M. Victoria Moreno-Arribas
Begoña Bartolomé Sualdea *Editors*

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Summary

From the point of view of chemical and sensory complexity and human health, wine is a model product that has been a focus of extensive research and relevant findings over the last years, exciting the interest of winemakers, researchers, and consumers. The aim of this book is to describe emergent investigations related to wine safety and quality, connecting with preferences by consumers and with a special emphasis in the beneficial effects of wine to human health. The first part of the book describes the most relevant aspects of wine safety, emphasizing the advances offered by new technologies and biotechnological progress as well as the impact of the global climate change on wine safety. The second part deals with wine consumer preferences, a topic little discussed in previous texts but that has gained current attraction not only from the scientific point of view but also at the industrial and social level. Finally, the last section provides an opportunity for deeper recapitulation of the beneficial effects of wine and its components on human health, including novel experimental approaches and data interpretation.

Preface

Wine is a traditional food that has been linked to human life since ancient times, especially present in the Western world, and that has been assessed and developed from multiple viewpoints including economic, social, artistic, and literary, complementary with each other. Regular moderate wine intake is recognized among the major characteristics of the Mediterranean diet, which constitutes a unique model, recommended by many specialists and several dietary guidelines in different countries.

In recent years, the topic "wine and health" has aroused much interest, although not absent from certain controversy. A large number of studies and scientific contributions have been carried out within this area. To date, increased and improved knowledge from a huge number of studies investigating wine components that can negatively affect the health of moderate wine drinkers has provided us useful solutions to decrease or to avoid their presence in wines. As a consequence, specific knowledge is currently available for winemakers to control and/or prevent the formation of harmful compounds in wine. Additionally, new issues related to the increase of wine alcohol content most likely due to climate change and other environmental awareness are of growing interest to the wine industry as well as to consumers. Wine, both from biotechnology and nutrition understandings, is at the forefront of "-omics" field progress. In the coming days, the "-omics" approaches will provide insights for designing metabolic processes in new-generation wine yeast that need to warrant consumer acceptability, as well as for determining human metabolic traits derived from moderate wine intake.

Wine is considered a hedonic product. One of the main motivations of consumers when consuming the product is the pleasure generated, which is linked to perceived quality. Research about consumer behavior (perception, attitudes, perceived quality factors) and especially about consumer's preferences for new values (sustainability, Mediterranean diet, health) remains a gap in the science of wine and represents a potential barrier to the winemaking sector when marketing wines. However, it is now apparent that different factors acting together can affect aroma perception during wine consumption, which provides us enormous opportunities to improve our understanding in this area. It is well documented in scientific studies published more than three decades ago that moderate wine consumption as part of a diet and

healthy lifestyle is associated with lower risk of developing and dying from diseases such as cardiovascular disease, certain cancers, diabetes, and neurodegenerative diseases such as dementia, Alzheimer's and Parkinson's. Most of these advances have been focused on the study of wine phenolic compounds, confirming their key role in some healthy aspects derived from wine consumption.

From an integrated perspective, the purpose of this book is to provide a state-of-the-art overview of what is known about wine safety and health-related considerations together with the perception of the product from the prospect of the consumer, and to summarize the ways in which such knowledge may be used.

It is hoped that *Wine Safety, Consumer Preference, and Human Health* will be a useful tool for researchers and educators working in both the private and public sectors. Above all, however, it will be a valuable resource for those starting out on the fascinating journey through the world of wine science.

Coordinated by M. Victoria Moreno-Arribas and Begoña Bartolomé Sualdea from the Spanish National Research Council (CSIC), this book brings together a unique collaboration of contributors from a range of experts on the chemistry, microbiology, and nutritional aspects of wine working in universities, research centers, hospitals and medical centers, and government agencies. The editors would like to express their thanks to Springer and all the authors who contributed their expertise and know-how to the success of this book.

Madrid, Spain

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Part I
Wine Safety

Chapter 1
Undesirable Compounds and Spoilage Microorganisms in Wine

Aline Lonvaud-Funel

1.1 Introduction

Wine is the result of the complex transformation of grape juice by the activity of a multitude of yeast and bacteria strains that live on the berry skins. Wine was already produced spontaneously from grapes in Antiquity. As Louis Pasteur proclaimed, "Wine is the healthiest and most hygienic of beverages." Indeed, from the microbial point of view, the environment is so harsh that very few microorganisms can develop. There is virtually no chance of pathogenic microorganisms developing, as is the case in some foods or other beverages. The role of yeast and bacteria in the fermenting grape must is to change the acidic and sugary medium into wine via the key mechanisms of alcoholic and malolactic fermentations. However, just as grape juice is not simply sugar and water, wine is not just an alcohol solution. The finished wine is composed of compounds produced by hundreds of biochemical reactions. The enzymes of yeast and bacteria catalyze the transformation of a complex mixture of grape substrates into wine components. This is how the wine aromas and flavors are developed, giving the wine its typical features, which depends on the grape varieties, the quality of the grapes at the harvest, the production area, and winemaking practices.

However, a few of the thousands of biochemical reactions that take place during winemaking may be detrimental to wine quality. Some of these are related to specific species or strains, qualified as spoilage microorganisms. Others result from the activities of usual strains that develop at an inappropriate moment in the process. For enologists and winemakers, wine quality is above all a question of sensory qualities. Spoilage microorganisms comprise those yeast and bacteria that produce

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off-flavors, such as the ethyl phenol-producing yeast *Brettanomyces bruxellensis*, or the lactic acid bacteria strains responsible for the "wine diseases" described by Pasteur, such as bitterness, "*tourne*" and ropiness. However, other transformations are undesirable from a health standpoint. To date, two problems have been identified: the production of ethyl carbamate and of biogenic amines. The first is the indirect product of the metabolism of *Saccharomyces cerevisiae* during alcoholic fermentation; the others are specific products of only a few strains of lactic acid bacteria. Their presence in wine is not surprising, as they are produced by microorganisms on the grape berry surface, which use grape substrates for growth. Both are usually below the detection limits or at very low concentrations, but may still be considered undesirable under certain circumstances. Many studies devoted to these problems have led to some recommended practices during and after winemaking, in order to reduce the amounts present in the finished wine. This chapter reviews the current knowledge about the presence of undesirable compounds (i.e., ethyl carbamate and biogenic amines) in wine, including pathways and microorganisms involved in their formation, influence of environmental and winemaking conditions, and strategies for minimizing their concentration.

1.2 Ethyl Carbamate in Wine

1.2.1 General Considerations

Ethyl carbamate (EC) is found in several fermented foods and beverages (Dennis et al. 1989; Canas et al. 1989), with the highest concentrations in distillated stone-fruit spirits. It is present in wines at variable but much lower concentrations (Battaglia et al. 1990). After ingestion, EC is metabolized; the majority (90–95 %) is degraded by liver esterases into ethanol, ammonia, and CO_2, which are excreted. The carcinogenic properties reported in a number of studies on several animal species are subject to debate (Schlatter and Lutz 1990; Zimmerli and Schlatter 1991). It is classified as a "probable human carcinogen" (group 2A in 2010) by the IARC (International Agency for Research on Cancer). The mutagenic effect comes from bioactive compounds, nucleic acids adducts resulting from the reaction between DNA (RNA) and EC, or, more probably, vinyl carbamate and vinyl carbamate epoxide, formed by EC oxidation (Gupta and Dani 1989; Park et al. 1993). Reports on the effect on mice are somewhat controversial concerning the simultaneous effects of ethanol and EC: in some instances the risk increased (Beland et al. 2005), while others suggested that the effect of EC was attenuated (Sotomayor and Collins 1990). Interestingly, the mutagenicity of EC decreased when wine was delivered to the mice together with EC, suggesting that other wine components may offset the impact of EC (Stoewsand et al. 1991).

To date, there is no international regulation on EC but some countries have set limits for alcoholic beverages, such as wines and spirits. No limits have been set in Europe, but in Canada the limit is 30 µg/L for wine, 100 µg/L for fortified wines,

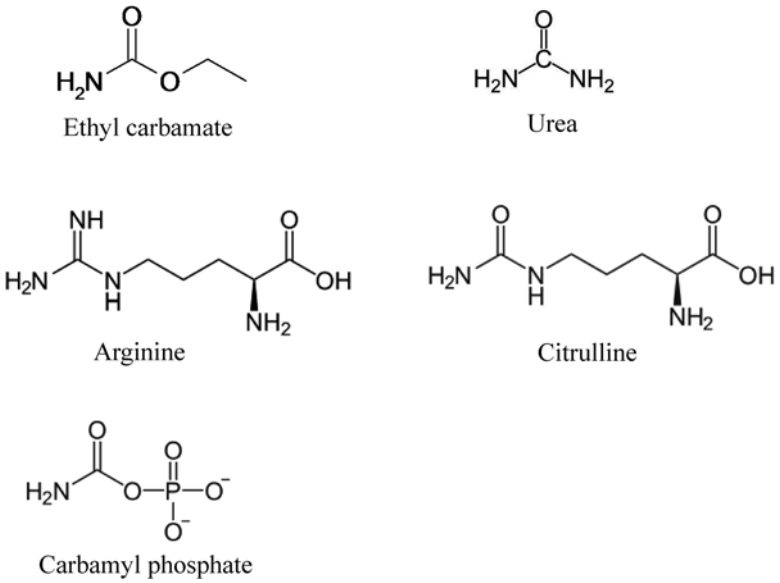

Fig. 1.1 Molecular structure of ethyl carbamate and its precursors in wine

150 µg/L for distilled spirits, and, 400 µg/L for fruit spirits, while in the USA, the figure is 15 µg/L in wine containing under 14 % alcohol, 60 µg/L for those with more than 14 % and 125 µg/L for spirits.

EC accumulates in wine during storage due to the reaction of ethanol with EC precursors, mainly produced by microorganisms: urea, citrulline, and carbamyl phosphate (Fig. 1.1). All these molecules are produced from arginine, one of the main amino acids in grape must, both by yeast and some lactic acid bacteria (LAB). However, the main origin of EC is urea produced by yeast. This is due to the fact that, firstly, the urea concentration is higher than that of citrulline secreted by bacteria and, secondly, the reaction rate of citrulline ethanolysis is lower.

