types (formally called α, β) and γ-gliadins have molecular weights of 30 000–45 000 Da and have tertiary and higher structure involving disulfide formation between cysteine residues. The ω-gliadins have atomic weights of around 50 000 Da, with few cysteine residues. The gliadins are found in the endosperm of wheat seeds and pass into wort at the mashing stage of brewing (Section 2.6.2). The same applies the hordein proteins of barley. The presence of gliadins or hordeins in the diet can cause gluten sensitive enteropathy, the most severe form of which is celiac disease, caused by autoimmune reaction of the cells lining the small intestine. On the other hand, the presence of ω-gliadins in the diet can cause wheat dependent (WD) exercise induced anaphyloxis and WD urticaria (skin rashes) in sensitive individuals. Symptom types and severity vary between individuals, with about 1% of Indo-Europeans being affected, although the condition may be underdiagnosed.

During the brewing process, gliadins and hordeins extracted into wort from the malt or grains are largely removed on the grains after mashing (Section 2.6.2) and in the trub after boiling (Section 2.6.3). More will be removed by the action of fining agents, filtration or centrifugation (Section 2.6.9). The presence of celiac positive proteins in beer has not been proven conclusively (Hughes and Baxter, 2001), partly because of limitations in the analysis of beer proteins. The prolamin content of several beers was determined by sodium dodecylsulfate gel electrophoresis (SDS-PAGE) immunoblotting, as well as enzyme linked immunosorbent assay (ELISA). Wheat beers were included, but most of the beers were barley based, with varying amounts of maize or rice adjuncts. One beer, describing itself as 'gluten free' was brewed using buckwheat and rice, with no barley (Kanerva *et al.*, 2005). Proteins were found in the molecular weight ranges 8000–17 000 Da and 38 000 Da in reduced and nonreduced samples, respectively. ELISA results suggested much higher levels of prolamins in the wheat beers, although the ELISA kit antibody was designed to measure gliadins and not hordeins and so may not be well matched for determination of the latter.

5.11.4 Mycotoxins

Mycotoxins are secondary metabolites formed during the growth of various species of fungus on infected crops, such as cereals and fruit. There are many such toxins, which vary widely from species to species; the major ones found in alcoholic beverages made from infected crops are shown in Figure 5.11.3. The most serious mycotoxins are the aflatoxins, a family of structurally related compounds produced by *Aspergillus flavus* growing on crops in tropical climates. This fungus can infect a large variety of crops – from peanuts to maize – but needs a very warm moist climate to thrive. Hence it is not normally found on cereals growing in the temperate beer producing countries, like those of Northern and mid-Europe or the Northern and Mid-West states of the USA. However, there are legal controls in many countries to guard against the importation of infected produce. In the EU, the limit is 4 mg/kg for total aflatoxins and 2 mg/kg for the most toxic member of this family of mycotoxins: aflatoxin B1. This compound is a potent carcinogen, with a rat oral LD_5o value of 0.4 mg/kg body weight.

Aflatoxins are only infrequently found in wines from temperate zones. An investigation of 41 wines using an HPLC method (Section 4.3.3) that was sensitive to 0.02 mg/l of individual toxins, found aflatoxin B2a (one of the less toxic aflatoxins), at concentrations of 0.3 mg/l and 0.05 mg/l, in two wines (Takahashi *et al.*, 1997) An earlier study of 150 German wines, using a less sensitive thin layer chromatography (TLC) method (Section 4.3.1), revealed no aflatoxins (Lemperle *et al.*, 1975).

Alcoholic beverages thus provide an insignificant contribution to dietary intake of aflatoxins in temperate climate zones. In tropical (or at least warm, moist) climates, significant contamination of a wide variety of crops can occur, including those, like maize, that form the staple diet. Eating foodstuffs that are consistently infected with *A. flavus* or *A. parasiticus* has been correlated with incidence of primary liver cancer in several African locations (Sibanda *et al.*, 1997), although there was no link with alcoholic beverages. Aflatoxin M_1 have been found in the biological fluids of individuals in these locations. This substance intercalates into DNA and alkylates bases (notably guanosine) via its epoxide moiety. This is thought to cause p53 gene mutation, an important gene that prevents cell cycle progression following DNA mutation.

Ochratoxin a (OTA: 7-(L-β-phenylalanylcarbonyl) carboxy-5-chloro-8-hydroxy-3,4-dihydro-(3*R*)-methyl isocoumarin) (Figure 5.10.3) is a secondary metabolite produced by many species of fungus growing on cereal and fruit crops in temperate climates. Thus crops infected by *Pencillium verrucosum*, *Aspergillus ochraceus*, or *A. niger* can produce alcoholic beverages containing quantities of ochratoxin A, depending on the level of infection and the processing conditions. Although OTA is considered to be less toxic than, say, aflatoxin B1, it is classed as a possible carcinogen to humans (Group 2B) by the International Agency for Research on Cancer (IARC) (IARC, 1993); it has been shown to produce kidney and liver tumors in mice and rats (JECFA, 2001d).

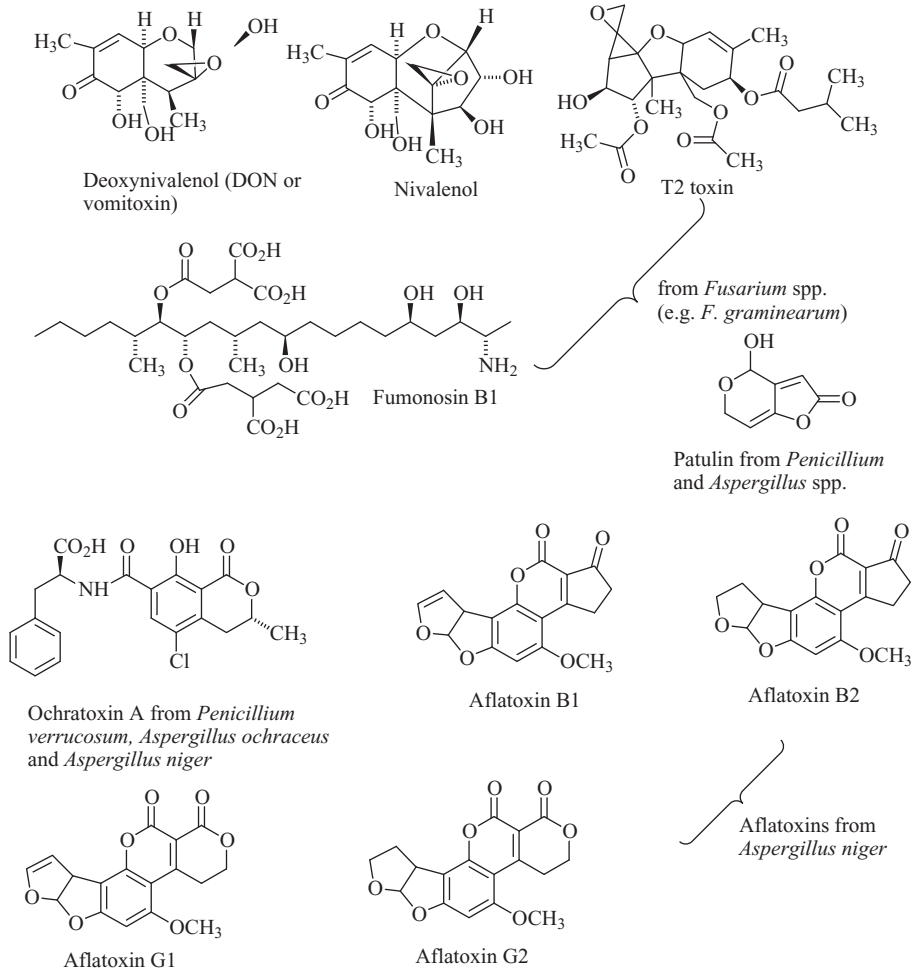

Figure 5.11.3 *Some mycotoxins sometimes found in alcoholic beverages.*

The panel on Contaminants in the Food Chain (CONTAM) of the European Food Safety Authority (EFSA, 2006) has suggested a provisional tolerable weekly intake of OTA of no more than 120 mg (0.12 mg) per kg of body weight. Regulatory limits for OTA content of beverages are in force in many countries; the EU has set a maximum allowable concentration of 2.0 mg/l in wine (EC, 2005). Likewise the EU has set maximum levels for OTA in cereal crops at 3 mg/kg. Wine is acknowledged to be a major dietary source of OTA in Europe (ECSCOOP, 2002), although most wines have been found to have levels of OTA well below the maximum allowed level of 2.0 mg/l (Bellí *et al.*, 2004; Aresta *et al.*, 2006; Mateo *et al.*, 2007 Valero *et al.*, 2008).

In general, wines from warmer climatic zones are higher in OTA content than those from cooler areas, and red wines have higher contents than rosé or white wines Bellí *et al.*, 2004; Mateo *et al.*, 2007). Interestingly, the levels of OTA in wine seem to have a definite dependence on specific production processes, especially with regard to dessert wines (Valero *et al.*, 2008). Wines made using fortified must (giving early stopped fermentation, such as Muscat VDN – see Section 2.10.9), those made from sun dried grapes (such as certain

Sherry and Montilla wines – see Section 2.10.2) and those made from fresh chamber dried grapes (such as Amerone) tend to have higher OTA contents. On the other hand, wines made using flor (e.g. fino Sherry – Section 2.10.3), fortified wines having had long ageing in cask (e.g. Madeira, Port, Sherry – Sections 2.10.5, 2.10.7 and 2.10.2, respectively), Marsala (Section 2.10.8), wines made with warm chamber-dried grapes (e.g. vin santo) and dessert wines made from botrytized (nobly rotted) grapes or late harvested grapes tend to have lower OTA contents (Valero *et al*., 2008).

Harvesting grapes with minimal damage, cool storage, rapid processing and sanitary winery conditions all help to minimize postharvest OTA contamination (Hocking *et al.*, 2007). Flash pasteurization of the must or addition of bisulfite prevents fungal growth and OTA formation (Roset, 2003). Up to 80% of OTA is lost on separation of the wine or juice from the skins (Fernandes *et al.*, 2007). However ochratoxin A is not metabolized by yeast, but some can be lost, via adsorption on yeast cells during fermentation, by racking the wine off the lees (Caridi, 2006). The action of malolactic bacteria, the use of fining agents and storage for over 10 months may also help to reduce the OTA content of wine (Fumi *et al.*, 2005; Leong *et al.*, 2007). In the vineyard prior to harvest, spraying with fungicides minimizes the growth of *Penicillium* and *Aspergillus* species.

Beer appears to be less affected by OTA residues, probably because the conditions under which barley and other cereals are stored and malted are unsuitable for the growth of *Penicillium* and *Aspergillus* species (Hughes and Baxter, 2001). For example, barley seeds for malting are dried to 12–14% moisture content and stored at low temperature until required. After malting, the moisture content of the grains is usually closer to 5%. It is known that the above fungi require a moisture content of at least 16% at ambient temperatures (say, *ca.* 20 °C) to grow on cereal grains. Nevertheless, recent investigations have shown that a high percentage of beers contain OTA, although often at levels below 0.2 mg/l; very much below the EFSA limit of 120 mg/kg of body weight/week (Mateo *et al.*, 2008), which means that beer is not a relevant contributor of dietary OTA. On the other hand, some of the indigenous brews of Africa (Section 2.7.2) have been found to possess in excess of 3 μg/l of OTA, with one having 2.3 mg/l – possibly the highest ever reported OTA content for a grain based alcoholic beverage (Odhav and Naicker, 2002). See also the review of Sibanda *et al.* (1997).

Of rather greater concern to maltsters and brewers is infection of grains by *Fusarium* species (eg. *F. graminearum*). These fungi, which are responsible for the scab (ear rot or Fusarium head blight) disease of cereal crops, produce a number of secondary metabolites known as trichothecenes, which includes deoxynivalenol (DON or vomitoxin), nivalenol and T-2 toxin (Figure 5.11.3). *F. graminearum*, along with *F. culmorum*, are important in temperate zones; they both produce trichothecenes. *F. arenaceum* is more common in Scandinavia and produces different metabolites (e.g. enniatins) (Uhlig *et al*., 2007).

The estrogenic (endocrine disruptor) zearalenone is also produced by *F. graminearum* on infected crops, although DON appears to be the mycotoxin produced in greatest quantity in scab-infected grain (Wolf-Hall, 2007). This compound seems to be very stable thoughout the normal brewing process, up to 93% remaining in the final beers (Schwarz *et al.*, 1996). Other *Fusarium* mycotoxins, such as zearalenone, were not detected in the final beer. Apart from its toxicity, DON in beer is undesirable; its presence has been correlated with 'gushing' – an overproduction of foam when the container of beer is opened (Schwarz *et al.*, 1996).

The most effective method to limit the occurrence of *Fusarium* mycotoxins in beer is to avoid the use of scab infected grains. Maltsters will refuse supplies of infected grains from growers, and malting companies in the USA have set an upper limit of 0.5 mg/kg in purchased grain. However, avoidance is not always feasible, and so with the present ineffective control of *Fusarium* head blight in the field, there are various ways of using part of the crop while keeping mycotoxin contamination to a minimum. These include separation of infected grains (e.g. the shriveled 'tombstone' grains of wheat), decontamination and prevention of further fungal growth (Wolf-Hall, 2007). It seems likely that beer provides an insignificant contribution of dietary

DON, since a survey of work carried out on mycotoxin contents of commercial beers indicated that these are found largely at trace (μg/l) levels (Scott, 1996).