1.2.2 The EC Precursors

1.2.2.1 Formation of Urea by Yeast

In yeast, arginine is hydrolyzed into urea and ornithine by arginase. This is the main origin of urea in wine (Fig. 1.2). Urea is in turn hydrolyzed into ammonia and CO_2 by the association of urea carboxylase and allophanate hydrolase (Cooper et al. 1980). During alcoholic fermentation, arginine, one of the main amino acids, is actively used by yeast and almost totally disappears. Urea accumulates inside the cell and either hydrolyzed to ammonia or excreted. Therefore, the urea

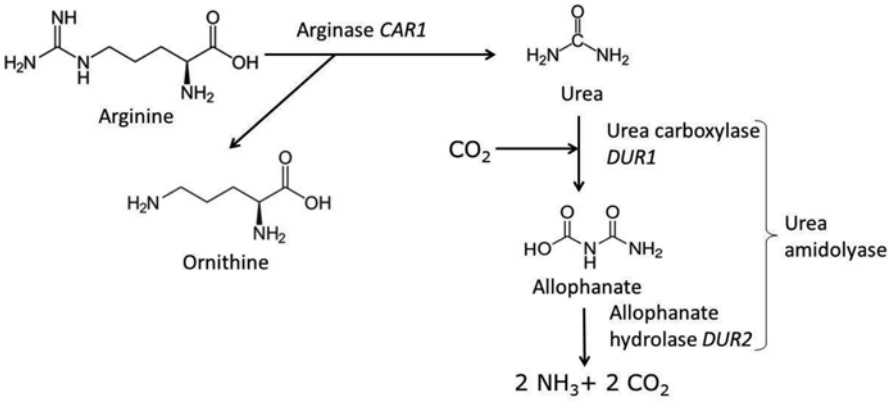

Fig. 1.2 Metabolic pathway of arginine by yeast

concentration increases until mid-fermentation, then decreases at a variable rate (Monteiro and Bisson 1991). Indeed, the excreted urea may be reabsorbed by the cell, thanks to an active transport and facilitated diffusion, and then hydrolyzed (Cooper and Sumrada 1975). In turn, it serves as a nitrogen source after the arginine has been completely exhausted during the active phase of alcoholic fermentation.

In the cell, the urea produced by arginase (CAR1) is metabolized by urea amidolyase, a bifunctional complex of successive activities of urea carboxylase (DUR1) and allophanate hydrolase (DUR2). Several hypotheses have been made to explain the accumulation of urea in fermenting must. Urea is excreted when the hydrolysis rate of arginine is higher than that of urea: it may be a question of delayed expression of the *DUR1,2* genes compared to *CAR1*, or excessively low urea carboxylase plus allophanate hydrolase. The balance depends on the yeast strain: some produce more urea that remains in the wine than others, under the same conditions (Ough et al. 1991; Monteiro and Bisson 1991). Presumably, they uptake and degrade arginine more rapidly before they reabsorb and hydrolyze urea. On the contrary, others do not excrete urea, either due to lack of a transport system or else because they hydrolyze urea rapidly, thus preventing it from accumulating.

The nitrogen source components of the grape also impact the final urea concentration. Supplementing grape juice with arginine leads to an increased urea concentration in the finished wine, in addition to changes in the relative concentrations of other amino acids (Monteiro and Bisson 1992). However, relatively less arginine is degraded when the nitrogen sources are diverse and at high concentrations. Urea accumulation is mainly controlled by gene regulation under nitrogen catabolic repression (NCR), so preferential nitrogen sources are used before the others, including urea. Several papers have described the crucial role of ammonia. Some of the earliest suggested reasons for urea accumulation: ammonia inhibits the utilization of urea after its excretion (An and Ough 1993) and represses the *DUR1,2* genes in fermenting must, leading to the higher urea excretion (Genbauffe and Cooper 1986). The addition of diammonium phosphate (DAP), a usual winemaking practice,

lowers or delays amino acid assimilation, especially that of arginine. This is explained by the downregulation of the *GAP1* gene (general amino acid permease) and *CAN1* (arginine specific permease), as shown by the transcription profile of a *S. cerevisiae* strain in a fermenting must supplemented with DAP and the downregulation of *GAP1*, *DUR1,2*, and *CAR1* (Marks et al. 2003). More recently, Zhao et al. confirmed that the preferred ammonia, glutamine, and asparagine repress urea utilization via downregulation of *DUR1,2* and *DUR3,* as shown by qPCR (Zhao et al. 2013). Due to the complex regulation of the urea metabolism, it is not surprising that even the time when DAP is added significantly influences the potential EC and, moreover, that the effect is strain-dependent (Adams and Van Vuuren 2010).

1.2.2.2 Formation of Citrulline by Lactic Acid Bacteria

Some wine LAB form citrulline from arginine via the arginine deiminase (ADI) pathway (Fig. 1.3) and excrete it (Liu et al. 1994). The activity of these three enzymes has been evidenced in all heterofermentative wine lactobacilli (*Lactobacillus hilgardii, Lactobacillus brevis*) and some *Oenococcus oeni* strains, but not in homofermentative lactobacilli and pediococci (Liu et al. 1995). Interestingly, one *O. oeni* strain was not able to degrade arginine but converted citrulline into ornithine and ammonia, thus having the positive effect of minimizing the EC precursor (Arena et al. 1999; Arena and Manca de Nadra 2005). *L. hilgardii* is frequently present in wines from warm regions. It also dominates LAB in fortified wines at relatively high ethanol concentrations (over 18 %), such as Douro port wines from Portugal (Couto and Hogg 1994). In such wines, irrespective of the concentration of degraded arginine, the citrulline produced is strictly proportional to the potential maximum EC formed during storage (Azevedo et al. 2002).

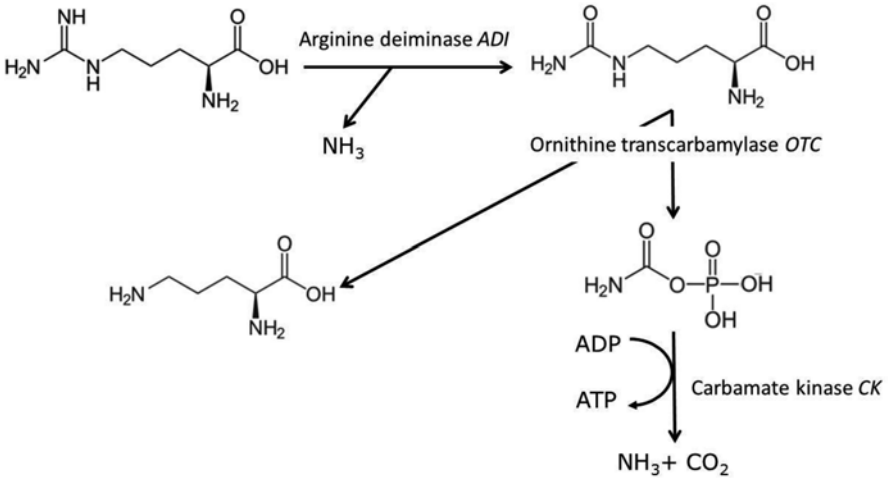

Fig. 1.3 Metabolic pathway of arginine by lactic acid bacteria

In *O. oeni,* the genes coding for the three enzymes needed for arginine degradation are organized in the *ArcABC* gene cluster, preceded by a gene coding for *ArcR*, a CRP-like regulator. Arginine induces the expression of the whole cluster (Tonon et al. 2001). Downstream of this cluster two genes, *ArcD1* and *ArcD2*, code for two arginine–ornithine antiports and complete the ADI genome sequence of more than 9 kb. Downstream from *ArcD2*, *argS2* encodes a putative arginyl-tRNA synthetase and is also induced by arginine. The *ArcABCD1* genes are transcribed in one mRNA, unlike *argS2*, which may play a role in regulation. The whole cluster of more than 10 kb is present in *O. oeni* strains capable of arginine degradation, but not in the others. Genome comparison of ADI-positive and -negative strains revealed that it was inserted/deleted like a mobile element (Divol et al. 2003; Nehme et al. 2006). This explains the earliest findings on the variability of arginine utilization by *O. oeni* strains. Comparison of ADI-positive and -negative strains of *O. oeni* led to a significant result regarding adaptation to wine. Arginine addition to starved viable but non-culturable cells only induced ATP synthesis in ADI^+ strains. Furthermore, it enhanced their growth and induced the revival of declining cells following arginine and carbohydrate exhaustion (Tonon and Lonvaud-Funel 2000). Moreover, it significantly suppressed the decline phase of ADI^+ strains under acidic conditions. Finally, incubating ADI^+ cells in the presence of arginine protects them from stress when they are added to wine (Nehme 2004). Possibly, not only heterofermentative lactobacilli, mainly represented by *L. hilgardii*, but also *O. oeni* may be implicated in citrulline production in wine.

1.2.3 EC Accumulation Factors

1.2.3.1 Precursor Formation Parameters

EC is formed by the spontaneous chemical reaction of ethanol and precursors. Therefore, the main factors that influence the final EC concentration are related to the accumulation of precursors, urea, and to a much lower extent, citrulline. In the late 1980s, when EC started to be a real concern, investigations were commissioned in several countries to investigate the problem. It was quickly noted that the extent of the problem was not only linked to microorganisms. Grape varieties and wine producing areas also influenced the final concentration of EC. As, for example, in France, where a survey showed that wines produced in the north-east (Alsace, Bourgogne, Champagne) contained more EC than those from other regions (Ingargiola 1992). In addition, within a given region, the grape variety also had an influence, as some are richer in amino acids (Larcher et al. 2013). However, the data are difficult to interpret, since amino acid concentrations in must also depend on the rootstocks, grape growing practices, fertilization of the vines, and climatic conditions during ripening. Furthermore, overripe grapes have higher concentrations of sugars and lower amino acid levels.

Fertilization is one of the factors that can be controlled. Excessive nitrogen levels generate a significant increase in amino acids in the grapes. The arginine in must is proportional to fertilization: indeed, levels over 1000 mg/L act as an indicator of over-fertilization (Butzke and Bisson 1997). For example, fertilizing a vineyard with 100 kg/ha for 2 years increased the total nitrogen in Merlot grape must, resulting in arginine concentrations 460 mg/L higher on average than the control (Bertrand, unpublished).