Patulin (4-hydoxy-4H-furo-(3,2c)-pyran-2-(6H)-one (PAT) (Figure 5.11.3) is a mycotoxin produced by *Penicillium expansum* growing on infected fruit and cereals. Apples are particularly susceptible to infection, so that apple juice and products such as cider may become contaminated by patulin. The substance is genotoxic, although carcinogeneticity studies on animals have proved inconclusive. The World Health Organization (WHO) have recommended a maximum allowed level of 59 mg/l for apple juice, and in the EU the maximum allowed level for patulin in foods containing or derived from apples (including ciders) has been fixed at 50 mg/kg. Contaminated apple juice can be detoxified in several ways, including heat treatment (not fully effective), the addition of sulfites or ascorbates and treatment with ozone gas or γ-irradiation. It was found that just 100 ppm of SO_2 was sufficient to reduce the PAT content of grape juice to 50% in 15 minutes (Ough and Corison, 1980). In a model solution of pH 6 held at 100 °C for 1 h, 50% of the patulin content was degraded, the main degradation product being 3-keto-5-hydroxypentanal (Collin *et al.*, 2008).

Avoidance of contamination of cider by patulin residues can be effected by the rejection of moldy apples; indeed the patulin content of apple must can be used as an indicator of fruit quality. Such control is not possible when using apple juice concentrate (AJC), although analysis of samples of imported AJC should reveal levels of patulin below regulatory limits. Although some of the detoxification methods mentioned previously could result in negative organoleptic changes, it appears that PAT can be totally removed from apple juice within three weeks, by the addition of ascorbate with no significant effect on organoleptic quality (Lai *et al.*, 2000).

Considering the precautions and treatments that are available, it is not surprising that patulin levels are consistently low in commercial ciders.

5.11.5 Other Trace Substances

Ethyl carbamate (EC) and *N*-nitrosamines are the most important trace substances in alcoholic beverages that arise from natural processes during the manufacturing procedure.

Ethyl carbamate is a noted carcinogen. The Joint Expert Committee on Food Additives (JECFA) of FAO/WHO has advised that although intake of EC was generally of low concern, there is a definite need to continue to seek ways of lowering the levels of this compound in certain alcoholic beverages (JECFA, 2005). Few countries have yet set acceptable limits, but in Canada these are 30 μg/l (table wines); 100 μg/l (fortified wines); 150 μg/l (distilled spirits); 200 μg/l (sake) and 400 μg/l (fruit spirits and liqueurs).

Ethyl carbamate is a byproduct of fermentation and is found in many drinks, notably distilled spirits, stone fruit spirits and liqueurs, Asian rice wines and wine. It is formed in two major ways:

- Reaction of nitrogenous compounds (e.g. citrulline or urea) with ethanol
- Reaction of cyanide with ethanol.

Nitrogenous compounds – amines, amino acids and ammonia – are found in both must and fermented liquors. Some yeasts have the ability to metabolize certain of these compounds to urea during fermentation and certain lactic acid bacteria are able to metabolize arginine and glutamine to citrulline and carbamyl phosphate, two possible precursors of EC (Section 2.3.9).

Reaction between urea and ethanol, especially at elevated temperatures, produces small quantities of ethyl carbamate (Figure 5.11.4). High temperatures are used in the processing of various alcoholic beverages, notably distillation to produce spirits (some of which is used to make fortified wines) and various wine

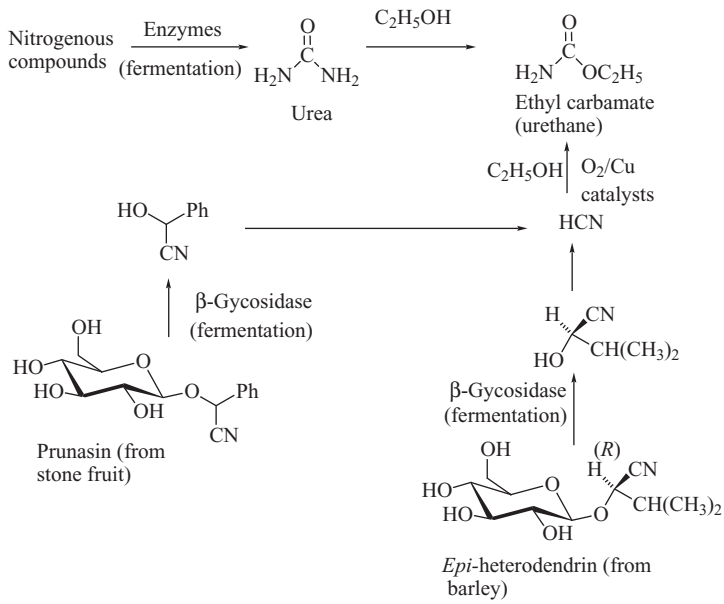

Figure 5.11.4 *Pathways for the formation of ethyl carbamate during the production of alcoholic beverages*

baking or grape drying processes used to make fortified wines such as Madeira (Section 2.10.6) and Sherry (Section 2.10.2).

Hydrogen cyanide is produced by the hydrolysis of natural cyanogenic glycosides during alcoholic fermentation (Figure 5.11.4). These glycosides are found in most barley varieties and are released into the wort on mashing of the malted barley grains in the production of beer and whisky (Swanston, 1999). In the brewing of beer these glycosides are hydrolyzed and the small amounts of hydrogen cyanide are driven off during the boiling stage (Section 2.6.3). However, in the production of whisky there in no boiling stage (Section 3.2.2) and so the glycosides survive into the fermentation stage, where they are hydrolyzed by yeast β-glucosidases to a heat labile product. This product is hydrolyzed to HCN during distillation, which then produces ethyl carbamate by reaction with ethanol (in the presence of O_2 and Cu^{2+}) during distillation. Ethyl carbamate is also formed from cyanide precursors in the kernels of stone fruit during the production of fruit brandies and liqueurs (Figure 5.11.4).

A survey of EC in alcoholic beverages sold in the UK during 2004, (Hasnip *et al.*, 2007), it was found that spirits, fruit brandies and fortified wines had the highest concentrations, wine had intermediate levels and beer (as well as the single cider sample) had none (i.e. in this study <10 μg/kg). Two sake (Section 2.7.1) samples had high levels of ethyl carbamate, and although the data was from just two samples it was in agreement with earlier work (Kitamoto *et al.*, 1991). Ethyl carbamate concentrations of all the wines that proved to be positive were quite close to the lowest quantifiable level of 10 μg/kg, with the highest concentration being 24 mg/kg. An earlier survey in Canada showed 74 wines contained up to 84 mg/kg and 155 stone fruit spirits had up to 4330 mg/kg of EC (Zimmerli and Schlatter, 1991). A survey of Korean liquors indicated very low levels of ethyl carbamate (close to the limit of detection) in fermented beverages and rather higher, though modest levels (up to 15.4 mg/kg) for indigenous distilled beverages (soju) (Section 3.4.4) (Ha *et al.*, 2006). Although concentrations of ethyl carbamate in alcoholic drinks appear to be erratic, the big majority of cases possess levels below those generally regarded as acceptable limits. Nevertheless, as JECFA (2005)

suggested, there is still a potential for reduction of levels via changes in production methodology. The use of genetically modified yeasts that do not produce urea during fermentation may be of particular value, provided that the organoleptic qualities of the beverage are not impaired. Sake fermented by such yeasts does not have quantifiable levels of ethyl carbamate (Kitamoto *et al.*, 1991). In the production of stone fruit spirits (Sections 3.8.3 and 3.8.4) or liqueurs (Section 3.9.2), rejection of some or all of the kernels may give beverages with lower ethyl carbamate levels, although this may also give altered flavor profiles. In the production of whisky, selection of barley varieties that do not produce *epi*-heterodendrin during malting is highly desirable. A gene marker for non-*epi*-heterodendrin producing varieties of barley was described by Swanston *et al.* (1999), which has been used by some barley breeders to develop recombinants, such as Decanter. Application was limited, however, because the marker (Bmac213) was some distance from the gene. More recently a direct gene marker that can reliably identify nonproducers of *epi*-heterodendrin has been developed by Hedley's group at the Scottish Crop Research Institute (Rae *et al.*, 2006). Aspects of this are discussed further in Section 3.2.2.

N-nitrosamines, sometimes present in the environment and in a wide range of foodstuffs, are known to be carcinogenic, mutagenic and teratogenic in a substantial number of cases. Of particular interest are the volatile nitrosamines, especially *N*-nitrosodimethylamine (NDMA), shown in Figure 5.11.5, as most of them are highly carcinogenic. As with ethyl carbamate, not all countries have set regulatory limits on the levels of *N*-nitrosamines in foodstuffs and present regulatory limits vary widely. The maximum level of total *N*-nitrosamines in beers in set at 5 mg/kg in the USA, 0.5 mg/kg in the EU, 0.5 mg/kg in Switzerland and 15 mg/kg in Russia (Sung, 2004). The carcinogenic potency of these volatile *N*-nitroso compounds lies in their ability to provide strongly electrophilic alkylating agents in a biological environment. These alkylating agents react with nucleophilic sites on nucleic acid bases and proteins, thus leading eventually to mutations and ultimately to carcinogenesis.

Surveys of the *N*-nitrosamine content of alcoholic drinks have generally revealed that only certain beers and whiskies contain one or more of these compounds at quantifiable levels (Jurado-Sánchez *et al.*, 2007), where

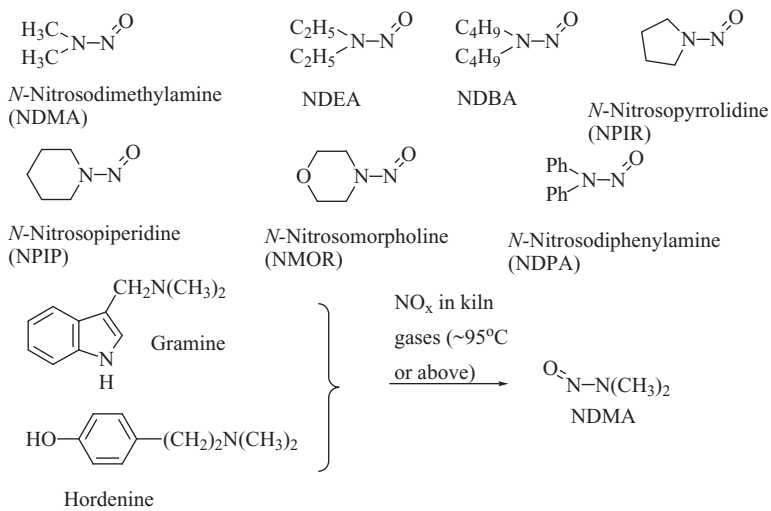

Figure 5.11.5 *Some volatile* N*-nitrosamines sometimes found in alcoholic beverages, and showing the formation of NDMA from NOx and barley amines*

NDMA and *N*-nitrosopiperidine appear to be the most common. In this recent survey, the total *N*-nitrosamine contents of beers (including nonalcoholic beers) were all below 1 μg/kg.

The major route to *N*-nitroso compounds such as NDMA in beer is via reaction of oxides of nitrogen (NO_x) and amines in the malted barley grains (Figure 5.11.5). This occurs during kilning through contact between the kiln gases and grains. In modern malting plants the kiln is fired externally and hence kiln gases do not come into direct contact with barley grains. Consequently, over the years there has been a general drop in the levels of NDMA and other nitrosamines in beer (Sen *et al.*, 1996).

Other routes to *N*-nitrosamines in foodstuffs involve the presence of nitrite salts (Sung, 2004). As far as alcoholic beverages are concerned, nitrite ions are most likely to originate in water used during the production process. There are strict regulatory limits on the nitrite content of water destined for human consumption (Section 5.10.4), but nitrite salts, originating from extensive use of nitrogen fertilizers in agriculture, are present in many public and private water supplies. Hops can also be a source of small amounts of nitrite salts in beer wort (Hughes and Baxter, 2001). In the brewery, continued use of a yeast strain can sometimes lead to bacterial infection. Some spoilage bacteria, notably *Obesumbacteria proteum*, can reduce nitrate to nitrite during the fermentation process. In the acidic fermenting wort, nitrite can react with secondary amines to from *N*-nitrosamines. These *N*-nitroso compounds are not necessarily volatile (like NDMA) and they have not been so extensively characterized as their volatile cousins: they are known generally as ATNC or apparent total *N*-nitroso compounds (Hughes and Baxter, 2001).

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Appendix 1

Units of Measurement and Interconversions

Note that the text uses SI (metric) units, with occasional inclusion of the corresponding US or British units. Nevertheless, readers may find the following SI–US–British unit conversion factors useful.