For several years, yeast nitrogen requirements have received much attention from researchers investigating slow or stuck fermentation. Roughly speaking, demand appears to vary according to the strain. Once the yeast assimilable nitrogen (YAN), including ammonium and free amino nitrogen, has been determined, DAP may be added in appropriate amounts to promote yeast population growth. More recently, the influence of YAN and, in particular, amino acids on the aromatic profile has been reported by several authors. Commercially available organic nitrogen sources, mainly yeast derivatives, affect both the fermentation rate and, to some extent, the aroma profile. However, no published data is available to date on the possible effect on urea and EC of an overdose resulting in excessive arginine concentrations.

Lees contact gradually delivers yeast components. These include peptides, which are hydrolyzed by yeast and provide a source of free amino acids. This has not been identified as a cause of an increase in EC precursors, but this effect presumably depends on the yeast strain used for alcoholic fermentation (Tegmo-Larsson and Henick-Kling 1990). In general, lees contact after alcoholic fermentation enriches wine in amino acids and other growth factors for LAB, which explains why malolactic fermentation is easier under these conditions. The risk is greater when malolactic fermentation occurs in wine during in extended contact with lees. Arginine utilization by heterofermentative lactobacilli and ADI$^+$ *O. oeni* strains promotes growth (Tonon and Lonvaud-Funel 2000; Terrade and Mira de Orduña 2006). Indeed, any practices that affect the initial arginine concentration may also influence the final concentration of citrulline after malolactic fermentation, if the bacteria survive. However, the impact of bacteria is much lower than that of yeast via urea.

1.2.3.2 Influence of Environmental Factors on the Production of EC from Its Precursors

The reaction between ethanol and precursors is spontaneous and takes place over time during aging. In fact, the potential EC content should be evaluated, rather than just the current level. EC concentrations may be nil or low at the end of alcoholic fermentation, but this does not mean that it will not increase to excessive levels over time. It is possible to predict the maximum amount of EC that will form during months of storage from the urea concentration in wine and the storage conditions.

Like any chemical reaction, its rate depends on the concentrations of molecules and temperature. The Arrhenius plots for urea and citrulline may be used to predict the rate of EC formation; it is lower with citrulline than urea (Ough et al. 1988). The maximum possible EC can be evaluated by heating the wine at 80 °C for 48 h. The

effect of temperature, pH, and wine type was investigated during 2 years' storage. At plausible initial concentrations of urea and citrulline, the most striking factor was temperature; a 10 °C increase multiplied EC production by 3. The authors concluded that urea concentrations should be under 5 mg/L and temperatures below 24 °C to limit EC to reasonable levels (Stevens and Ough 1993). Low-temperature storage of bottled wines at prevents EC synthesis, while it may reach undesirable levels in the same wine at 40 °C, for example (Larcher et al. 2013). Interestingly, EC production has been used as an indicator for measuring the accumulated heat exposure of wines during shipping (Butzke et al. 2012), which also causes other deteriorations. EC formation was also studied for 3 years with time and temperature as variables, according to the initial concentrations of urea and citrulline. An equation was obtained for predicting EC concentrations on the basis of times and temperature (Hasnip et al. 2004).

1.2.4 How to Limit the Production of Precursors and EC

Preventive and curative methods for reducing EC concentrations in wines are directly based on knowledge about production conditions. In prevention, as the main precursor is urea via the arginase pathway of yeast, any practice that would minimize the quantity of arginine metabolized, arginase activity, and the urea transporter, would be a possible solution. If the EC potential is too high, the alternative is to hydrolyze urea after it has been accumulated. However this cannot solve the problem of the bacterial origin via citrulline. All the precautions, from nitrogen fertilization in the vineyard to temperature control of during storage, are summarized in the "Preventive action manual" (Butzke and Bisson 1997). They are all based on vineyard and winemaking practices recognized as parameters in EC synthesis, as described above. Some of them are simple and winemakers just need to adapt the process. Other possibilities have been studied and require further evaluation, but some have already led to applications.

1.2.4.1 Urease Treatment

The addition of urease to reduce the urea content was suggested very early on, when the urea problem was first identified in sake wines (Yoshizawa and Takahashi 1988). The first experiments with killed *Lactobacillus fermentum* cells demonstrated the effectiveness of the treatment. In the pH range of wine, urea can even be totally removed. However, the effectiveness of this treatment depends on wine composition, with malic acid being one of the strongest inhibitors (Ough and Trioli 1988; Trioli and Ough 1989). A survey of a significant number of wines showed that the EC potential was reduced on average by 44 % for dessert wines and 84 % for table wines (Fujinawa et al. 1990). Urease treatment is allowed when the urea concentration is over 1 mg/L in wines that are to be aged.

1.2.4.2 Arginase Suppression

If arginine is not used by yeast, the main EC precursor is avoided. Based on this observation, a sake yeast was engineered to disrupt the two copies of the *CAR1* gene encoding for arginase in this diploid strain. The mutant strain was used in laboratory-scale sake brewing, thus proving the stability of the disrupted locus. The resulting sake did not contain urea and the general chemical analysis was the same as that of the control sake, fermented using the parent strain, but the arginine concentration was higher and that of ornithine was lower (Kitamoto et al. 1991). Using this mutant, the authors established a protocol to isolate arginase mutants from populations of sake and wine yeast strains, to avoid the use of engineered strains (Kitamoto et al. 1993). Arginase inhibition was also obtained by the antisense method in order to repress the expression of the *CAR1* gene, but no fermentation tests were conducted (Park et al. 2001).

1.2.4.3 Enhancing Urea Hydrolysis by Yeast Urea Amidolyase

As shown in Fig. 1.1, urea is carboxylated to form allophanate, which is then hydrolyzed into ammonia and carbon dioxide. The bifunctional urea amidolyse enzyme is encoded by the *DUR 1,2* genes. Since urea is toxic for yeast at high concentrations, it is exported into the medium when nitrogen conditions repress *DUR1,2*. The nitrogen metabolism is regulated in *S. cerevisiae* by a nitrogen catabolism repression (NCR) system, which impacts the *CAR1* and *DUR1,2* genes. Excess urea accumulates if the latter are repressed, thus reducing urea hydrolysis. On the contrary, the constitutive expression of *DUR1,2*, by integrating a copy of the genes between the suitable signals (*PGK1* promoter and terminator), makes it possible for the enzyme to be synthesized under conditions where normally it is not. This was achieved in a laboratory strain, and then in a commercial wine strain. The engineered wine strain was genetically stable, and hydrolyzed urea efficiently, so that the maximal potential EC of Chardonnay wines produced with it decreased by 90 % (Coulon et al. 2006).

1.2.4.4 Improvement of Urea Reabsorption

Urea is transported inside the cell by a facilitated diffusion coded by *DUR4* and an energy dependent transporter coded by *DUR3,* under the control of the NCR system. In another approach to reduce the urea content in wine, the *DUR3* gene was inserted between the same two signals as for *DUR1,2*, so that it was expressed constitutively. This engineered DUR3 strain plus the DUR1,2 strain and the DUR1,2/DUR3 strain were used to ferment Chardonnay and compared to the parental strain. The potential EC in the wine was reduced to nearly the same extent, i.e., about 81 % (83 %, 81.5 %, and 80.5 %, respectively). The other important result was that no impact was noted on the alcoholic fermentation capacity of the strain. (Dahabieh et al. 2009). Two sake yeasts were modified using the same approach and the results were exactly

the same (Dahabieh et al. 2010). The engineered strains of both the wine and sake yeasts were similar to the parents in terms of genotype and transcriptome except, of course, for the presence of the cassette comprising the DUR genes, the promoter and terminator. This made these strains more acceptable for commercialization.

1.2.4.5 Selection of Starters

Considering that yeast or malolactic starters can take over from the indigenous population, at least in the most active phases of alcoholic and malolactic fermentation, the EC problem may possibly be controlled by choosing the catalogue strains that produce the least urea or citrulline. Indeed the ability of *S. cerevisiae* or *O. oeni* to produce EC precursors is strain dependent. Regarding yeasts, some authors concluded that significant differences existed according to the strain (Ough et al. 1991). The result also depends on the grape variety (Larcher et al. 2013), but the urea production of selected commercial strains is not documented. Winemakers cannot, therefore, include this parameter among the criteria used to choose starters, unless information is available on previous use.

Regarding malolactic starters, the situation is much clearer. As explained above (Sect. 1.2.2.2), *O. oeni* strains have the genes for arginine degradation and citrulline production or not. It is easy to detect citrulline-producing strains using PCR by focusing on the *Arc* gene cluster. Regions of the genomic sequence are conserved in several wine bacteria species and provide PCR primers. Inoculation with selected ADI-negative malolactic strains reduces the risk. The beneficial effect of arginine on the adaptation and growth in wine should also be considered, since the efficiency of starters is still not fully proven. It is possible that ADI-positive strains may be more efficient, but this has not been evaluated. Moreover it must be emphasized that citrulline is not the main EC precursor.

1.3 Biogenic Amines

1.3.1 General Considerations

1.3.1.1 Impact on Health

Many foods, especially fermented foods, contain biogenic amines (BA) in variable concentrations, depending on the raw material, process, and possible microbial contamination. As is the case in wine, strains that are normally involved in fermentation may produce BA. Some LAB strains, which are part of the whole microbial system produce BA during or after malolactic fermentation. BA are a risk factor for intolerance and toxicity at high concentrations. Sensitivity to their effects depends on the person and their state of health. Adverse effects are mainly due to a defect in detoxification by monoamine and diamine oxidase activities. Drugs and ethanol can act as

inhibitors (Marquardt and Werringloer 1965; Sattler et al. 1985). Acetaldehyde, an intermediate in the ethanol metabolic pathway, competes with aldehyde metabolites of histamine and inhibits its elimination (Zimatkin and Anichtchik 1999).

Some foods contain much higher BA concentrations than wine. Toxicity depends not only on BA concentrations, but also on the quantity of food and beverages ingested, as well as any drug intake. Histamine, tyramine, and putrescine are the most likely to trigger intolerances. They are not generally the most abundant in wine, but cadaverine and, above all, putrescine, frequently at higher concentrations, are said to potentiate the toxicity of the other compounds. Today there is still much controversy on the topic. Observations were conducted on a population of wine-intolerant persons who ingested wines with low and high histamine concentrations. The intolerance was noted for nearly all of them, irrespective of the histamine concentration. Blood analysis and clinical findings suggest that another wine component is implicated in the intolerance (Kanny et al. 2001).