Units of Length

SI: 100 cm = 1 m; 1000 m = 1 km
US/British: 12 in = 1 ft; 3 ft = 1 yd; 1760 yd = 1 mile

Interconversions

1 cm = 0.3937 in (1 in = 2.540 cm)
1 m = 39.370 in = 3 ft 3.370 in
1 km = 1093.6 yd = 0.62137 mile (1 mile = 1.6093 km)

Square Measure

SI: 10 000 m^2 = 1 ha; 100 ha = 1 km^2
US/British: 1 acre = 4863 yd^2 = 0.001570 $mile^2$

Interconversions

1 ha = 2.471 acres (1 acre = 0.4047 ha)

Cubic Measure

SI: 1000 cm^3 = 1 dm^3; 1000 dm^3 = 1 m^3
US/British: 1728 in^3 = 1 ft^3; 27 ft^3 = 1 yd^3

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Interconversions

1 $cm^3 = 0.061025\ in^3$
1 $dm^3 = 0.035315\ ft^3$
1 $m^3 = 1.30795\ yd^3 = 61\ 024\ in^3$

Weights

SI: 1000 g = 1 kg; 1000 kg = 1 tonne
US/British: 16 oz = 1 lb; 2240 lb = 1 ton

Interconversions

1 g = 0.035274 oz (1 oz = 28.350 g)
1 kg = 35.274 oz = 2.203 lb (1 lb = 0.4539 kg)
1 tonne = 0.984 ton = 2204 lb

Capacity or Volume

SI: 10 ml = 1 cl; 100 cl = 1 l; 100 l = 1 hl
US/British: 8 pints = 1 gallon
US: 16 fl oz = 1 pint; British: 20 fl oz = 1 pint

Interconversions

1 l = 2.113 US pints = 1.760 British pints = 0.2641 US gall = 0.2200 British gall = 33.81 US fl oz = 35.20 British fl oz
1 hl = 26.41 US gall = 22.00 British gall
1 US gall = 3.785 l; 1 British gall = 4.546 l
1 US gall = 0.8326 British gall (1 British gall = 1.201 US gall)
1 US fl oz = 1.041 British fl oz (1 British fl oz = 0.9606 US fl oz)

Energy

The **SI unit** is the joule (J) (kg m^2/s^2).

1 cal = 4.184 J

Note: 1 Cal = 1000 cal or 1 kcal.

Appendix 2

Table 1 *Comparison table of hydrometer scales for beer wort or fruit juice*

Specific gravity at 15 °C	°Oechsle	°Brix (Balling, Plato)	°Baumé	%Sugar content (w:v)	Potential %ethanol (v:v)
1.000	0	0.0	0.00	0.0	0.0
1.004	4	1.0	0.55	0.7	0.03
1.010	10	2.5	1.4	1.6	0.08
1.020	20	5.0	2.8	2.3	1.4
1.024	24	6.0	3.3	3.2	1.9
1.028	28	7.0	3.9	4.5	2.6
1.032	32	8.0	4.4	5.4	3.2
1.036	36	9.0	5.0	6.6	3.9
1.040	40	10.0	5.6	7.8	4.6
1.044	44	11.0	6.1	8.7	5.1
1.048	48	12.0	6.7	9.5	5.6
1.050	50	12.4	6.9	10.3	6.0
1.053	53	12.9	7.2	11.3	6.5
1.055	55	13.5	7.5	11.6	6.8
1.057	57	14.0	7.8	12.2	7.2
1.060	60	14.6	8.1	13.0	7.6
1.063	63	15.3	8.5	13.8	8.1
1.065	65	15.8	8.8	14.3	8.4
1.067	67	16.2	9.0	14.8	8.7
1.070	70	16.9	9.4	15.6	9.2
1.073	73	17.6	9.8	16.4	9.6
1.075	75	18.0	10.0	17.0	10.0
1.078	78	18.7	10.4	17.8	10.5
1.080	80	19.2	10.7	18.3	10.8
1.083	83	19.8	11.0	19.1	11.2
1.085	85	20.4	11.3	19.6	11.5

(*Continued*)

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Table 1 (*Continued*)

Specific gravity at 15 °C	°Oechsle	°Brix (Balling, Plato)	°Baumé	%Sugar content (w:v)	Potential %ethanol (v:v)
1.088	88	20.8	11.6	20.4	12.0
1.091	91	21.6	12.0	21.2	12.5
1.093	93	22.1	12.3	21.8	12.8
1.095	95	22.5	12.6	22.3	13.1
1.098	98	23.4	13.0	23.1	13.6
1.100	100	23.8	13.2	23.7	13.9
1.105	105	24.8	13.8	25.0	14.6
1.110	110	26.0	14.45	26.4	15.4
1.115	115	27.0	15.0	27.7	16.1
1.119	119	27.8	15.45	29.0	16.8
1.129	129	30.0	16.6	31.7	18.3

Table 2 *Temperature correction table for must or cooled wort specific gravity measurement*

Temperature/°C	Correction	Temperature/°C	Correction
10.0	−0.6	21.0	+1.1
11.0	−0.5	22.0	+1.3
12.0	−0.4	23.0	+1.6
13.0	−0.3	24.0	+1.8
14.0	−0.2	25.0	+2.0
15.0	0	26.0	+2.3
16.0	+0.1	27.0	+2.6
17.0	+0.3	28.0	+2.8
18.0	+0.5	29.0	+3.1
19.0	+0.7	30.0	+3.4
20.0	+0.9		

Approximate Relationships

°Brix, Balling or Plato = 1.8 × °Baumé
% sugar content (v:v) = (°Oechsle/4) – 2.5
% alcohol (v:v) = °Balling (°Brix or °Plato) × 0.55

Appendix 3

Pressure and Temperature Unit Conversions, and Numerical Values of Constants

Pressure Units

The official (SI) unit of pressure is the pascal (Pa); 1 Pa = 1 N (newton)/m^2 = 1 kg/ms^2

1 bar = 10^6 dyn/cm^2 = 10^5 Pa
1 atmosphere (atm) = 1.01325×10^5 Pa = 760 mm Hg
1 Torr = 1 mm Hg = 1.333×10^{-3} bar = 133.3 Pa = 0.001316 atm
1 psi (pound per square inch) = 0.07 atm (1 atm = 14.97 psi)

Temperature Units

The SI unit of temperature is the Kelvin (K), but °C (Celsius) is in common use, and in the USA °F (Fahrenheit) is often used.

°C = K − 273.15
°C = 0.5556 (°F – 32) (°F = 9/5 °C + 32)

Important Constants

The gas constant $R = 0.0802$ l atm/K mol or 8.314 J/K mol or 1.987 cal/K mol
Planck's constant $h = 6.626 \times 10^{-34}$ J s
Speed of light $c = 2.998 \times 10^8$ m/s

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Glossary

A

Acescence. Sourness, probably due to the presence of extraneous acetic acid.

ADY. Active dried yeast for winemaking, brewing and cider production.

Etiological. Scientific description of cause of disease.

Alembic (Alambic). A kind of pot still with a bulbous lower part for heating the liquor, and a well-fitting upper head with a long tube for condensing and collecting the distillate. Nowadays, the name is sometimes used loosely to indicate a pot still.

Alquitara. A type of ***alembic*** still (see above) used to distil wine pomace or wine, especially in the northern provinces of Spain.

Amylopectin. A highly branched polymer of α-D-glucose, found as a food reserve in seeds and plants. It is one of the two components of starch, the other being the linear polymer amylose.

ANOVA. Analysis of variance; statistical methods in which observed variance is divided into components due to different sources of variation.

Apéritif. Generally, any alcoholic beverage that is taken before a meal. It can be dry (e.g. Champagne or fino Sherry) or sweet (e.g. ruby Port). More specifically, the term refers to a range of flavored, fortified alcoholic drinks often of ethanol content close to 20% (v:v), such as Dubonnet, Lillet, vermouth and many others. See also ***bitters***.

ApoE knockout mouse. Mutant strain of mouse lacking the gene for synthesis of ApoE, an ***apolipoprotein*** that plays an essential role in the transport and catabolism of triglyceride esters. ApoE knock-out mice suffer from ***atherosclerosis*** from an early age and are often used to study that condition.

Apolipoprotein. A lipoprotein without its fatty inner part (e.g. cholesterol, cholesteryl esters or triglyceride esters, enveloped in a phospholipid monolayer). The synthesis of apolipoproteins in vertebrates is essential for the transport of lipids between organs via the blood stream. See also HDL and LDL.

Apoptosis. The process of programmed cell death, apoptosis is essential for the wellbeing of multicellular organisms. Insufficient apoptosis is a contributing factor to cancer and excessive apoptosis causes atrophy (tissue wasting), such as ***ischaemic*** damage. Apoptosis is controlled by many cell signalling processes, involving many proteins, including caspases, which eventually bring about destruction of the cell into membrane bound fragments that are eliminated by shedding or by ingestion by ***phagocytes***.

Artificial neural networks (ANNs). ANNs are computational models that simulate structural and functional aspects of biological neural networks. Generically, the method is a nonlinear statistical data modeling tool for investigating complex relationships between input (x) and output ($f(x)$) or to find patterns in data. It does this by imitating a neural network, with nodes or axons, as shown below, where $f(x)$ is a component of other functions, $g_i(x)$, which in turn are defined as components of other functions, $h_i(x)$.

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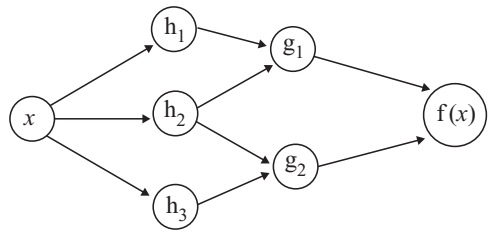


Atherosclerosis. Sometimes called arteriosclerotic vascular disease, atherosclerosis refers to hardening of the arteries caused by the formation of atherotomatous plaque. This is produced initially by immunoresponsive reply to damage to the arterial walls caused by oxidized ***LDL***. White blood cells (***macrophages*** and T-***lymphocytes***) are unable to fully destroy the oxidized LDL particles, leading to a gradual build up of plaque (consisting of oxidized LDL, cholesterol, foam cells and other fatty deposits, with concurrent increase in inflammation. The condition is compounded by inhibition of ***HDL*** to collect cholesteryl esters and transport them to the liver for processing. In an inflamed artery, muscle cells enlarge and form a fibrous layer over the inflamed area, thus constricting the artery and giving rise to increased blood pressure. The plaque is often unstable, with ruptures resulting in complete blockage, thereby denying blood (and hence oxygen) supply to organs, such as the heart or brain.

Autolysis. Self-digestion of dead or dying cells by the action of their own enzymes, released by the lysosomes. In yeast alcoholic fermentation, yeast cell death occurs when nutrients are short and when the alcohol level is high (∼15% ABV, but higher or lower for some strains). Autolysis releases polysaccharides, proteins and amino acids into the beverage, if 'lees contact' time is prolonged (i.e. with no racking or filtration), which can be detrimental (as in the production of many beers and wines) or beneficial (as in Champagne production).

B

Bacteriophage. A virus that selectively infects bacteria (such as lactic acid bacteria), sometimes causing loss of malolactic activity.

Barrel. This term is often used loosely to describe a cask. More strictly, it refers to a cask of 36 Imperial gallons (∼160 l), once a standard cask size for cask-conditioned beer. Other cask sizes related to this are kilderkin (18 Imp. gall.), firkin (9 Imp. gall.) and pin (4.5 Imp. gall.).

Bilirubin. Sometimes known as hematoidin, bilirubin is a normal yellow breakdown product of heme catabolism. It is a potent *in vitro* antioxidant and is probably a major cellular antioxidant.

Bitters. Liquors, usually of alcoholic strength greater than 20% (v:v) and flavored with various bittering botanicals, such as cinchona bark, gentian or wormwood. They are used to make a wide range of cocktails, but some can be used as apéritifs or digestifs. Examples include Abbot's, Amer Picon, Angostura Bitters (containing essence of gentian), Boonekamp, Campari, Fernet Branca, Law's, Orange Bitters, Pommeranzen, Secrestat, Toni Kola, Underberg, Unicum and Welling's.

Botrytis cinerea. A fungus that belongs to the *Ascomycota* phylum and is characterized by hyaline conida (asexual spores) carried on grey tree-like conidiophores. It infects a variety of fruiting plants causing diseases such as ***grey mold*** with resultant loss of quality and yield. However, infection of fully ripe white grapes is usually beneficial, causing ***noble rot*** (pourriture noble, Edelfäule) and producing some of the greatest sweet wines of the world.

Brij 35. A surfactant, polyoxyethylene[23]dodecanol, used to form micelles in micellar liquid chromatography, thus enhancing the selectivity of the method.

Butt. A size of wine cask. It is equivalent to two hogsheads (= 108 Imperial gallons, or 480 l). It is also known as a 'pipe.'

C

cfu. Colony forming unit is an estimate of the number of viable microorganisms (bacteria or fungi) or basal cells. It is usually quoted as CFU/ml or CFU/g.

C-reactive protein. A member of the pentraxin family of proteins, which are involved in lipid metabolism. C-reactive protein is an acute phase reactant – its concentration (e.g. in blood) rises dramatically during inflammatory processes – hence it is used as a marker of inflammation (e.g. of arterial walls).

Calcofluor White. A tissue stain reagent used to produce fluorescence (green or bluish) in the presence of viable fungal cells, upon receiving incident light of the correct wavelength (440 nm). It binds to the cellulose and chitin on the cell walls. It is the disodium salt of 4,4′-bis-[4-anilino-bis-diethylamino-*s*-triazin-2-ylamino]-2,2-stilbene disulfonic acid (CAS registry number: 4404-43-7).

Canonical variation analysis (CVA). Statistical method for analysis of group structure in multivariate analysis. Sometimes known as canonical discrimination analysis (CDA), it is a one way multivariate analysis of variance, often used to study change of variables with time.

Cardiomegaly. Enlargement of the heart. The pathological form of cardiomegaly is caused by response to stress or disease. The increase in heart weight is the result of increased collagen content, which instead of contributing to the pumping rate, can lead to dysfunction and failure.

Cardiomyopathy. Relates to the deterioration of the myocardium (heart muscle) function, which is often caused by ischaemia (restriction of blood supply), caused either by local thickening of arterial walls (vasoconstriction) or via local blockage by a blood clot (thrombosis) or via blockage by a blood clot transported from elsewhere (embolism).

Catabolism. The oxidative degradation of nutrient molecules (carbohydrates, fats and proteins) obtained from the environment or from cellular reserves. Catabolic pathways are generally overall energy yielding.