1.3.1.2 Origins of BA in Wine

Grapes contain BA, the most abundant being generally polyamines, including spermidine, putrescine, and spermine. Histamine and tyramine concentrations are usually much lower. But, as in the case of amino acids, this is highly dependent on the grapes: variety, ripeness, and the nitrogen fertilization (Bach et al. 2011; Smit et al. 2014).

Concentrations of some BA increase slightly during alcoholic fermentation, while others, like polyamines, decrease. Wine yeasts do not generally produce BA. *S. cerevisiae* and non-*Saccharomyces* strains isolated from wines and used to induce fermentation do not cause significant increases in BA (Marcobal et al. 2006; Landete et al. 2007). However, conflicting reports are not surprising, in view of the extreme diversity of yeast, the variability of grape must composition, particularly its nitrogen content, and the practical conditions of fermentation. BA-producing activity in a single species like *S. cerevisiae* varies according to the strain. In some cases, wines obtained using indigenous yeast had even lower BA concentrations than those obtained with selected starters (Torrea and Ancin 2002). In some instances, although the concentrations were low, *S. cerevisiae* and *B. bruxellensis* produced more BA than other non-*Saccharomyces* yeasts (Caruso et al. 2002). *B. bruxellensis,* which is mostly feared for its off-flavors, has a confirmed capacity to produce amines. However, it releases small amounts of polyamines, rather than the more undesirable histamine and tyramine (Vigentini et al. 2008). Other minority non-*Saccharomyces* species, such as *Zygoascus hellenicus, Issatchenkia orientalis, Issatchenkia terricola, Pichia manushurica,* and *Metschnikovia pulcherrima*, have variable amino acid decarboxylase activities that release BA, depending on the strain (Tristezza et al. 2013).

The first works on BA in wines focused on histamine (Lafon-Lafoucade 1975), then extended to others, like tyrosine and putrescine, which are often more abundant. BA were gradually determined in all wine-producing countries, first providing an overview of the situation in each producing area, and then attempting to relate the results to viticultural and winemaking practices. Dozens of papers are now avail-

able. Roughly speaking, they all reach the same conclusions: red and white wines contain varying quantities of BA. Concentrations are usually very low, not exceeding the limit of 10 mg/L set in the past by Switzerland. Today, there are no official regulations on the histamine content of wine, but importers or buyers may set their own limits.

A survey of the literature on the topic reveals that BA concentrations are highly variable, but the common point is that they always increase after malolactic fermentation, implying that the main source of BA in wines is the LAB activity during malolactic fermentation or even afterwards, if they survive (Soufleros et al. 1998; Lonvaud-Funel 1999; Lonvaud-Funel 2001; Marcobal et al. 2006; Moreno-Arribas and Polo 2008).

1.3.2 BA-Producing Pathways

1.3.2.1 Overview

As early as 1965, the origin of BA in wines was suspected to be "bacterial infection" (Marquardt and Werringloer 1965). Although wines contain several BA, the first works dedicated to the topic focused on histamine, concluding that few wine LAB were capable of producing this substance (Lafon-Lafoucade 1975; Radler 1975). However, the results remained very controversial for some time. Then histamine-producing strains of *L. hilgardii* and *O. oeni* were isolated from Argentinean and French wines (Farias et al. 1993; Lonvaud-Funel and Joyeux 1994), as well as tyramine-producing strains (Moreno-Arribas and Lonvaud-Funel 1999). More recently attention has been paid to putrescine, which is the most prevalent BA in wine (Mangani et al. 2005). Most of the research in recent years has focused on the genetics of BA-producing pathways and significant results have been obtained. However, the crucial question of the conditions required for bacteria to accumulate BA in wines is still unresolved; indeed it is much more difficult to identify the environmental parameters involved and understand how they interact, than to identify the genes. Therefore BA-producing strains are needed but predicting the risk of their undesirable activity is not yet fully possible.

1.3.2.2 Histamine Production

The microflora harvested following centrifugation of a wine with a high histamine content after malolactic fermentation is able to produce histamine in a sterile wine. Histamine is produced in larger amounts if the environmental conditions for growth are unfavorable (low pH, high alcohol) and when lees are added. *O. oeni* strains were isolated from the LAB population and histidine decarboxylase (HDC) activity was identified (Fig. 1.4). Most of the strains lost their activity in subcultures

(Lonvaud-Funel and Joyeux 1994). These were the first results on the topic, which initiated the genetic approach. The HDC of an *O. oeni (Leuconostoc oenos)* strain was purified to homogeneity and the kinetics parameters determined. A pyruvoyl-dependant enzyme is specific to histidine and, thus, unable to decarboxylate the other amino acids. The gene (*hdcA*) coding for the protein (HDC) was sequenced. Data analysis showed that the protein was synthesized as a proenzyme π, activated by serinolysis to form α and β subunits, that is active as a hexamer $(\alpha\beta)_6$ (Coton et al. 2010). PCR primers based on this sequence were designed and then used for an extensive survey of the histamine-producing bacteria in wines from many countries. Quantitative PCR revealed that up to 10^7 HDC^+ strains/mL may develop during winemaking, while excessive histamine concentrations seemed to be produced by 10^3 HDC^+ strains/mL (Lucas et al. 2008). The *hdcA* gene, like the ability to produce histamine, also exists in other wine LAB genera, such as pediococci and lactobacilli, but, like *O. oeni* strains, they can lose their phenotype. This instability was studied in a strain of *L. hilgardii* isolated from a red wine. It was explained by the instability of a plasmid, which was lost under favorable growth conditions and maintained under poor nutritional and acidic conditions. The plasmid carried a locus comprising the *hdcA* gene, an *hdcP* gene coding for a histidine/histamine exchanger upstream, and two *hdcB* and *His RS* genes downstream (Lucas et al. 2005). The putative functions of the proteins encoded by the latter two were activation of the HDC proenzyme and a histidine-tRNA synthase. The nucleic sequences are notably well conserved in the various wine LAB species.

HDC^+ strains have a growth advantage under adverse conditions, since they benefit from the metabolic energy provided by decarboxylation coupled with the histidine/histamine exchange. Some strains seem to have integrated the gene cluster in the genome, while others still carry it on a plasmid. The cluster is present in many strains of *O. oeni*, the dominant species during malolactic fermentation. However, in this species, it is probably more unstable than in others which explains why none of the selected commercial starter strains are HDC^+, following a probable loss of the plasmid during the numerous stages in cultures.

1.3.2.3 Tyramine Production

Tyramine is produced in a one-step reaction by decarboxylation of tyrosine (Fig. 1.4). The tyrosine decarboxylase (TDC) activity of wine LAB was first evidenced in *Lactobacillus brevis* and *L. hilgardii* and shown to involve a pyridoxal phosphate-dependent enzyme, which was confirmed by protein and gene sequencing (Moreno-Arribas and Lonvaud-Funel 1999; Moreno-Arribas et al. 2000; Lucas and Lonvaud-Funel 2002). All the proteins needed for the activity of the *L. brevis* strain studied were coded by the TDC operon, which, in addition to the *tdc* gene comprised the *tyrP* gene coding for TyrP and a gene coding for tyrosyl tRNA synthase. TyrP catalyzes the tyrosine/tyramine exchange and the role of tyrosyl tRNA synthase was not demonstrated. Tyrosine decarboxylation and the exchanger are

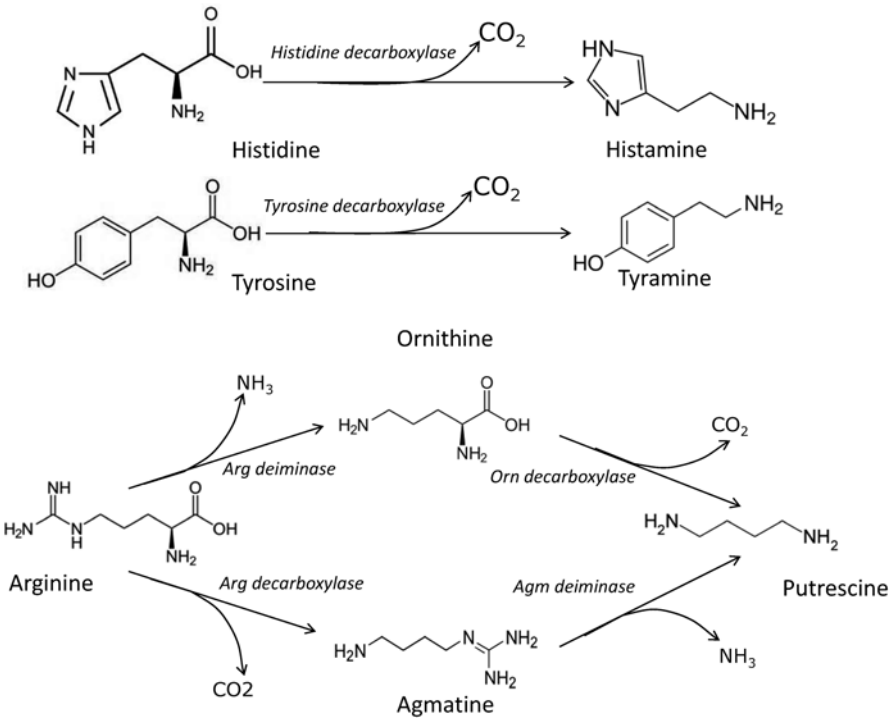

Fig. 1.4 Biogenic amines production pathways by lactic acid bacteria

beneficial to the cell by providing energy. Like the decarboxylation of other amino and organic acids by LAB, the alkalinization resulting from decarboxylation plays a role in pH homeostasis and tolerance to acid stress (Lucas et al. 2003; Wolken et al. 2006).

1.3.2.4 Putrescine Production

Putrescine is the most abundant BA in wine. It is produced by LAB, either by decarboxylation of ornithine by ornithine decarboxylase (ODC), or from arginine, which is generally much more abundant in grape must and wine. In this second case, there are two possible routes, each depending on the successive activity of two enzymes: either ADI plus ODC or arginine decarboxylase plus agmatine deiminase (AgDI), which functions very similarly to ADI (Fig. 1.4), via carbamoyl putrescine. Therefore, strains which carry both the ADI and ODC systems can produce putrescine from both arginine and ornithine, according to their availability in the medium. In a study of more than 100 wines, the ODC^+ populations reached a higher level than AgDI. The closer correlation between the putrescine concentration and the ODC^+ population, rather than the AgDI population, suggests that the former are

mainly involved in wine spoilage (Nannelli et al. 2008). On the contrary, the AgDI$^+$ strains seem to be related to the cider environment (Coton et al. 2010).