Chemiosmotic. Pertaining to chemiosmosis – diffusion of ions across a selectively permeable membrane. For example, chemiosmosis of H^+ is important in the production of ATP by ATP synthase in ***mitochondria*** or plasmids, during respiration.

Chronopotentiometry. An electrochemical method, by which the rate of change of electrical potential (at constant current) is measured versus time.

Cocktail. A mixed drink, dating from the early nineteenth century, usually made with a distilled spirits base (e.g. brandy, cachaça, gin, rum, tequila, vodka and whisk(e)y and other ingredients, frequently fruit juices, but also sometimes ***bitters***, fruit, honey, liqueur, milk (or cream) or spices. Some cocktails are based on beer or sparkling wine (e.g. Buck's Fizz). There are many cocktails, and although each has a specific name, flavor can vary considerably due to differences in composition and method of preparation. Cocktails are usually made to order at a bar, but particularly over the past few decades, many have become available as ready to drink items, but even these can be further mixed (e.g. Pimm's No. 1 cup). Cocktails are usually served cold or with ice, and with a slice of lemon or lime, or an olive or Maraschino cherry as garnish.

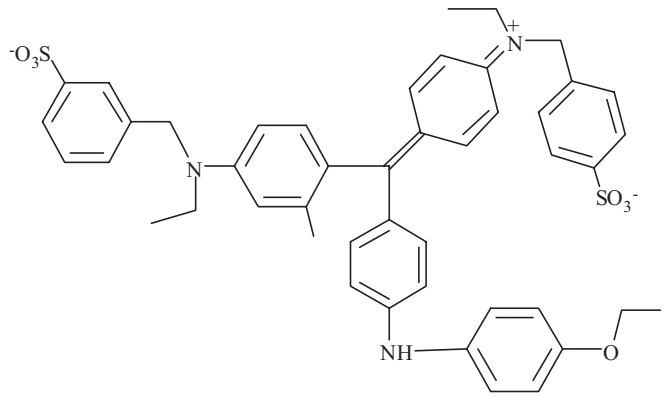
The following table gives a short list of cocktails with a summary of ingredients. They are grouped according to the major distilled spirit base.

Cocktail base	Examples
Brandy	Brandy Manhattan (as Manhattan, but with brandy in place of rye whiskey), Brandy Sour (brandy, curaçao, sugar, lemon juice and soda), Pisco Sour (Pisco, syrup, lime, egg white, bitters)
Cachaça	Batida (cachaça, fruit juice, sugar), Caipirinha (cachaça, lime, sugar)
Gin	Martini (gin, dry vermouth), Monkey Gland (gin, orange juice, absinthe, grenadine), Pall Mall (gin, dry and sweet vermouth, white crème de cacao), Pink Gin (gin, Angostura Bitters), Vesper (gin, vodka, Lillet Blanc)
Rum	Bacardi (white rum, lemon or lime, grenadine), Daiquiri (as Bacardi, except with gomme syrup – sugar, water, gum arabic – in place of grenadine), Piña colada (white rum, crème de cacao, pineapple juice), Planter's Punch (dark rum, fruit juices, soda, curaçao, Angostura Bitters)
Tequila	Margarita (tequila, Cointreau, lemon or lime juice), Tequila Sunrise (tequila, orange juice, grenadine)
Vodka	Bloody Mary (vodka, tomato juice, lemon juice), Harvey Wallbanger (vodka, Galliano, orange juice), Screwdriver (vodka, orange juice), Vodka Martini (as Martini, but with vodka in place of gin).
Whisk(e)y	John Collins (Bourbon, lemon juice, sugar syrup, soda), Manhattan (rye or Canadian whisky, red vermouth, bitters), Mint Julep (Bourbon, mint leaves, sugar), Rob Roy (Scotch, sweet vermouth, Angostura Bitters), Sazerac (Rye whiskey, Bitters, sugar, absinthe)

Celiac disease. This is an autoimmune disorder of the small intestine that occurs in genetically predisposed persons in response to the presence of ***prolamins*** in the diet.

Conchae. Organ structures that resemble a shell in shape. Nasal conchae provide humidity for the proper functioning of the olfactory epithelium.

Coomassie Brilliant Blue. A Coomassie type dyestuff used to stain (visualize) proteins in SDS-PAGE analysis. It interacts with proteins via ionic and ***van der Waals forces***. The structure of Coomassie Brilliant Blue G-250 is shown below.



CAS registry number: 6104-58-1.

Copigmentation. Interaction of pigment molecule (e.g. an anthocyanin or derivative) with a cofactor (e.g. a polyphenol) via **π-π *attractions***, causing stacking of molecules. The color of black grapes, red wine must

and new red wine is due largely to copigmentation, which decreases during maturation, leading to marked color changes.

Cyanogenic glycosides. Cyanohydrin {NC-C(R)(OH)-} derivatives of sugars, found in cereal kernels (e.g. *epi*-heterodendrin in barley), cereal roots (e.g. dhurrin in sorghum), nuts (e.g. amygdalin in almonds) and stone fruit (e.g. prunalin). They are natural plant defence compounds. The cyanohydrin moiety is easily cleaved from the sugar and can be hydrolyzed chemically or enzymically to produce HCN.

Cyclic AMP. Cyclic adenosine monophosphate is a second messenger that is important in signal transduction. Its participation is important in many biochemical processes, such as glycogen, sugar and lipid metabolism, via the activation of certain ***protein kinases***.

Cyclooxygenases. Members of the COX family of enzymes, which catalyze reactions that produce ***prostaglandins*** and related molecules from arichidonic acid. COX-1 and COX-2 are the best known. Sometimes known as prostaglandin endoperoxide synthases.

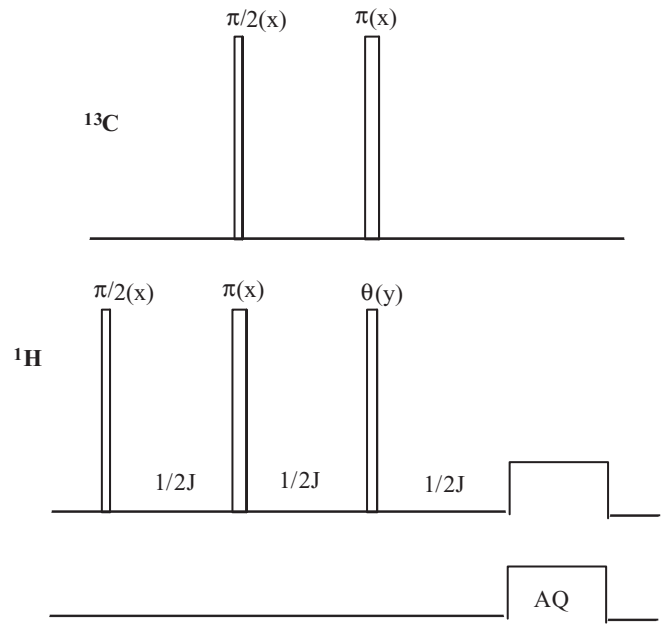
Cytochrome isoenzymes. A superfamily of heme containing enzymes in the endoplasmic reticulum membrane of cells. They catalyze the oxidative metabolism of many endogenous and exogenous molecules (including drugs). They are known as isoenzymes (or isoforms) because each one is derived from a different gene; in humans, 21 families, 20 subfamilies and 57 genes have been so far described. CYP 2E1 is the main component of ***microsomal*** ethanol oxidizing system.

Cytolysis. Sometimes called osmotic lysis, cytolysis refers to cell rupture due to osmotic imbalance, and is caused by extraneous water entering the cell.

D

Density function theory (DFT). A quantum mechanics based theory used to study the electronic structure (distribution of electron density) of the ground states of atoms and molecules, and also of condensed phases, particularly solids. The time dependent version can be used to describe excited states.

DEPT. This is short for **D**istortionless **E**nhancement by **P**olarization **T**ransfer, an NMR spectral editing technique that indicates ^{1}H–^{13}C connectivity in molecules. The pulse sequence is shown below.



The final proton pulse is often set at $\pi/2$ (giving a 'DEPT-90' spectrum, indicating CH) and $3\pi/4$ (giving a 'DEPT-135' spectrum, indicating CH again, and CH_3, as negative peaks). Methylene and unprotonated carbons can be found by difference.

Digestion. With respect to atomic spectroscopy, digestion refers to the method of dissolution of the sample in a concentrated acid medium, such as aqua regia ($HCl + HNO_3$). The elements in complex molecules of the sample are thus converted to simple ionic species.

Diploid. A diploid cell is one that contains two sets of homologous chromosomes, one donated from each parent. *Saccharomyces cerevisiae* is a single cell eukaryote with both diploid and *haploid* (with single chromosome set) forms.

Dipole–dipole forces. Attractive (stabilizing, energy lowering) forces between opposite ends of molecular or covalent bond dipoles, either in the same molecule (intramolecular) or in different molecules (intermolecular). Hydrogen bonding, where one of the involved atoms is hydrogen, is considered by many to be a special kind of dipole–dipole force (e.g. $>C^{\delta+}=O^{\delta-} \ldots . {}^{\delta+}H\text{-}^{\delta-}O\text{-}$). Bond strengths are low (2–10 kJ/mol, but higher for some hydrogen bonds, compared with 200–400+ kJ/mol for most covalent bonds), but these forces can be significant if the number of interactions is large.

Diuretic. A drug that increases the rate of excretion of water from the body, by increasing the urination rate. They are used to treat kidney diseases, heart failure and hypertension.

Dysplasia. A condition in which cell maturation and differentiation are delayed, resulting in increased numbers of immature cells with respect to mature cells of the same type. It is often an indication of an early neoplastic (cancerous) process.

E

Ebulliometry. The precise measurement of the boiling points of solutions, which can be used to determine the composition (e.g. the ethanol content) of the solution. It is based on the colligative properties of matter; the presence of a solute increases the boiling point of a solvent (by lowering the vapor pressure) in accordance with the concentration of the solute.

Eigenvalue and eigenvector. An eigenvector is a nonzero vector x, such that $Ax = \lambda x$, for a particular matrix A. The eigenvalue of x and the matrix is λ.

Electrokinetic. This term relates to the motion of particles or fluid that results from or produces an electrical potential difference. Electrokinetic motion is of special importance in electrophoretic methods of analysis, where application of an electrical potential causes the tangential motion of the diffuse layer of a fluid with respect to an adjacent interfacial double electrical layer.

Electrophiles. Electron deficient species, such as cations (e.g. H^+, R_3C^+), molecules containing group IIA or IIIA elements, and positive ends of polar groups (e.g. $>C^{\delta+}=O^{\delta-}$). They react with ***nucleophiles***.

ELISA. Enzyme linked immunosorbent assay is a method used mostly to detect the presence of an antibody or antigen in a sample. There are several variations of the method, but briefly, a sample containing an antigen is immobilized on a solid support (usually a polystyrene microtiter plate). The detector antibody (usually linked to an enzyme) is added, forming reactive complexes with the antigen molecules. An enzymic substrate is then added, whose reaction with the immobilized enzyme is accompanied by change in color (absorbance) or produces fluorescence that can be related to the concentration of the antigen in the original sample.

Ellman's reagent. This is 5.5-dithio-bis(2-nitrobenzoic acid), a sensitive reagent for the detection of sulfydryl (thiol; SH) groups in samples, by forming a yellow product that absorbs strongly at 412 nm. CAS registry number: 69-78-3.

Endosymbiosis. A theory that postulates that all mitochondria and plastids (organelles in eukaryotic cells) are evolved from prokaryotic bacteria that once lived in symbiosis with their host cells.

Endotoxins. These are toxins associated with certain bacteria; they are secreted when the cells are lysed (ruptured) or during normal cell division. Many are lipo-oligosaccharides, some are proteins, but all cause

immunoprotective responses. Their presence in the blood stream can lead to septic shock if the immune response is pronounced.

Enterocytes. This is the name for intestinal absorptive cells, which are simple columnar epithelial cells found in the small intestine and colon. They are responsible for the uptake of nutrients and the excretion of ***immunoglobulins***, such as IgA, which provide protection against microbes.

Estrogenic. Any substance that produces physiological behavior like that of estrogen is called an estrogenic substance. It disrupts the physiological action of endogenous estrogen, by binding to cellular estrogen receptors, thus up regulating the expression of many genes. More generally, endocrine disruptors influence the balance of body regulation by interfering with hormone action.

F

FAN. The free amino nitrogen (FAN) content of wine must or beer wort is a measure of the nitrogen available to yeast as nutrition during alcoholic fermentation. It is often related to the α-amino acid concentration, but ***yeast assimilable nitrogen*** (*YAN*) measurement takes into account ammonium salts.

FPD. A flame photometric detector is a largely specific GC detector, used mainly to determine P and S containing compounds. It works on the principle of excitation of atoms in a hydrogen flame. Light emitted by the excited atoms can be related to the concentration of the substance that gives rise to those atoms. Band pass filters are used to detect P (526 nm) and S (394 nm) with very high sensitivity; other elements can be detected, but at much decreased sensitivity.

Ferredoxins (ferrodoxins). Redox proteins containing iron–sulfur clusters that mediate electron transfer in a wide range of metabolic reactions and organisms. They act as 'biological capacitors,' being able to accept and donate electrons. Some ferredoxins, such as chloroplast-like, mitochondrial adrenodoxin type and thioredoxin types, have an Fe_2S_2 cluster linked to four CYS units, whereas others, such as bacterial types, possess an Fe_4S_4 cluster bound to four CYS units.