The first *O. oeni* strain able to produce putrescine from ornithine was isolated from a wine containing BA (Coton et al. 1999, 2010). This was later confirmed by others and the *odc* gene of an *O. oeni* strain was identified (Guerrini et al. 2002; Marcobal et al. 2004). However *odc*-carrying gene strains are rare in *O. oeni* laboratory collections, indicating that the genes may be lost during successive laboratory cultures. ODC seems to be more present in lactobacilli, namely *L. brevis.*

In *L. brevis* and *O. oeni,* the ODC operon comprises the *odc* gene and the downstream *potE,* which encodes the ornithine/putrescine exchanger protein PotE. These ODCs can also decarboxylate lysine and diaminobutyrate, leading in the first case to the formation of cadaverine, a component of the BA pool in wines. However, the affinity and catalytic efficiency are much higher for ornithine, especially in *O. oeni* (Romano et al. 2012). The PotE transporter of *O. oeni* also has an affinity for lysine and cadaverine.

The AgDI pathway is probably often involved in the production of putrescine. A strain of *L. hilgardii,* isolated from wine, decarboxylated ornithine and arginine, respectively, to form putrescine and agmatine, which, in turn, was deaminated (Arena and Manca de Nadra 2001). Similarly to ADI, in the AgDI pathway, deiminase yields ammonia and carbamoyl putrescine; then putrescine transcarbamylase is phosphorylated to produce carbamoyl phosphate and putrescine. Finally, carbamoyl phosphate combines with carbamate kinase to produce CO_2 and NH_3, as well as ATP. Like the ADI pathway, this provides energy for the cell and contributes to pH control. In an *L. brevis* isolated from wine, the AgDI cluster of 6 genes coding for the proteins required was identified: *AgmP* agmatine/putrescine exchanger, *AgDI* (two homologous genes), putrescine transcarbamylase, *Arc C* for carbamoyl kinase, and finally a regulator (Lucas et al. 2007). This strain produced large amounts of putrescine from agmatine, but not from arginine. *AgmP* is specific to agmatine/putrescine and unable to transport arginine and ornithine. The AgDI pathway seems to be common in wine isolates of several LAB species, but not in all strains of the same species.

1.3.2.5 Common Considerations on BA Pathways

The physiological interest of the BA-producing pathways is their contribution to the response to acidic stress tolerance and cell energetics. Decarboxylation and deamination participate in intracellular pH homeostasis. The transmembrane potential and exchange of substrate/product generates a supplement of ATP. In a medium deprived of primary energy sources, such as sugar, which is the case in wines, especially during aging, this should be highly advantageous for biogenic amines producing strains (BA$^+$). Genomic approaches using several strains revealed that decarboxylating pathways were encoded by clusters of genes acquired by horizontal gene transfer (Romano et al. 2014). In an *L. brevis* wine strain, the three gene clusters coding for the malolactic reaction (MLF), AgDI, and ODC are assembled in a genomic region of approximately 20 kb, while in an *L. casei* the region comprises MLF, AgDI, and

TDC, and, finally, in another *L. brevis* the locus comprises only MLF and AgDI. AgDI and TDC are widespread in lactobacilli and are often present in the same strains. The clustering of these physiologically important functions may be understood as a result of evolution in the wine environment.

1.3.3 Essential Factors for BA Production

Wines always contain free amino acids or peptides and proteins that can be hydrolyzed to amino acids. On the other hand, except for "sterile wines," they also host microorganisms. BA synthesis requires BA^+ bacteria and amino acids. The level of BA^+ bacteria and their activity is crucial. To the best of present knowledge, a risk can be predicted as soon as this specific population exceeds 10^3 CFU/mL (Nannelli et al. 2008). However a linear relationship between the concentration of bacteria and amines is not obligatory. In addition to the concentration of precursors, numerous environmental factors may control bacterial activity and require further study.

Some wines do not contain any amines while others generally have most of them: histamine, tyramine, putrescine, cadaverine, and the minor phenyl ethylamine. This implies that in this latter group, the population comprises a variety of BA^+ strains, and/or strains with several BA-producing activities, as amino acid decarboxylation is specific to the substrates. For example, some strains have all the enzymes needed to produce tyramine and putrescine.

In winemaking practice, it should be noted that wine may contain BA although no BA^+ bacteria have been detected. The explanation is very simple, but is not often considered: the BA concentration at a given time does not preclude any microbial activity in the past. The BA^+ population may have grown and produced BA before declining; consequently, at the time of analysis, the amines are still present but the bacteria are no longer detectable. This simple observation may explain some controversial reports on the relationship between the bacterial population and the final amine concentration.

1.3.3.1 Occurrence of BA-Producing Strains in Wine

Extensive work on the BA pathways has identified the enzymes and genes. The genes coding for HDC, TDC, ODC, and AgDI have been sequenced and it is now possible to select oligonucleotides to provide specific primers. The first PRC test was developed for histamine-producing strains and proved its reliability for detecting the bacteria directly in wines (Le Jeune et al. 1995; Coton et al. 1998a). Other primers were then selected for *hdc* and *tdc, odc,* and *agdi* genes in turn, which differed to varying degrees according to the authors (Lucas and Lonvaud-Funel 2002; Marcobal et al. 2004; Lucas et al. 2007). Primers were also chosen to develop multiplex tests adapted to the simultaneous detection of all the possible BA^+ bacteria (de Las Rivas et al. 2005; Sciancalepore et al. 2013).

Similarly, primers for quantitative PCR are now available (Nannelli et al. 2008). It was, therefore, possible to quantify the BA^+ population throughout the winemaking process. Results showed that, from the grape must to the finished wine, BA^+ populations developed in exactly the same way as other LAB. They multiply after alcoholic fermentation and carry out malolactic fermentation, possibly reaching populations as high as 10^7 CFU/mL.

It must be emphasized that the gene sequences coding for the BA pathways are very similar in all species. The judicious selection of primers focused on conserved regions has made possible to produce PCR tests that are really specific to the BA pathways, regardless of the species. For example, any *O. oeni* strain and any species of *Lactobacillus* or *Pediococcus* able to produce BA can be detected. In practice, it is obviously very important to assess the risk.

1.3.3.2 Influence of Vintage, Vineyard Management, and Winemaking

The final BA concentration in wine depends on the availability of the precursor amino acids and the BA^+ population. Both are determined by environmental conditions, on a large scale in the vineyard and on smaller scales in the cellar, tanks, and barrels.

In poor climatic conditions, with cool temperatures and high humidity, and when storms have damaged the pellicle, grape berries carry more genera of fungi, yeast, and bacteria on their surface. This highly complex ecosystem favors the diversity of spoilage microorganisms, particularly BA-producing LAB. It is the opposite if the weather is warm and dry. This partly explains why the vintage is so important (Martín-Álvarez et al. 2006). In a given area, the prevalence of BA^+ bacteria is extremely variable from 1 year to another.

The variation in BA concentrations with vintage also depends on initial total nitrogen content and ripeness (Ortega-Heras et al. 2014). Nitrogen fertilization of the grapevines increases the concentration of nitrogen compounds in grape must, including both amino acids and biogenic amines, such as aliphatic polyamines (agmatine, cadaverine, diaminobutane, spermine, and spermidine). This has a positive impact on alcoholic fermentation rate and completion, as yeast growth and activity depend on assimilable nitrogen. However, high nitrogen levels may have side effects by providing more substrates for undesirable bacteria, such as promoting the production of EC by yeast (see Sect. 1.2.3.1). Not only do LAB grow more easily, but also, thanks to the BA metabolic pathways, the BA^+ strains take advantage of amino acid degradation to remain viable and active in the wine for longer. Briefly, fertilization increases the concentration of amines in musts and wines (Smit et al. 2014).

Long maceration, to enhance the extraction of grape components, may also have an influence (Martín-Álvarez et al. 2006), due to the increased availability of amino acids and precursors, like peptides and proteins. However, not all the results are in accordance, probably because all the maceration factors cannot be taken into account, most importantly the state of the grapes.

After alcoholic fermentation, free amino acids are those that have not been consumed by yeast, as well as those released by yeast. The variety can be even wider in wine than in grape must. Yeast autolysis enriches the wine in free amino acids, peptides, and proteins. All of them are present in even higher concentrations as the contact time with the yeast lees is extended, with final levels depending on the yeast strain (Lonvaud-Funel and Joyeux 1994; Rosi et al. 2009). Earlier works on the subject noted that BA concentrations in wines increased during aging in barrels, tanks, and, possibly, bottles. This has been confirmed more recently by other authors (Alcaide-Hidalgo et al. 2007). This is not surprising, as undesirable strains that are capable of growing during fermentation are certainly able to survive afterwards. These particular strains have been gradually selected from the original diverse LAB population in grape must and their remarkable adaptation to wine results, at least partly, from the BA producing pathways, which provide them with energy and acid tolerance.

The major factor in the spontaneous selection of bacteria from grapes to wine is pH. The higher the pH, the greater the diversity in strains and species, and the higher the LAB concentrations. It is well known that *O. oeni* constitutes the major population in most wines, but Lactobacilli and Pediococci participate in malolactic fermentation and may be the dominant bacteria in wines with a high pH. Their BA pathways are more frequent and stable than those of *O. oeni*, which explains why wines with a pH higher than 3.6 contain more BA. This is related not only to the region of production but also to the degree of ripeness. Overripe grapes usually produce musts with a relatively high pH. In addition, in high pH wines, all species of bacteria develop much faster and reach higher concentrations so their total activity in converting amino acids is enhanced.

1.3.4 How to Avoid or Minimize BA Production

Recommendations for decreasing the risk consist of achieving the opposite conditions to those observed to increase it (Sect. 1.3.3). If BA^+ populations have been detected, or are suspected, due to problems in previous years, some simple practices should be implemented to minimize BA production.