Filiform papillae. Swallow tail-like papillae with hair-like structures found in most areas of the upper tongue surface. They are the most common papillae, their action being mechanical (e.g. trapping food particles to assist in taste), rather than related to taste as such, since they do not possess taste buds.

Foliate papillae. Leaf shaped papillae found mostly on the sides of the upper tongue surface at the back. They contain taste buds.

Fungiform papillae. Mushroom shaped papillae on the upper tongue surface, found mostly at the front and middle. They contain taste buds.

Friability. This is the ability of a solid object to be reduced to small pieces upon the application of a modest pressure or frictional force. High friability is a desirable property of malted barley and other grains used in the brewing of beer and production of whisky.

Fourier transform (FT). A mathematical operation that transforms one complex valued function of a real variable to another. In signal processing used in FT spectroscopy (e.g. IR, MS, NMR), a time domain function is transformed into a frequency domain function.

Freon. Generic name of chlorofluorocarbons (CFCs), very useful extraction solvents for low to mid polarity compounds from an aqueous matrix or solids. However, due to environmental considerations, large-scale manufacture of Freons is being phased out. In the usual molecular nomenclature (e.g. Freon-12 or CFC-12) there are three possible digits: the left most indicates the number of carbons minus 1; the middle digit gives the number of hydrogens plus 1 and the right most digit indicates the number of fluorine atoms. Thus all CFCs based on methane have only two digits, e.g. CF_2Cl_2 is Freon- or CFC-12.

G

GABA. Gamma-aminobutyric acid is a γ-amino acid (not found in proteins). It is the main inhibitory neurotransmitter in the central nervous system of mammals. In vertebrates, GABA acts at inhibitory synapses in the brain by binding to transmembrane receptors, thereby regulating ion channel function.

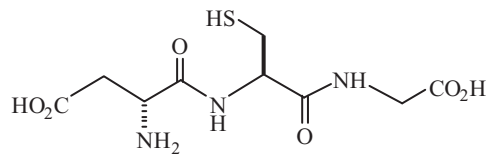
G proteins. Guanine nucleotide binding proteins are involved in the regulation and control of a range of cellular processes, such as function of ion channels and transcription. They are essentially signal transducers (they convert an extracellular signal into intracellular action), they are associated with cell membranes and are activated by G protein coupled receptors. Their action involves the interconversion of GDP and GTP.

Glassy carbon disk (disc). A type of electrode used in electrochemical analysis. The disks, typically 5.0 mm diameter × 4.0 mm thickness, are cut from glassy carbon rods, often using submicron alumina powder to achieve a mirror polish.

Glucogenolysis. This term is usually referred to the metabolic (catabolic) processes that generate glucose from noncarbohydrate sources, such as proteins and fats contained in body tissues, such as muscle. It is usually significant following vigorous exercise in the absence of dietary amino acids.

Gluconeogenolysis. The process of conversion of glycogen polymers (animal energy reserves) to glucose monomers, via hydrolysis of glycosidic bonds, following initial phosphorylation of a terminal glucose unit.

Glutathione. A tripeptide cell antioxidant that helps protect cells from damage by reactive oxygen molecules. It is present in healthy cells entirely as the monomeric reduced form (GSH – see below; it is oxidized to glutathione disulfide, GSSG) at concentrations of around 5 mM.



Glycoside. This is the generic name given to molecules in which a noncarbohydrate moiety (e.g. a phenol or a ***terpenoid***) is bonded to a sugar moiety (usually a monosaccharide or disaccharide) through the latter's anomeric carbon atom, via a glycosidic bond.

Grain neutral spirit. GNS refers to spirit of 85–95% ABV that has been produced by repetitive distillation/condensation cycles of a wash derived from cereal grains. It is thus almost pure ethanol–water, with only a very small concentration of flavor compounds (congeners), such as esters and higher alcohols. It is nearly always produced using continuous (usually two column) stills.

Gravimetric analysis. An analytical method for the quantitative determination of analyte based on accurate and precise weighing, and usually involving the conversion of a soluble analyte into an insoluble form of known composition via a precipitation reaction.

Grey mold. Often known as grey rot or botrytis bunch rot, grey mold is a disease of many plant species caused by *Botrytis cinerea* infection of the tissues, particularly in warm, humid conditions. Fruit are affected, resulting in loss of yield, quality and (in the case of colored fruit, such as black grapes) color. Other parts of the plant (e.g. leaves and stalks of grape vines) may also be seriously affected).

Gushing. The term given to the excessive, even violent frothing of carbonated alcoholic drinks, notably beer, upon opening the container. The phenomenon can be caused by the presence of mycotoxins from fungus infected grains used in brewing.

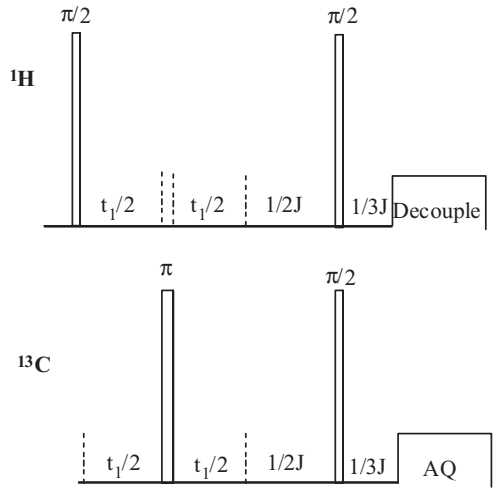
H

Haploid. The haploid number (n) is the number of chromosomes in a gamete (a germ cell), or alternatively, a haploid cell or organism is one that has half the number of chromosomes in somatic cells (normal body cells). In humans, sperm or egg cells (germ cells) contain only one of each of the 23 chromosomes of the human genome. In contrast, somatic cells are ***diploid*** and contain 2 × 23 chromosomes.

HDL. High density lipoproteins belong to one of five major groups of lipoproteins that transport lipids through the bloodstream. They are composed of ***apolipoproteins*** ApoA-I or ApoA-II wrapped around a phospholipid monolayer, collecting or surrendering lipids (cholesteryl esters or triglyceride esters), with the

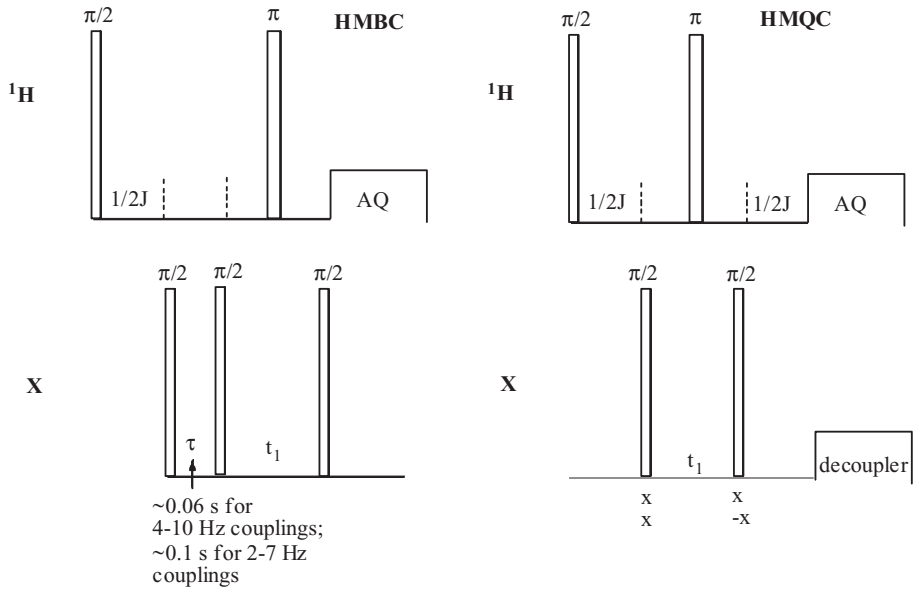
help of various transfer proteins. In particular, HDL contributes to the transport of lipids from cholesterol laden foam cells of ***atherosclerotic*** arteries to the liver for excretion into the bile duct.

HETCOR. This is the name of a family of 2D NMR **HET**eronuclear **COR**relation techniques that are used to demonstrate connectivity between different magnetic nuclei (e.g. ^{1}H and ^{13}C) via transfer of polarization. See also HMBC and HMQC. The pulse sequence used in HETCOR spectroscopy is shown below.

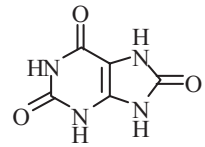


HGCA. The UK's Home Grown Cereals Agency, which is responsible for 'the production, wholesomeness and marketing of UK cereals and oilseeds, so as to increase their competitiveness in UK and overseas markets in a sustainable manner.' HGCA board members are appointed by the UK agricultural ministers.

HMBC and HMQC. Heteronuclear **M**ultiple **B**ond **C**oherence and **H**eteronuclear **M**ultiple **Q**uantum **C**oherence are 2D NMR inverse (usually proton-^{13}C) correlation techniques, giving atomic connectivity information. HMBC gives long-range (2–4 bond) couplings, whereas HMQC is selective for direct one bond (such as C–H) coupling. The pulse sequences for HMBC and HMQC are shown below.



Hyperuricaemia. This term refers to the condition where the level of uric acid (below) in the blood is abnormally high (>400 μmol/l for men). Crystallization of this substance may sometimes occur at joints, tendons and surrounding tissues, giving rise to the painful condition known as gout. A major cause of hyperuricaemia (though not necessarily of gout) is excessive alcohol consumption, which results in both increased production and decreased excretion of uric acid.



Hypocholesterimic. An agent (e.g. a specific substance such as niacin or a foodstuff such as garlic) that lowers the concentration of cholesterol in general in the bloodstream.

Hypoglycaemia. This is the medical term for a condition where an abnormally low level of glucose is present in blood. A principal cause is a complication of treatment of diabetes mellitus, from increased levels of insulin.

Hypokalaemia. This describes a condition in which the blood potassium level is abnormally low (≪3.5 mEq/l). It may be caused by a number of factors, including gastrointestinal loss, inadequate uptake and excessive secretion of K^+ ions, which in turn can be influenced by high alcohol consumption. Symptoms include cardiac arrythmias and impairment of muscle function.

I

Ideal. With reference to gases, an ideal gas is one that obeys the ideal gas law ($PV = nRT$). Ideal gases are hypothetical gases in which the interparticle forces are zero and the particles have zero volume, but at lower pressures and higher temperatures, some real gases (e.g. H_2, He and Ne) exhibit very close to ideal behavior. Many other gases (e.g. N_2, CO_2) behave approximately like ideal gases under these conditions.

Interleukins (IL). Sometimes known as *cytokines* or interferons, interleukins are small proteins that are secreted by certain cells of the immune system in order to carry signals locally between cells. For example, the two forms of IL-1 (IL-1α and IL-1β) are proinflammatory cytokines involved in the body's defence mechanism against infection. It is produced by ***macrophages***, ***monocytes*** and other cells and it functions by increasing expression of adhesion factors on endothelial cell walls, thus enabling transmigration of ***leukocytes*** (cells that destroy pathogens) to sites of infection. It also resets the hypothalamus temperature regulation center, causing fever.

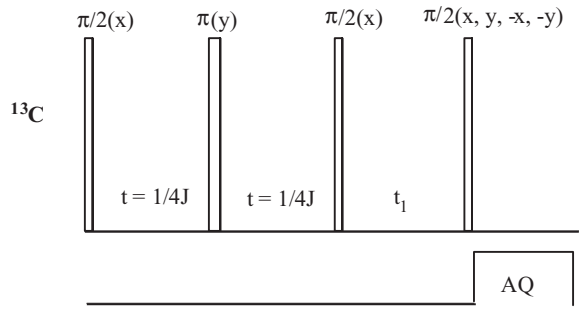
Iodometry. This is the name of an important method of volumetric chemical analysis and involves redox titrations in which appearance or disappearance of I_2 occurs suddenly at the equivalence (end) point. Usual reagents are sodium thiosulfate as titrant, starch or polyvinyl alcohol as indicator and an iodine compound that is either reduced (e.g. iodate, IO_3^-) or oxidized (e.g. iodide, I^-) by the analyte being studied. For example, iodometry can be used to determine the chlorine content of water used in brewing, distilling or winemaking and the sulfur dioxide content of beverages.

Isoprenoids. Often called ***terpenoids***, they constitute a very large and diverse group of natural products found throughout the plant and animal kingdoms. Their structures are based upon that of isoprene (2-methyl-1-butene), but biochemically they are synthesized either from acetate via isopentyl pyrophosphate and the mevalonic acid pathway or via the 2-*C*-methylD-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway. They are generally prone to complex rearrangements in mildly acidic conditions or upon mild heating and many are important flavor compounds in alcoholic and other beverages.

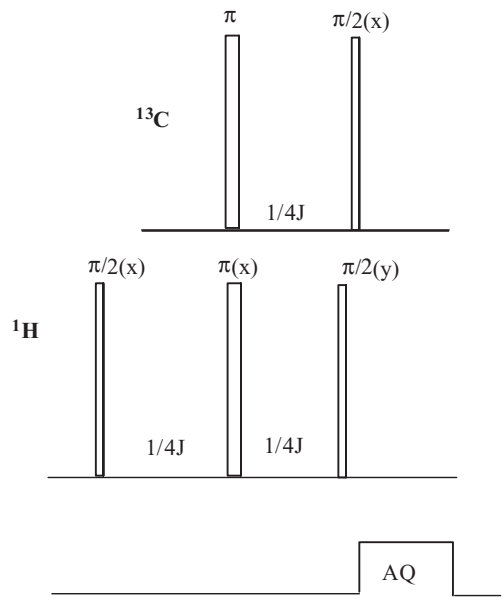
Immunoglobulins (Ig). Also known as antibodies, immunoglobulins are glycoproteins found in blood and other body fluids, such as saliva, mucus and colostrum. They are used by the immune system of vertebrates to identify and destroy foreign entities (immunogens or antigens), such as parasites, bacteria and viruses.