Obviously it is not possible to eliminate undesirable indigenous bacteria, which form an integral part of the wine microbiota. It is, therefore, necessary to minimize their presence, as far as possible, during the process. The only simple solution is the use of malolactic starters (Nannelli et al. 2008; Hernandez-Ortes et al. 2008; García-Marino et al. 2010). After inoculation, the selected strain becomes dominant during malolactic fermentation. The indigenous bacteria do not have the time to grow significantly, since malolactic fermentation is usually accelerated. They remain at too low a level to produce a significant quantity of amines. However, the malolactic starter does not eliminate them, so that they can multiply afterwards. If wine is to be aged for a long time, microbial analysis is necessary, including the detection of undesirable strains. They are normally eliminated by sulfiting, but this is often less efficient than expected. The first reason is that the strains capable of surviving all

the winemaking steps are those best adapted to "wine stress" (including pH, ethanol, sulfur dioxide, fatty acids, and all toxins that have yet to be identified). The second reason is that free sulfur dioxide is less active at high pH and this is exactly the case in wines that host a wide diversity of populations.

The other recommendation is to avoid the practices that increase amino acid and peptide concentrations. As already noted above, nitrogen fertilization of the vine induces higher amino acid concentrations in grape must. This is positive for yeast growth and, consequently, for the completion of alcoholic fermentation. However, it has other, less desirable, effects: in addition to possible deterioration of sensory quality, the wine contains higher levels of amino acids (Bell and Henschke 2005). This also implies that LAB have more substrates, not only for producing BA but also to survive better and longer after malolactic fermentation. It is the same with DAP and other nitrogen supplements, added before alcoholic fermentation to promote yeast growth, as well as yeast autolysates added afterwards to promote malolactic fermentation (González Marco et al. 2006). These should only be added if really required, on the basis of an analysis of assimilable nitrogen. Also, for the same reason, if BA^+ bacteria are suspected, it is recommended to separate the wine from the lees as soon as the alcoholic and malolactic fermentations are completed.

Until now, there has been no useful treatment for reducing BA concentrations after they have accumulated in wine. However, it has been shown that rare wine LAB strains have amine oxidase activities that degrade histamine, tyrosine, and putrescine (García-Ruíz et al. 2011). They do not seem to be sufficiently effective to really reduce BA in wines. Nevertheless, this phenomenon may explain some of the unusual, sporadic observations of slight spontaneous decreases in BA in wine during aging.

1.4 Conclusions

In our current state of knowledge, EC and BA are the only undesirable compounds of microbial origin in wine that raise potential health concerns. Even if not all wine-producing countries have established limits, concentrations should be reduced as far as possible. Excessive concentrations may be detrimental to trade. The concentrations of these substances in the finished wine depend mainly on the microorganisms present and the availability of amino acids and peptides. However, the concentration and variety of nitrogen compounds also have a significant impact on fermentation rates and completion. This is why winemakers are prompt to supplement must or wine with ammonium or yeast derivatives, despite the fact that an excess of organic nitrogen indirectly induces an increase in EC and BA.

EC is produced by a post-fermentation chemical reaction. Its precursors, urea and citrulline, are released by yeast and arginine-metabolizing LAB, respectively. However, the main difference between the two microorganisms is that all yeast strains degrade arginine and produce urea, while only some LAB produce citrulline. Urea production depends on the dominant *S. cerevisiae* strain during alcoholic fer-

mentation: some produce more than others. Citrulline production also depends on the strains present, but LAB should not really be considered as a risk factor for EC.

Despite a great deal of research, including toxicological studies, it has not been scientifically proven that BA are responsible for serious health problems (EFSA 2011). Ultimately, the most important finding was the discovery of the importance of these pathways as adaptation mechanisms to the oenological niche. All the BA pathways provide energy to cells and participate in pH homeostasis. They are determined by similar gene clusters that have been integrated in all wine LAB genera by horizontal gene transfer and are more or less stabilized in the genomes according to the species. Among other characteristics, they are examples of adaptation mechanisms to a harsh environment.

Finally, it is possible to issue recommendations to winemakers to avoid excess EC and BA, on the basis of research results. Oenological practices, including nitrogen addition, contact with grape solids and lees, the use of starter cultures, etc. must take into account the interactions among all the elements in the ecosystem.

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Chapter 2
Utilisation of Natural and By-Products to Improve Wine Safety

Francisco M. Campos, José António Couto, and Tim Hogg

2.1 Introduction

As a chemical definition, *natural products* are small molecules derived from biological sources, most often being secondary metabolites (Anon 2007). The study of such natural products is therefore largely directed at the biological and chemical properties of isolated molecules. In this chapter the natural products definition is widened to include proteins and other larger molecules that can have a role in wine safety and quality. By-products, on the other hand, refer to mixtures of compounds that are left after the extraction of primary value from biological, normally agricultural material. Thus, by-products are not distinct from natural products; rather they are complex mixtures of them in a matrix that will also often contain many other categories of molecules and higher structures.

Natural products and their vehicles such as by-products, when they possess relevant biological activities, are attractive candidates as food additives. Their natural status confers on them a ready acceptance amongst consumers and where they fulfil the function that would otherwise be taken by a synthetic additive this can be used in product communication, often to great effect.

Safe food is that that does not provoke pathologies provided that it is prepared and consumed according to its intended use (Codex Alimentarius 1997). In the case of wine, *intended use* infers *moderate consumption*, since it is abundantly clear that excessive ethanol consumption has serious negative effects on health. However where wine is consumed healthily it is also often consumed regularly and this regularity of consumption also has great significance in the potential health effects of wine, both positive and negative.

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Wine is a globally transacted product category both in its finally packaged form and in bulk. This global exposure means that wine must comply with the safety concerns of the importing countries in many different economic environments. These concerns might be legally defined or might simply be applied by commercial actors in the distribution network. In this way, independent of the real risk that any hazard might represent in a wine, a number of specific chemical hazards are controlled in commercially transacted products. As has been established elsewhere in this book, the inherent acidity and alcohol content of wine assures that pathogenic or toxigenic bacteria are not a relevant food safety hazard in wines, the microbially derived hazards being limited to biogenic amines and ethyl carbamate (Chap. 1). From the point of view of food safety, and in spite of the fact that controls are applied, wine must still be considered very much a low-risk product.

Controlled chemical hazards in wine can be grouped into two broad categories; those that are incorporated into grape material due to poor agricultural practices, environmental contamination or fungal contamination and growth and those that are introduced into the product as part of the winemaking process and that might present a risk to certain sensitive consumers. The first group includes heavy metals, phytosanitary products and mycotoxins whilst the second group includes sulphur dioxide and proteic fining material, both of which can provoke acute reactions in sensitive individuals. From a food safety perspective chemical hazards differ from microbial hazards in a number of aspects, most relevant here being that they cannot be inactivated by a (normally) thermal process step. The control of chemical hazards is achieved by assuring that they are not incorporated into the product and, where possible, their removal through technologies based on adsorption or precipitation/complexation. The latter option is most often extremely difficult to achieve without altering the organoleptic quality of the product, which is obviously a key issue in wine.

The risks that these hazards represent can be managed to a large extent by a combination of good agricultural and manufacturing practices and by informing consumers of the potential risks. However were it possible to intervene technologically to attenuate the risk this would be highly desirable. Indeed considerable effort is being made in the research community to identify natural alternatives to sulphur dioxide and animal proteins for fining and many of these alternatives can potentially influence organoleptic and other quality characteristics as well as food safety parameters. Similarly a number of approaches have been proposed and studied for the removal of mycotoxins, heavy metals and phytosanitary products.

2.2 Sulphur Dioxide and Its Role in Winemaking.

The case of the search for substitutes for sulphur dioxide (SO_2) is worthy of special mention both because it is such an intrinsic part of modern winemaking and because it has mobilised the research community so very strongly. Replacement of SO_2 is not the only health-directed role for natural additives and intrinsic solutions and

it is certainly not the only quality-directed one. Even in conventional winemaking, employing SO_2 within the current legal and technical framework, wines often suffer from oxidative and microbial deterioration and new sources of protection are needed. However SO_2 reduction and replacement is a major driver in the search for new antimicrobial and antioxidant agents, of which those discussed in this chapter form an important part, and thus deserves a particular treatment in order to outline the challenges.

Sulphur dioxide is industrially produced by the burning of sulphur in air and its application as a wine additive represents a minor use of what is a key industrial chemical. However, in solution and particularly in a complex solution such as wine, SO_2 distributes itself into in a variety of ionised and non-ionised molecular species each having distinct properties (Rose 1993). These properties include a number of antioxidant mechanisms (including oxidase inhibition and oxygen scavenging), antimicrobial activity and involvement in the stabilisation of colour (Guerrero and Cantos-Villar 2015). The abundance of each form depends on the specific chemical environment that that particular wine represents (Rose 1993). SO_2, or some other chemical vehicle that delivers it, is added at various times during the winemaking process from harvest right through to final bottling. At each stage of addition the technological objectives are different, or at least the emphasis on those facets of its activity that are valued at that stage is different. It is this variety of functions that have led to an almost complete dependence on SO_2 in winemaking in the modern era. Our very expectations of what an unspoiled, unoxidised wine should look and taste like is formed by the SO_2 treated wines we have been exposed to. The main driving force behind the reduction of SO_2 use in wine and in the rest of the food chain is the adverse health effects it produces in a small but significant proportion of the population (Guerrero and Cantos-Villar 2015). Amongst the general population the proportion of people with some clinical sensitivity is estimated at about 1 % although this number is between 3 and 10 % amongst asthmatics (Vally et al. 2009). It is not certain the mechanism of sensitivity although the symptoms and consequences are certainly similar to allergic reactions (Vally et al. 2009). It is not within the scope of this chapter to discuss in detail the medical aspects of SO_2 in wines and interested readers can find excellent recent reviews that can lead them to further literature on this (Guerrero and Cantos-Villar 2015). Whatever the medical situation underlying society's concerns, SO_2 is highly regulated in foods and wines all across the world. Regulations generally cover aspects of obligatory labelling and maximum permitted levels in different foodstuffs. Once more, the complex legal framework of the legal governance of SO_2 in wines is not the focus of this chapter and the same review article cited above presents a thorough overview and extensive references for those interested in this.

The medical and subsequent legal motivations for the reduction of SO_2 levels in wines have led to extensive studies aimed at finding alternative solutions for the various roles that it plays in winemaking. Some of these are based on physical methods such as UVC-irradiation (Fredericks et al. 2011) and high hydrostatic pressure treatment (Morata et al. 2012). In these cases the function of interest is antimicrobial. Isolated natural products and other preparations in which active ingredients are

naturally present represent another highly studied source of replacement for SO_2. In these cases it is normally either the antimicrobial or one of the antioxidant roles that is the target activity.