Millions of immunoglobins exist, each with slightly different tip structures (for binding with specific antigen receptors), but with overall very similar structures, thus allowing the recognition of a huge range of antigens. Immunoglobulins can act as part of a cell membrane (e.g. up to 100 000 surface Ig sites on effector B cells) or can be secreted into mucus or serum without their transmembrane regions. When Ig molecules bind to surface antigens on a bacterium, a 'complement cascade' is activated, in which the tagged microbe is either attacked by effector cells (mast cells, natural killer cells, neutrophils or *phagocytes*), which are attracted to the microbe by the presence of complement molecules produced in the cascade, or more directly by a membrane attack complex of complement system components and antibody molecules.

INADEQUATE. A 1D or 2D NMR experiment that reveals carbon–carbon connectivity. The 1D form provides a means of measuring (natural abundance) ^{13}C–^{13}C coupling constants and for establishing carbon–carbon connectivity on the basis of coupling magnitudes that are common to the two particular carbon atom types. The 2D form uses the pulse sequence below, where the fixed time period t_1 is used to encode the double quantum frequency domain (that of the satellite resonances, caused by ^{13}C–^{13}C coupling; $^1J \sim 30$–70 Hz), and the phase of the final pulse is cycled through the directions shown. The 2D plot shows no diagonal peaks (unlike COSY), but connectivity is obtained from cross peaks after drawing in a diagonal.



INEPT. This is short for **I**nsensitive **N**uclei **E**nhancement by **P**olarization **T**ransfer. The pulse sequence shown below results in enhanced signals for insensitive nuclei, such as ^{13}C and ^{15}N.

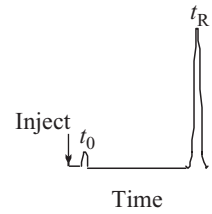


Ischaemic. Local lack of blood supply, caused by narrowed arteries (vasoconstrction) or by a local blockage by some obstacle.

K

***k′* (‘*k* prime’).** In HPLC, k′ is known as the retention or capacity factor; it measures the retention time (t_R) of a particular analyte under a particular set of conditions with respect to the solvent front or an unretained solute (t_0).

$$k' = \frac{t_R - t_0}{t_R}$$



Its optimum value is 2–10, giving optimum resolution ($R_s = {}^1/_4\ (\alpha - 1)\ \sqrt{N}\ \{k'/(1 + k')\}$, where N is the efficiency factor and α the selectivity factor), but without inconveniently long retention times.

Kinetic stabilization. This term describes the stabilization of a molecule due a high transition state energy of a particular reaction, causing that reaction to be very slow. It is often provided by the presence of bulky groups around a reaction center (‘steric hindrance’), thus causing the reaction transition state to be too high in energy, because of serious repulsive interaction.

L

Landrace. This term refers to domesticated plants that have adapted to the local natural environment in which they exist. They are often of variable appearance, but are morphologically identifiable and have a certain degree of genetic integrity. Landraces are grown from seeds that have been systematically selected for desirable characteristics.

LD_{50}. A measure of the toxicity of a substance; it is the dose required, usually expressed as mg/kg body weight, to kill 50% of the animals (usually rat, but should be specified) on oral or other administration (which should also be specified) of the substance.

LDL. Low density lipoprotein is, like ***HDL***, one of five main groups of lipoproteins and is responsible for the transport of cholesteryl esters through the bloodstream. The ***apolipoprotein*** is Apo B-100 (molecular weight ~514 000 D), which combines with a phospholipid monolayer and cholesterol to trap cholesteryl ester molecules in its center, giving LDLs of differing sizes and densities. The smaller, more dense LDL particles are thought to pose a greater risk factor than larger particles, but high LDL content in general is associated with increased risk of cardiovascular disease.

LOD and LOQ. Limit of detection (LOD) is the lowest quantity or concentration of analyte that can be distinguished from a blank (containing no analyte), within a defined confidence limit, usually 1%. Instrumental detection limit is usually taken as 3 × standard deviation (SD) of the noise level, whereas method detection limit is derived from the SD near the LOD and the one sided *t* distribution. The limit of quantification (LOQ) is the limit at which the difference between two different values can reasonably be detected. It is usually 10 × SD of noise level.

Leukocytes. This is the general name for white blood cells involved in the body’s immune defence system. The major groups are basophils, eosinophils, ***lymphocytes***, ***monocytes*** and neutrophils.

Leukoplakia. (leukoplasia). White patches on skin of say, mouth, which are the result of mucus membrane dysfunction and can be caused by alcohol abuse. They are sometimes associated with a precancerous condition.

Leukotrienes. Unsaturated compounds produced by degradation of arachidonic acid and other unsaturated membrane long chain fatty acids. They are local chemical messengers involved in inflammation; they help regulate the state of blood vessels and airways, and they influence the activity of ***leukocytes***. High levels of serum leukotrienes have been correlated with oxidative stress and increased risk of cardiovascular disease, which sometimes can be linked to excessive alcohol consumption.

Lignins. This is the general name for large, complex phenolic molecules, often derived from woody materials. Lignin breakdown products (phenols) are often important in the wood maturation of wines and spirits. Lignins form an integral part of plant cell walls, being covalently bonded to hemicellulose and they are found sandwiched between cellulose, hemicellulose and ***pectin*** components.

London dispersion forces. The induced dipole/induced dipole attractive forces that contribute to the ***van der Waals*** intermolecular forces are known as London dispersion forces. They are very weak forces ($\sim$1 or 2 kJ/mol), but nevertheless play a fundamental role in the formation of condensed phases and they can be important in attractive interaction between or within large molecules, such as proteins and nucleic acids, or between an enzyme and its substrate.

Lymphocyte. There are several types of white blood cells involved in immune response; lymphocytes are one type, being composed of two subtypes: large granular lymphocytes, including natural killer cells (NKC), and smaller lymphocytes, which include B cells and T cells. All are involved in the vertebrate immune system, offering protection from a wide range of foreign entities, including bacteria, cancer cells and viruses.

Lymphokine. These proteins belong to a subclass of cytokines, proteins produced by ***lymphocytes*** (certain types of cells of the immune system), such as T cells. Lymphokines aid immune response through intercellular signaling.

Lyophilized. Refers to freeze drying used to preserve or to concentrate perishable material, making it more suitable for long keeping and transportation. Lyophilization (also known as cryodesiccation) is a dehydration process that is achieved by the sublimation of the water content of frozen material at low pressure.

M

MAPK. Mitogen-activated protein kinases are SER/THR specific ***protein kinases***. They respond to extracellular stimuli, such as stress, proinflammatory agents, cytokines and mitogens – compounds that stimulate cell division. They regulate many cellular processes, including ***apoptosis*** (cell death), differentiation, gene expression, mitosis and proliferation.

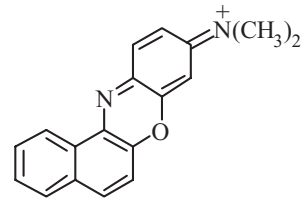
Marangoni current. A flow (convection) in liquids from areas of low surface tension to those of higher surface tension. The surface tension gradient may be formed by temperature differences in a pure liquid or by concentration differences in a solution containing water, ethanol and other solutes, for example.

Macrophages. These important cells of the vertebrate immune defence system are produced by stimulated differentiation of ***monocytes*** at the site of infection. As stationary cells (e.g. in the lungs) or as mobile cells, they nonspecifically engulf cell debris and foreign bodies (such as bacteria), breaking them down into simpler products. They also stimulate ***lymphocytes*** and other more specific immune cells to respond to the presence of a pathogen.

Matrix metalloproteinases. MMPs are zinc dependent endopeptidases; they hydrolyze proteins at nonterminal amino acids. They can degrade extracellular matrix proteins, such as collagen; in fact they are the only known proteases able to hydrolyze the triple helix of collagen. MMPs are known to be involved in a number of cellular processes, including the cleavage of cell surface receptors, the release of apoptotic

proteins (such as FAS) and cytokine activation/deactivation. Hence, they play a role in cell ***apoptosis***, cell differentiation, cell migration/adhesion and cell proliferation. MMPs are also known to be involved in angiogenesis (production of new blood vessels). They are deactivated by proteins known as tissue inhibitors of matrix metalloproteinases (TIMPs).

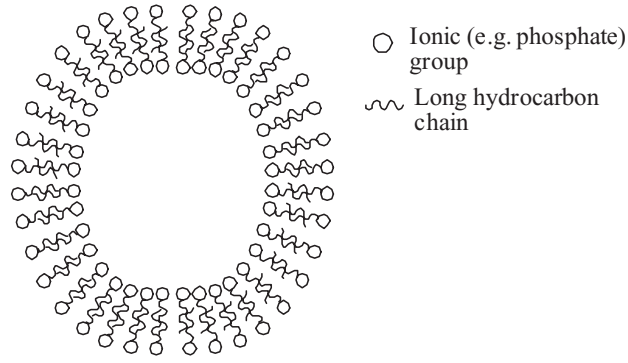
Meldola's Blue. Often known as Basic Blue 6, Meldola's Blue (CAS registry number 7057-57-0; see below) is sometimes used, along with metal oxides such as ZnO as a coating on electrodes (e.g. ***glassy carbon electrodes***) in electrochemical analysis. The dye is strongly adsorbed on ZnO surfaces.



Microalbuminuria. A condition that involves the leaking of small amounts of albumin proteins into urine. The presence of albumins in urine can be used as an indicator of not only kidney disease, but also cardiovascular disease and endothelial dysfunction. The condition can result from alcohol abuse.

Mitochondrion (pleural: mitochondria). This is an important membrane enclosed organelle found in most eukaryotic cells. Mitochondria generate most of the cell's ATP, itself a major store of energy required for cell metabolism, and are involved in aerobic respiration, the catabolism of glucose and other molecules being used to generate ATP.

Microsomal. This term refers to microsomes, vescicle-like (spherical bilayer micelles – see below for a schematic representation of a section through a microsome) structures formed from the endoplasmic reticulum when eukaryotic cells are broken up by mechanical action. They can trap globular enzymes, such as ***cytochrome*** P450 in their hydrophilic centers.



Monocytes. Immunological or white blood cells, that are produced by the bone marrow and circulated throughout the bloodstream, about half of them being stored in the spleen. Their basic function is to respond to inflammation signals, moving quickly to the site of infection, where they differentiate into ***macrophages*** or dendritic cells (antigen binding/presenting cells) to illicit a full immune response.

Myofibillar. Relates to myofibrils, cylindrical organelles found in muscle cells. They are composed of proteinaceous filaments running from end to end and are attached to the cellular membrane at both ends.

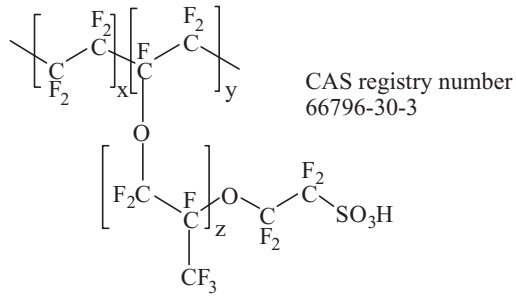
The filaments are of two types – thin, consisting of proteins actin and nebulin, and thick filaments, consisting of proteins myosin and titin (connectin).

Myelin. This is the name for the electrically insulating sheath that protects axons of the nervous system and regulates the conduction of electrical impulses. It is composed of about 80% lipid (mostly galactocerebroside and sphingomyelin) and 20% protein, including myelin basic protein. In the peripheral nervous system (as in the olfactory system), ***Schwann cells*** are responsible for wrapping a myelin sheath around neuron axons.

Myoglobinuria. This describes a condition that is characterized by the presence of myoglobin in urine. It is usually associated with muscle deterioration or ***rhabdomyolosis***, both of which can result from alcohol abuse.

N

Nafion. This is the trade name (Du Pont) for a sulfonated tetrafluoroethylene (Teflon) based fluoropolymer copolymer, containing many perfluorinated ether side chains terminating with sulfonate groups (see below). It is used as a proton exchange membrane, and as a 'superacid' catalyst. It is also used in the production of sensors.



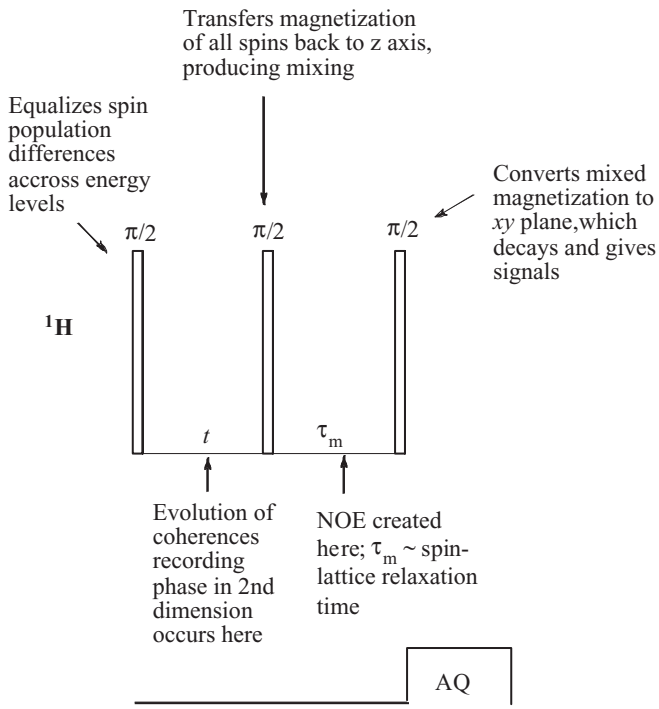
Necrosis. The term used to indicate premature cell (or tissue) death. It is caused by external factors, such as bacterial infection, cytotoxins, trauma or viral infections – in contrast to programmed or naturally occurring cell death (***apoptosis***). The lack of signals from prematurely killed cells to the immune system prevents detection and treatment by immune cells (***macrophages***, ***monocytes***, etc.) and hence leads to build of dead tissue, which must be removed.