In the scientific and technical literature, any proposed alternative almost always has its performance measured against an SO_2 treated wine or wines. Performance in this respect often covers sensory and analytical chemical evaluations of the wines produced. The recent review of Guerrero and Cantos-Villar (2015) gives an exhaustive treatment of this subject to date. These reviewers appeal for more standardised and therefore comparable methods, the absence of which, make the identification of the most promising technologies considerably more difficult. Of course it is possible to produce a wine without the addition of SO_2 or any other exogenous antimicrobial or antioxidant agents but such wine will always be distinct from classically produced examples. Indeed the so-called "natural wine" movement defends wines produced in such a way, although not using SO_2 is not itself a sufficient motive to be classed a "natural wine". These are valid products in themselves and should not be judged against the same criteria as classically produced wines (Legeron 2014).

2.3 Phenolic Compounds

2.3.1 General Properties of Phenolic Compounds

Wines (and red wines in particular) are naturally rich in phenolic compounds which contribute to the sensory characteristics and chemical qualities of wine both directly and indirectly, through their interactions with other molecule types, e.g. proteins, polysaccharides and other polyphenols. Indeed, phenolic compounds are responsible for the colour of wines, originating pigments with distinctive hues ranging from yellow to blue. Also, some phenolic compounds impart bitterness to wines while other high-molecular compounds are known to precipitate with salivary proteins causing "astringency" which is particularly important for the taste of red wines.

Phenolic compounds are also well known for their antioxidant activity in biological processes being able to scavenge free oxygen radicals or Reactive Oxygen Species (ROS). The interaction of the hydroxyl groups with the π-electrons of the benzene ring allows the generation of free radicals where the radical is stabilised by delocalisation. The formation of these more stable and long-lived radicals is a major feature of the antioxidant role of phenols in biological processes.

Besides their impact on perceived wine quality, there is increasing evidence that wine phenolic compounds interfere with the activity of wine microorganisms. Indeed, some of these compounds are known to inhibit the growth of wine microorganisms while others can be actively metabolised by them.

Phenolic compounds are normally extracted to the wine from grape materials (skins, seeds and stalks) and from wood used for storage of wines but can also be added during the winemaking process in the form of oenological tannins. The phenolic composition of wines is much diversified ranging from simple one-ring aromatic

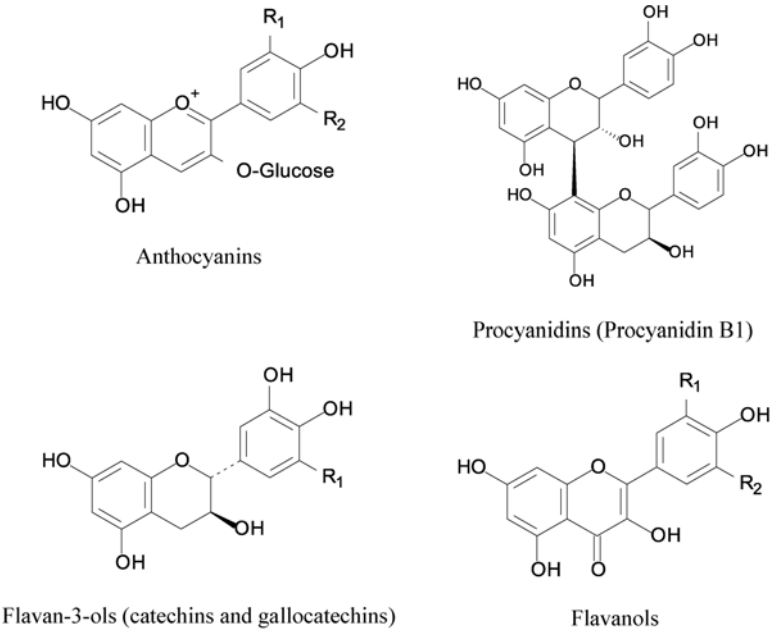

Fig. 2.1 Examples of flavonoid phenolic compounds found in wines (R_1 and R_2 represent different ring substituents)

structures to complex polymers with multiple aromatic rings. Phenolic compounds are usually divided broadly into two groups, according to their chemical structure: flavonoids, which possess a distinctive flavan (C_6-C_3-C_6) structure which includes anthocyanins, flavonols, flavan-3-ols, proanthocyanidins and condensed tannins, and non-flavonoids—compounds that lack the flavan structure—which includes phenolic acids, aldehydes, and alcohols and other compounds such as coumarins, stilbenes and "soluble" tannins (Figs. 2.1 and 2.2).

2.3.2 Antimicrobial Properties of Phenolic Compounds

Besides differing in their structural formulae and complexity, phenolic compounds also exhibit different properties regarding their antimicrobial activities.

Phenolic acids have been used for a long time in their pure state as preservatives in the food industry due to their relatively low toxicity and solubility in both aqueous and lipidic moieties. Benzoic acid was one of the first authorised preservatives for use in the food industry to attain Generally Regarded As Safe (GRAS) status. However, due to its relatively high pK_a (around 4.2) the effective use of benzoic acid as a preservative is limited to acidic foods with pH in the range 2.5–4.0.

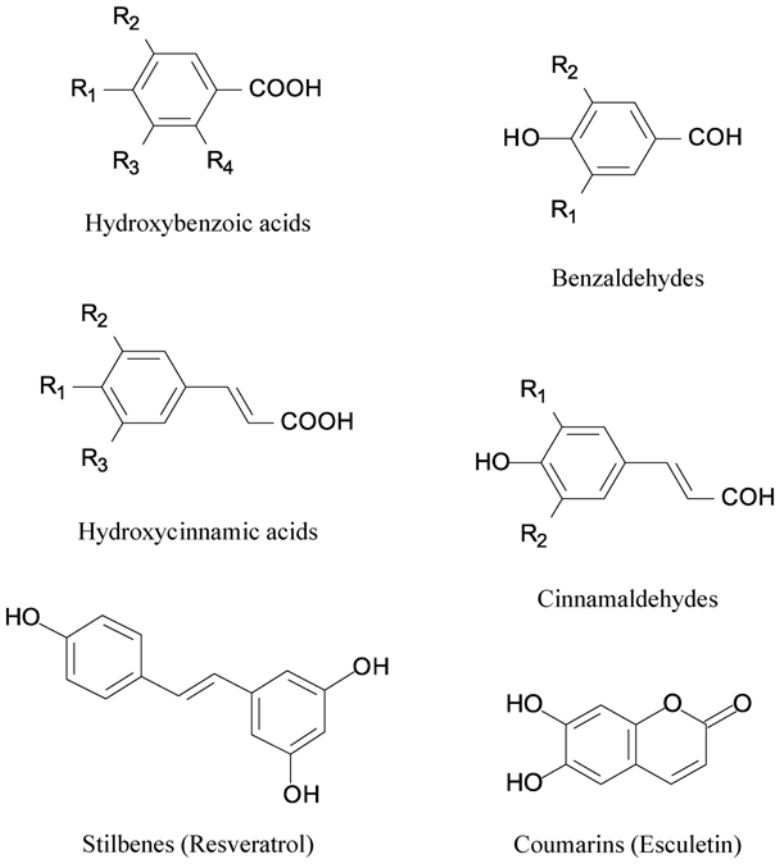

Fig. 2.2 Examples of non-flavonoid phenolic compounds found in wines (R_1 to R_4) represent different ring substituents)

Due to their lipophilic nature, it is thought that phenolic acids (and their derivatives) can cross the bacterial cell membrane in its undissociated form and acidify the cytosol, eventually causing protein denaturation and interfering with cellular activity.

Several authors have compared the antibacterial effects of benzoic and cinnamic acids. In most studies, cinnamic acids showed a stronger effect than benzoic acids (Ramos-Niño et al. 1996; Campos et al. 2003; Vaquero et al. 2007b). Amongst hydroxycinnamic acids, p-coumaric and ferulic acids seem to be particularly inhibitory towards bacteria (Stead 1993; Ramos-Niño et al. 1996; Campos et al. 2003; Cueva et al. 2012).

Besides phenolic acids, some phenolic aldehydes may also show inhibitory effects against microorganisms. The activity of cinnamaldehyde, a component of many essential oils, against pathogenic bacteria has been extensively studied (Gill and Holley 2004; Valero and Giner 2006; Vaquero et al. 2007b). Benzaldehydes

have antagonistic properties against pathogenic bacteria including *Campylobacter jejuni*, *Escherichia coli, Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* sp. (Ramos-Niño et al. 1996; Friedman et al. 2003).

Among benzaldehydes, vanillin has a particular industrial importance being a major flavour compound used in foods, beverages, perfumes and pharmaceuticals. There are many published works dedicated to the antimicrobial properties of vanillin against pathogenic bacteria such as *E. coli* (Fitzgerald et al. 2004), *Listeria* sp. (Fitzgerald et al. 2004; Delaquis et al. 2005), or *Bacillus cereus* (Valero and Giner 2006).

The antimicrobial properties of flavonoids have been reviewed by Cushnie and Lamb (2011) and Friedman (2014) and there is nowadays a growing interest in this subject in the scientific community due to the natural abundance of these compounds in all vegetables. The flavonols quercetin, kaempferol and myricetin were found to be inhibitory against several antibiotic-resistant pathogenic bacteria (Xu and Lee 2001). Quercetin has been shown to have an antibacterial effect against several pathogenic bacteria including *E. coli*, *S. aureus* and *Pseudomonas aeruginosa* (Rauha et al. 2000; Vaquero et al. 2007a). Puupponen-Pimiä et al. (2001) found that myricetin had inhibitory activity against some strains of *Lactobacillus rhamnosus* while quercetin had much more limited inhibitory effect on the same strains.

Some tea flavonoids and catechins (and particularly galloylated catechins) are active against pathogenic bacteria at nanomolar levels (Friedman 2007; Almajano et al. 2008). However, these compounds do not seem to negatively influence the growth of lactobacilli or bifidobacteria (Almajano et al. 2008).

The antifungal and antibacterial properties of tannins have been known for a long time and are well documented in the literature.

Tannic acid (a hydrolysable tannin composed of gallic acid esterified with glucose) has been shown to have inhibitory action towards a number of food-borne pathogenic bacteria (Akiyama et al. 2001; Funatogawa et al. 2004; Taguri et al. 2004).