NF-κ-B. 'Nuclear factor kappa light chain enhancer of activated B cells' is a protein complex found in most cells and is involved in cellular response to external stimuli, such as bacterial infection, cytokines, free radicals, oxidation of *LDL*, UV-radiation and viral infection. NF-κ-B plays a major role in the regulation of immune response to infection, but incorrect regulation has been linked to a number of pathological conditions, including autoimmune diseases, cancer and inflammation.

Noble rot. Otherwise known as Edelfäule, pourriture noble and muffa, noble rot describes the infection of ripe or over-ripe white grapes by *Botrytis cinerea*. It is a most beneficial condition for the production of most sweet wines, particularly Beerenauslese and Trockenbeerenauslese wines of Germany and Austria, Sauternes and similar wines of Bordeaux, the sweet Chenin wines of the Loire region and Tokaji aszú wines of Hungary. For noble rot to develop properly, alternating moist and dry (but warm) weather conditions are required in the autumn. Infection by *B. cinerea* at this late stage causes the grapes to shrivel, thereby concentrating the contents (but decreasing the yield!) and also producing beneficial components, such as glycerol and polyols (like sorbitol). The fungus dominates, thus minimizing infection by spoilage fungi, such as *Aspergillus* and *Penicillium* species.

NOE. The '**N**uclear **O**verhauser **E**ffect' is named after the physicist Albert Overhauser. In NMR spectroscopy, it describes the transfer of spin polarization from one nuclear spin population to another in the same molecule, via cross polarization, although originally the term was used to indicate the polarization transfer between electron and nuclear spins in metals. It is a through space effect, the magnitude (enhancement or diminution of signal) of which depends on the spatial proximity of interacting nuclei and hence the phenomenon can be used to determine complex structures, such as carbohydrates and large cyclic ***terpenoids***.

NOESY. '**N**uclear **O**verhauser **E**ffect **S**pectroscop**Y**' is just one (but an important) application of the *NOE*. It is used mainly as a 2D method, where the cross peaks in the spectrum connect signals from nuclei that are spatially close. A 1D version involves selecting a particular signal, then radiating it at its frequency, and observing the changes in signal intensity elsewhere in the spectrum. The 2D NOESY pulse sequence is shown below.



Norisoprenoids. This is the name for a large family of compounds that are biochemically derived from carotenoids or other compounds that have been biosynthesized from acetate via the mevalonic acid pathway. They contain 13 carbon atoms and are produced by the enzymic degradation of carotenoids to C13 ketones, which then undergo rearrangement to norisoprenoids. Over 300 of these compounds have been identified in wine, and some, like β-damascenone, make an impact on aroma.

Nucleophiles. Electron rich species, such as an anion (e.g. phosphate), a molecule with lone pair electrons (e.g. H_2O and NH_3) or a multiple bond (such as alkenic or aromatic C=C). They react with ***electrophiles***. Electrophile–nucleophile steps are important in a large number of biological reactions (e.g. phosphorylation, hydration, hydrolysis) and in many reactions involved in the maturation of alcoholic beverages (e.g. ester formation, polyphenol formation, hydrolysis of lignins).

O

Orbital. Wave mechanical representation of electrons in atoms and molecules; actually relating to allowed solutions of the Schrödinger or similar equations, and is most often given as a boundary surface, defining the volume of space in which an electron has a 95% chance of being found.

Organoleptic. Relating to the perception by a sensory organ or involving the use of sensory organs in an assessment of beverage character or quality. Hence the term refers to the sensory properties of a drink, involving color, odor, taste and feel. Organoleptic or sensory analysis involves an examination of a beverage using the above senses and is usually carried out and interpreted according to a set of protocols. Sensory analysis is often used in conjunction with analytical methods (e.g. GC, HPLC) at all stages of alcoholic beverage production.

Orocecal transit. This term refers to the movement of food through the alimentary tract. The transit rate can be estimated by the measurement of expired H_2 after ingestion of lactulose.

P

t-PA. Tissue plasminogen activator is a serine ***proteinase*** that catalyzes the conversion of plasminogen into plasmin, a fibrolytic protein, thus influencing blood clotting. This enzyme has been implicated in helping normal neural function, cell migration and tissue remodeling. It is used to treat stroke.

Pascal. The SI unit of pressure (Pa). Its basic units are N/m^2 or kg/ms^2.

Phagocytes. White blood cells that protect the host by first engulfing and then ingesting foreign entities, such as bacteria and dead or dying cells. There are many types, but the more aggressive kinds, those that attack directly, include dendritic cells, ***macrophages***, mast cells, ***monocytes*** and neutrophils. These have surface receptors that can recognize 'tagged' (ligand coated) particles that need to be ingested. Other phagocytes are more selective in their action; these include endothelial cells, epithelial cells, erythrocytes, fibroblasts and *lymphocytes*.

PDMS. Polydimethylsiloxane, an organosilicon polymer that is inert and nontoxic. It is used as a low to mid polarity sorbent in solid phase microextraction and stir bar sorptive extraction of organic compounds from aqueous or aqueous-alcoholic matrices. Its formula is $CH_3[Si(CH_3)_2O]_nSi(CH_3)_3$, and when n is large, the polymer is a viscous liquid or flexible semi-solid.

PLSR. Partial least square regression is a bilinear factor statistical method related to principle components regression. It finds a linear regression model by projection of predicted and observed (measured) variables to a new space.

Pectinase. Pectolytic enzymes, those that hydrolyze ***pectins***, are generally known as pectinase. They include pectolyase, pectozyme and polygalacturonase and (usually of *A. niger* origin) are useful in winemaking for increasing extraction efficiency, by helping to break down plant cell walls.

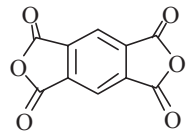
Pectins. Like cellulose and other carbohydrates, pectins form part of the primary cell wall structure in nonwoody parts of plants. They are also present in the middle lamella between plant cells, thereby helping to bond the cells together; most fruits used for winemaking have a significant pectin content. Pectins are not digested in the small intestine, but act as dietary fiber in the colon, where there is some decomposition by gut microflora, releasing beneficial compounds. There are several classes of pectins, including homogalacturonans, which are linear polymers (1–4 linked) of α-D-galacturonic acid and α-L-rhamnose, with branches containing L-arabinose, D-galactose and D-xylose. Pectins vary in the extent of methylation of the carboxylic acid groups and many contain acetylated or amidated carboxyl groups. In general, they are used as gelling agents in the food industry, but a high pectin content in wine is undesirable, hence the use of ***pectinase*** by some winemakers.

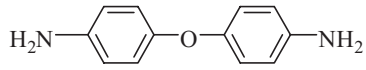
Peristaltic. This refers to pumps that operate by performing successive wave-like contractions and relaxations, usually by use of a rotor. Such pumps are used for pumping aggressive solvents and solutions, for example to the nebulizer in ICPMS.

π–π (pi–pi) attractions. Weak ***van der Waals*** type forces between multiple bonds, both aliphatic and aromatic. They are believed to arise from oscillating induced dipoles in the π-bonds, and although π–π forces between individual multiple bonds pairs are weak, if there is a stack of such bonds (as in DNA and stacked anthocyanins in fruit juice), then their influence is significant. Also, if they act in conjunction with other intermolecular forces, such as ionic forces and/or hydrogen bonding (as in protein–polyphenol interactions), they are likewise significant.

Plummer. This is the name given to a category of medium bodied pot still Jamaican rum that was much exported to the UK and formed an important part in numerous blends. It is still produced from sugar industry products and by-products and can be found, for example, in a 1:1 blend (with Wedderburn) of Smith & Cross, at 57% ABV ('navy strength').

Polyimide. Polymers that are frequently used as inner coatings ('cladding') of fused silica capillary GC columns, upon which the stationary phase is then coated. They are generally very strong, heat resistant and solvent resistant. Typical monomers include mellitic dianhydride and 4-oxydianiline (below).





Preservative. Any substance, that when added to a foodstuff, protects it from microbiological spoilage. The most widely used preservative in the alcoholic beverage production is sulfur dioxide, usually added as potassium or sodium metabisulfite. This particular preservative also offers some protection against chemical oxidation.

Prolamins. Plant storage proteins with high GLN and PRO content, found in cereal grains; avenin (oats), gliadin (wheat), hordein (barley), secalin (rye) and zein (maize). Some, like gliadin, induce celiac disease in genetically predisposed persons.

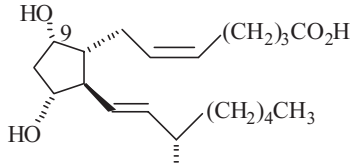
Promyelocytic. Refers to promyelocytes, immature granulocytes, which are a kind of ***leukocyte***. They are derived from stem cells in the bone marrow.

Protein kinases. These are enzymes that catalyze the nucleotide dependent phosphorylation of proteins at the OH function of SER, MET or TYR residues at specific sites. The presence of a large dianionic group on the protein causes a change in conformation, thus changing the reactivity of the target protein. Protein kinases constitute a superfamily of proteins that differ widely in size, subunit structure and localization within the cell. Many depend on cAMP and cGMP for activation and many, such as tyrosine kinases, are involved in cellular signaling pathways and may be influenced by the presence of ethanol (or acetaldehyde) and phenols in the diet. Other kinases, such as hexokinase, phosphorylate (with the aid of ATP) nonprotein OH groups, such as the 6-OH group of glucose, an important step in glycolysis.

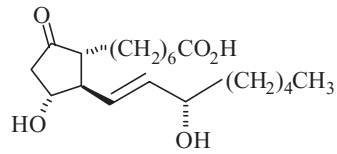
Proteinase. This is the generic name for enzymes that break down (catabolize) proteins by hydrolysis of peptide (amide) bonds. These enzymes are also known generally as proteases or peptidases. Proteinases that hydrolyze peptide bonds at specific site within the molecule are known as endopeptidases, those that hydrolyze terminal links are called exopeptidases. They are important in the digestion of dietary proteins and some proteinases also play a part in blood clotting.

Prostaglandin. A member of a group (along with leukotrienes and thromboxanes) of naturally occurring lipid compounds known as eicosanoids, which are derived from arichidonic acid and other cell membrane unsaturated fatty acids, via enzymic action. They contain 20 carbon atoms, a cyclopentane or cyclopentene ring, or sometimes other ring types (as in thromboxanes). Prostaglandin nomenclature is based on the type of five membered carbocyclic ring, ranging from PA to PI (nine types) and, where appropriate, a subscript

Greek letter is used to denote the carbocyclic ring C9 configuration; α indicating down and β indicating up. A further numerical subscript is used to denote the number of exocyclic alkenic double bonds. See examples, $PGF_{2\alpha}$ and PGE_1 below. Thromboxanes are given the symbol TX. Prostaglandins are short lived local chemical messengers with many functions; they are regulators of cell growth, inflammation mediators and regulators of smooth muscle tissue function and calcium transport.



$PGF_{2\alpha}$



PGE_1

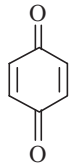
Prussian Blue. A dark blue pigment discovered in the early eighteenth century. It is $Fe^{III}{}_3[Fe^{II}(CN)_6]_3$. Prussian Blue is electrochromic, changing from blue to colorless upon reduction and hence is used in a colorimetric test for phenols. It is also used as an electrode coating in electrochemistry.

Pseudomolecular ion. In mass spectrometry, this term refers to ions that are related to the true molecular ion ($M^{+\bullet}$) by either inclusion of adducts (e.g. MH^+, $MCH_4{}^+$ or MNa^+) or by loss of hydrogen ($\{M - H\}^-$). These are sometimes known as quasimolecular ions.

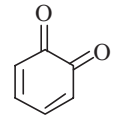
Q

Q (Quadrupole). In mass spectrometry, this term stands for quadrupole detector. It consists of four parallel metal rods (hyperbolic or more likely, circular), with each opposite rod pair electrically connected and a radiofrequency (alternating) voltage applied between both pairs of rods. A direct current is superimposed on the RF voltage. It is a much used detector in GCMS and also in LCMS.

Quinone. This term describes a fully conjugated diketone structure that can be derived from an aromatic structure (e.g. via the oxidation of a polyphenol). See also ***ubiquinones***.



1,4-benzoquinone
(cyclohexadienedione)



1,2-benzoquinone
(*ortho*-benzoquinone)

R

RAPD. Random **A**mplification of **P**olymorphic **D**NA is a form of polymerase chain reaction (PCR), a technique used to amplify one or a number of copies of a section of DNA across many orders of magnitude, thus giving millions of copies of a particular DNA sequence. Heat stable polymerases (such as Taq polymerase) are generally used along with a thermal cycling method, but there are many variants, one of which is RAPD, where the DNA segments to be amplified are random.

Repeatability. Refers to the closeness of agreement (precision) of measurements or test results using a particular method, the same equipment and the same experimenters, often performed sequentially. It is often reported as ***standard deviation*** or ***% relative standard deviation*** (%RSD) (coefficient of variance, CV).

Reproducibility. Refers to the ability of a method to produce results that may be closely reproduced by other experimenters at other times and in other laboratories.

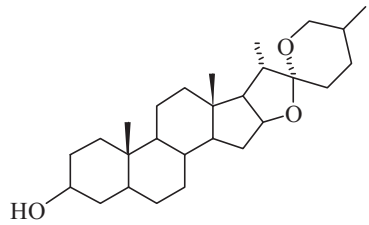
Rhabdomyolysis. This is a term for a medical condition that results when severe injury causes rapid breakdown of skeletal muscle. Breakdown products (including myoglobin) can be released into the bloodstream

and can cause kidney damage. The condition can be caused by the presence of an arterial thrombosis or embolism.

Root mean square error. This is a measure of the differences between values predicted by a model and the actual observed values. It is sometimes known as 'residual' error.

S

Saponins. These are C27 steroids with a spiroketal side chain; that is they contain six rings – the standard four carbocylic rings of steroids, plus two oxygen containing heterocyclic rings. As glycosides, they are present in plant tissues, such as roots, sometimes in substantial concentrations, but their biological functions are largely unknown. They have detergent properties (hence the generic name) and some are very toxic. The aglycosides are known as sapogenins (see below).



Sarsasapogenin

Schwann cell. A type of large cell in the peripheral nervous system, whose plasma membrane wraps spirally around the axon of a myelinated neuron to form a protective ***myelin*** sheath.

Sequelae. A pathological condition resulting from disease, injury or trauma. Typically, sequelae refers to a chronic condition that arises from an acute condition and is essentially a complication of that condition. For example, sequelae of traumatic brain injury include anxiety, dizziness, headache and personality changes.

Sorption. With respect to the analysis of the components of alcoholic beverages and their precursors, this term refers to the distribution of analyte between a liquid solution phase and a sorbent phase, according to the Nernst partition law. The latter phase is essentially a liquid phase (e.g. *PDMS*) coated or bonded to an inert material, such as silica particles, a fused silica microfiber or a stir bar.

Standard deviation and % relative standard deviation. These are two accepted ways of assessing the precision associated with a set of N experimental results.

$$\text{Standard deviation (s or } \sigma) = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N-1}}$$

x_i is a particular result
$\bar{x}$ is the arithmetical mean of the results
N is the number of results

$$\%\text{Relative standard deviation (\%RSD)} = \frac{100\,\text{s (or } \sigma)}{\bar{x}}$$

$\bar{x}$ and s or σ are defined as above

Percent standard deviation is sometimes known as the coefficient of variance (CV).

Steatosis. This term is used to describe the abnormal retention of lipids in a cell. It is also known as fatty charge, fatty degeneration or adipose degeneration. Steatosis is primarily associated with fatty liver disease, but it can also occur in the heart, kidneys and muscle. Chronic excessive alcohol consumption produces high levels of NADH, discouraging the cell to break down fatty acids and, at the same time, encouraging fatty acid synthesis.

Support vector machines (SVM). This term relates to a number of supervised learning statistical methods used for classification and regression. An SVM training algorithm builds a model from training examples, each belonging to two or more categories, and this model is used to predict the category to which a new example belongs. Various extensions to the basic method have been introduced fairly recently in order to include mislabeled samples or to reduce the effect of outliers on the outcome of the statistical analysis.

Supramolecule. This is a stable, organized molecular system formed by two or more molecules being held together by many intermolecular (noncovalent) interactions, such as dipole–dipole forces, electrostatic forces, hydrogen bonding and ***London forces***.

Synergesis. A term used to describe a situation where one factor helps another, so that the total result is greater than that of either of the two factors acting separately.

T

t_R. In chromatography, this is the abbreviation for retention time; it is the time between injection of the sample and detection of a particular analyte.

Terpenoids. See ***isoprenoids***.

Tetrapoid. This term refers to organisms that have four times the ***haploid*** number of chromosomes in the cell nucleus. Tetrapoidy is particularly common in the plant kingdom (as is polypoidy in general) as in the strain of wheat known as durum wheat, but also occurs in animals, such as salmon and salt water prawns.

Thermodynamic stabilization. Any physical effect or process that leads to an overall lower Gibbs free energy of the system. It can include stereochemical effects; proteins exist in certain preferred conformations because extensive intra- and intermolecular attractions are maximized, hence lowering the system energy.

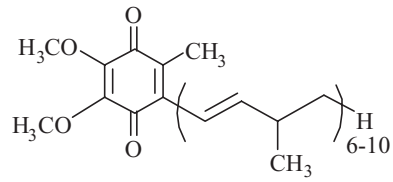
TOCSY. TOtal **C**orrelation **S**pectroscop**Y** is a homonuclear 2D NMR technique that shows the connectivity within an entire spin system, such as an isopropyl or butyl group. The TOCSY experiment as rather like the COSY experiment, except that on the second pulse, the magnetization is locked along the *y* axis, so that all the protons have the spin lock frequency. All coupled spins within a spin system become closely coupled and magnetization is transferred from one nucleus of the spin system to all the others. TOCSY is especially useful in the analysis of large molecules with specific repeating units, such as nucleic acids and proteins.

Transversely heated graphite tube atomizer. This refers to a kind of graphite furnace used in atomic spectroscopy in which the graphite tubes used to atomize the sample are heated transversely rather than longitudinally. This gives a more uniform temperature from end to end, so the method does not suffer from sensitivity losses and contamination caused by lower temperatures at the ends of the tubes. Moreover, the temperatures needed are substantially lower than those needed for their longitudinally heated equivalents, this giving extended tube life and saving energy.

Tumor Necrosis Factor. TNF belongs to a family of proinfammatory cytokines that are produced by ***macrophages*** and circulating ***monocytes***, and is involved in host defence and the pathogenisis of certain diseases. It is also involved in sleep regulation. TNF signaling can lead to three distinct results, each of which is initiated by different signaling pathway complexes; survival mode (gene induction), *apoptosis* mode and ***necrosis*** mode.

U

Ubiquinones. Sometimes called coenzyme Q, ubiquinones (below) are membrane coenzymes (especially in mitochondria) involved in aerobic cellular respiration, and participating in the electron chain mechanism and the generation of energy in the form of ATP. They are also antioxidants and their highest concentrations in the human body are in organs that need most energy, such as the heart. Their biosynthesis involves both the shikimate route (for the ***quinone*** unit) and the mevalonate route for the ***terpenoid*** side chain. Coenzyme Q_{10} is the best known, and is used as a dietary supplement.



Ultrasound. A cyclic sound pressure of frequency 20 kHz to over 200 MHz (i.e. greater than the upper frequency limit of human hearing). It can be used to aid various extraction processes in brewing and winemaking and is used in the measurement of fluid flow or liquid density.

V

V-PDB and V-SMOW. V-PDB stands for Vienna Peedee Belemnite Standard, which defines the carbon isotope composition of beleminite (marine cephalopods of the Cretaceous period) guard fossils found in the Pee Dee region of South Carolina, USA. It is used for calculating $\delta^{13}C$ of samples, usually by IRMS. V-SMOW stands for Vienna Standard Mean Ocean Water. It is a standard defining the isotopic composition of water and was set up by the International Atomic Energy Agency. It refers to pure water (distilled ocean water) with a fixed composition of H and O isotopes and is used as a reference standard for comparison of H and O ratios in samples, often determined using IRMS. $^{1}H/^{2}H = 155.76 \pm 0.1$ ppm; $^{3}H/^{1}H = 1.85 \pm 0.36 \times 10^{-11}$ ppm; $^{16}O/^{18}O = 2{,}005.20 \pm 0.43$ ppm; $^{16}O/^{17}O = 379.9 \pm 1.6$ ppm.

Vallate papillae. Large, flat papillae each surrounded by a trench, contained in a V shaped group close to the base of the upper tongue surface. Also known as circumvallate papillae.

Van der Waals forces. This is the collective name often given to attractive and repulsive intra- and intermolecular forces other than covalent and ion-ion forces. The attractive forces, all of which are relatively weak and of varying ranges, include ion–dipole (induced dipole) (Keesom) forces, dipole–dipole (induced dipole) (Debye) forces and induced dipole induced dipole (***London dispersion***) forces. Quadrupoles and higher can be involved too.

Volumetric methods. Analytical chemistry methods that involve accurate and precise measurement of volume. Titrations are the best known volumetric methods and require high precision volumetric equipment, such as burettes, flasks and pipettes.

Vomeronasal organ. An auxiliary olfactory sense organ located in the nasal cavity of many animals. It is sometimes known as Jacobson's organ. Although its level of development differs from animal to animal, the vomeronasal organ is a chemoreceptor organ whose main role is thought to be the detection of pheromones; chemical signals between individuals of the same species.

W

WALTZ. A modern form of broadband heteronuclear decoupling, used routinely in ^{13}C NMR spectroscopy. The decoupling irradiation is applied as a series of pulses ($\pi/2, -\pi, 3\pi/2$), in which phase cycling eliminates pulse imperfections. The minus sign in the second pulse indicates that this pulse is 180° phase shifted, i.e., magnetization is rotated counterclockwise about the x axis.

Wedderburn. A heavy style of Jamaican rum, produced using pot stills from a combination of sugar production products and by-products (cane juice, molasses, skimmings and 'syrup bottoms'), as well as dunder (the residue from previous distillations). Fermentation is traditionally carried out using wild yeasts and the rum is characterized by fruity, spicy notes. Wedderburn rum was one of the main types to be exported to the UK, where it was usually blended with other rum types.

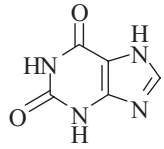
WET. This is an NMR spectroscopic shaped pulse sequence that is used to suppress solvent signals. There are several versions, but the composite pulse sequence $\pi/2(x)$, $\pi/2(y)$, $\pi/2(-x)$, $\pi/2(-y)$ and the '270' WET sequence (as the composite sequence, but with a 3/2 π pulse in the fourth scan) are common.

Wistar rat. A strain of albino rat that has been developed and bred specifically for scientific research. The strain was developed originally at the Wistar Institute (Philadelphia, USA) and is still much in use, although other strains have been developed from it.

X

XAD. The generic trade name for a group of cross linked polymer resins that are used in the purification of substances and in sample preparation for analysis (e.g. chromatographic analysis). They are highly porous macroreticular polymers based on styrene–divinylbenzene, acrylates or formaldehyde–phenol, with varying degrees of cross linking, hydrophobicity and selectivity. Macroreticular means that the pore size is stable and is not dependent upon swelling by an appropriate solvent.

Xanthine. A purine base found in tissues (see below). It is formed in the degradation of adenine and guanine to uric acid. Xanthine is also the generic name for compounds that possess the skeletal structure below, including methylated xanthines, such as caffeine, theobromine and theophylline.



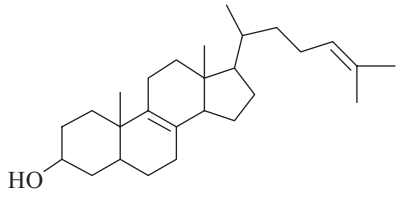
Y

YAN. Short for yeast assimilable nitrogen or 'fermentable' nitrogen. It is the total nitrogen as ammonia and free α-amino acids (***FAN***) in must or wort and is essential for the yeast in order to conduct a healthy fermentation.

Z

Zeeman correction. A background correction technique used in atomic spectroscopy to eliminate the broad background molecular absorption that can interfere with the measurement of the absorbance of the atomic element under investigation. The total absorbance is measured in the absence of a magnetic field and then remeasured in the presence of a magnetic field (e.g. applied to the atomizer) at the same wavelength. The Zeeman effect in the presence of the magnetic field splits the atomic line into two lines of different wavelengths. Thus this measurement gives the background absorbance and the difference between the two is the corrected atomic absorbance.

Zygosterol (zymosterol). This sterol is a precursor of ergosterol and, like ergosterol, is found in the cytoplasmic membrane of fungi, such as yeasts. See below.



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Handbook of Alcoholic Beverages: Technical, Analytical and Nutritional Aspects Edited by Alan J. Buglass

© 2011 John Wiley & Sons, Ltd.

ISBN: 978-0-470-51202-9

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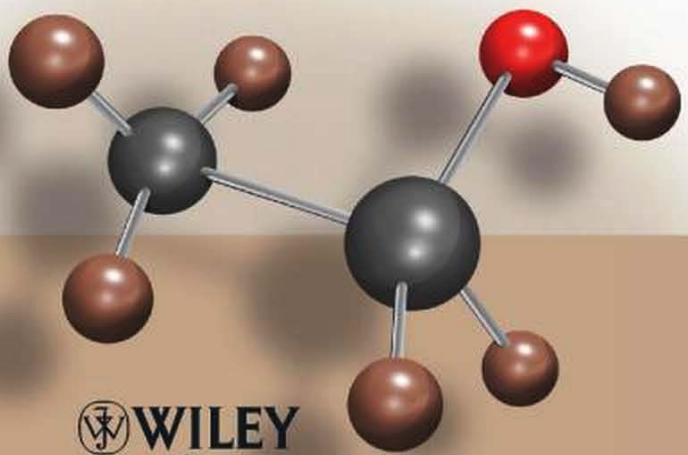

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Cover design: Gary Thompson

ISBN 978-0-470-51202-9