Polyphenols from berries are also known to have anti-adhesion activity against bacterial uropathogens (Puupponen-Pimiä et al. 2005a; Howell 2007) gastrointestinal pathogens such as *Helicobacter pylori* (Puupponen-Pimiä et al. 2005a) and oral pathogens such as streptococci (Smullen et al. 2007; Bodet et al. 2008; Muñoz-González et al. 2014). The presence of A-type oligomeric procyanidins seems to be particularly important for the anti-adhesion properties of these molecules (Howell 2007).

2.3.3 Mechanisms of Microbial Inactivation by Phenolic Compounds

Although the precise antimicrobial mechanism of phenolic compounds is not well known, there is growing evidence that their primary effect is to interfere with the cytoplasmic membrane, increasing its permeability and causing leakage of

intracellular constituents such as proteins, nucleic acids, and inorganic ions (Campos et al. 2009a; García-Ruiz et al. 2011). Generally, Gram-negative bacteria seem to be more resistant to phenolic compounds than Gram-positive bacteria. This resistance is thought to be due to the presence of the outer membrane of the former which may act as a natural barrier, protecting them from the action of these biocides.

Phenolic acids are weak organic acids ($pK_a \approx 4.2$) and their antimicrobial activity is considered to be dependent on the concentration of the undissociated acid. Due to their partially lipophilic nature, phenolic acids can cross the cell membrane by passive diffusion in their undissociated form, disturbing the cell membrane structure and possibly acidifying the intracellular cytoplasm, interfering with cellular activity and/or causing protein denaturation. The primary action of phenolic acids on the cell membrane has been supported by several published works (Ramos-Niño et al. 1996; Nohynek et al. 2006; Campos et al. 2009a; García-Ruiz et al. 2011).

Benzaldehydes are thought to act primarily on the external surface of bacteria combining with sulfhydryl groups of proteins (Ramos-Niño et al. 1996; di Pasqua et al. 2007). However other works have shown that these compounds can also cause cellular leakage (Gill and Holley 2004; Fitzgerald et al. 2004).

Flavonoids, due to their higher molecular weight and more complex structure are thought to act mainly at membrane level. Quercetin is known to increase the permeability of the bacterial membrane and cause a dissipation of the membrane potential (Cushnie and Lamb 2011). Xu and Lee (2001) have shown that myricetin can inhibit protein synthesis and found that the corresponding flavone or 3-glucoside did not have any activity on *S. aureus*, thereby concluding that the free C-3 hydroxyl group in the flavan-3-ol structure is important for its antimicrobial activity. This result is consistent with the reported absence of antimicrobial activity of rutin (quercetin-3-O-glucoside) against bacteria which were susceptible to quercetin (Rauha et al. 2000; Vaquero et al. 2007b). Flavones and flavonols (and particularly quercetin) have also been reported to inhibit bacterial DNA gyrases and topoisomerases (Plaper et al. 2003). This inhibitory effect is, however, conditioned by the ability of these compounds to cross the cell membrane and interact with these cytoplasmic enzymes. Several authors suggested that the antimicrobial activity of flavonols might increase with an increasing degree of hydroxylation of the B-ring (Puupponen-Pimiä et al. 2001; Xu and Lee 2001). Regarding flavan-3-ols (catechins), the bactericidal activities of galloylated catechins and gallocatechins also seem to be related to perturbations on the phospolipidic cell membrane (Uekusa et al. 2007; Sirk et al. 2008). Some authors (Sirk et al. 2008) discovered that catechins interact with the lipid bilayer via hydrogen bonding, with some of the smaller catechins being able to penetrate underneath the membrane surface.

The antimicrobial effects of tannins are generally believed to be due to four types of mechanisms: (a) cell-membrane interactions, (b) non-covalent binding with extracellular enzymes and other functional proteins (c) covalent binding of oxidised polyphenols with sulphhydryl groups of enzymes and (d) deprivation of substrates required for microbial growth (Scalbert 1991; Mila and Scalbert 1994; Akiyama et al. 2001; Puupponen-Pimiä et al. 2005a, b).

2.3.4 *Influence of Phenolic Compounds on Growth and Viability of Wine Lactic Acid Bacteria*

A substantial amount of research works has been published regarding the activity of wine phenolic compounds on growth and survival of wine lactic acid bacteria (LAB). One of the first investigations on this interaction was published by Cornu et al. (1984) who found that (+)-catechin and (−)-epicatechin could have a stimulatory or inhibitory effect on growth of *Oenococcus oeni* depending on the bacterial strain and concentration level of the phenolic compounds. The same authors discovered that in the presence of (+)-catechin, some strains of *O. oeni* consume preferentially fructose over glucose, metabolise L-malic acid more slowly and produce higher amounts of organic (lactic and acetic) acids than the control.

The inhibitory activity of hydroxycinnamic acids towards wine spoilage LAB was studied by Stead (1993) who established that high concentrations of p-coumaric, ferulic and caffeic acids inhibited the growth of *L. collinoides* and *L. brevis* while low concentrations stimulated their growth. On a subsequent study, the same author found a similar stimulatory effect of gallic acid on the growth of the same LAB species (Stead 1994).

Later, Vivas et al. (1997) studied the effect of phenolic acids and free anthocyanins on *O. oeni* and found that gallic acid and free anthocyanins stimulated growth and malolactic activity of the tested strain and enhanced its survival in phosphate buffer. On the other hand, vanillic acid had a negative effect on growth and viability while protocatechuic acid showed no effect on this bacterium.

In another study, Rozès and Peres (1998) found that hydroxycinnamic (caffeic and ferulic) acids affected negatively the growth of *L. plantarum* and increased the proportion of unsaturated fatty acids of the cell membrane.

The influence of oak wood and grape tannins on *O. oeni* was later studied by Vivas et al. (2000) who reported that oligomeric procyanidins from grape seeds were the most powerful inhibitors towards this bacterium, affecting bacterial viability in both growing and non-growing conditions. The oxidation state of the polyphenols also seemed to affect their antibacterial effect: while ellagitannins were only inhibitory if previously oxidised, the opposite occurred with procyanidins. In the same work, the authors have shown that tannins could be adsorbed to the bacterial envelope of *O. oeni* (Vivas et al. 2000).

In another work, Salih et al. (2000) studied the inhibitory effect of hydroxycinnamic acids towards *O. oeni* and *L. plantarum* and found a strong decrease in the growth rate and biomass production in *O. oeni* while only an apparent growth rate decrease was observed in *L. plantarum*. Ferulic acid had the strongest effect, followed by p-coumaric and caffeic acids. Reguant et al. (2000) also studied the activity of hydroxycinnamic acids towards growth and malolactic activity of *O. oeni* and found that these compounds (and p-coumaric acid in particular) were inhibitory at high concentrations; contrarily, catechin and quercetin had a stimulatory effect on MLF by this species. Similar results were obtained by Campos et al. (2003) who found that hydroxycinnamic acids (and particularly p-coumaric acid) were more

inhibitory towards *O. oeni* and *L. hilgardii* than hydroxybenzoic acids; on the other hand some phenolic acids showed a beneficial effect on growth of *L. hilgardii* (Campos et al. 2003).

Alberto et al. (2001) found that low concentrations (up to 200 mg/l) of gallic acid and (+)-catechin stimulated growth of *L. hilgardii* while at high concentrations (1000 mg/l) gallic acid was inhibitory to this bacterium. In later works, the same strain of *L. hilgardii* was shown to be able to metabolise both gallic acid and (+)-catechin producing some phenolic compounds with oxygen-scavenging capacity (Alberto et al. 2004).

Landete et al. (2007) reported that despite being quite resistant to wine phenolic compounds even at high concentrations, *L. plantarum* was more sensitive to hydroxycinnamic acids than to hydroxybenzoic acids and flavan-3-ols at the same molar concentrations; p-coumaric acid was again found to be the most inhibitory of the tested phenolic compounds. The same authors studied the effect of hydroxybenzoic acids on growth of *L. plantarum* from olives and found that salicylic and syringic acids have some inhibitory activity against this species (Landete et al. 2008).

Figueiredo et al. (2008) found that different condensed tannin fractions (particularly tetramers and pentamers) extracted from grape seeds diminished the cell viability of *O. oeni* and *L. hilgardii* (though the effect was less pronounced in the latter); the same authors found that several phenolic aldehydes and flavonols (quercetin and kaempferol) inhibited the growth of *O. oeni*.

Regarding flavan-3-ols, (−)-epigallocatechin gallate was found, by some authors (Theobald et al. 2008) to have a stimulatory or inhibitory effect on *O. oeni* growth depending on the concentration level. More recently, García-Ruiz et al. (2009, 2011) have also observed that flavonols and stilbenes had comparatively a stronger inhibitory effect towards wine LAB than phenolic acids at the same concentration levels. The same authors have confirmed that membrane damage occurred after exposure of bacterial cells to these compounds (García-Ruiz et al. 2011).

2.3.5 *Influence of Phenolic Compounds on Wine Lactic Acid Bacteria Metabolism*

Besides having an effect on growth of LAB, phenolic compounds were found to alter the carbohydrate and the organic acid metabolism of this group of bacteria. Early studies on the influence of phenolic compounds on the malolactic activity and organic acid metabolism by LAB suggested that gallic acid can stimulate the malolactic activity of some strains of *L. hilgardii* and *O. oeni* (Vivas et al. 1997; Alberto et al. 2001) and delay the production of acetic acid from citric acid in *O. oeni* (Reguant et al. 2000). On the other hand, other authors found that some phenolic acids delayed the conclusion of the MLF by *O. oeni* (Vivas et al. 1997; Reguant et al. 2000) and by *L. hilgardii* (Campos et al. 2009b).

Rozès et al. (2003) studied the effect of different mixtures of phenolic compounds (malvidin-3,5-diglucoside, phenolic acids and catechin) on the metabolism of glucose and citric acid by *O. oeni* and concluded that these compounds reduced the rate of sugar consumption and enhanced citric acid consumption, increasing the yield of acetic acid from glucose metabolism. Similar results were obtained by Campos et al. (2009b) who have shown that the malolactic activity of *O. oeni* was not strongly affected by these compounds, the opposite to what happened to *L. hilgardii*. Moreover, the addition of phenolic acids delayed the metabolism of glucose and citric acid in both bacteria and increased the yield of lactic and acetic acid production from glucose by *O. oeni* as previously reported by Rozès et al. (2003).

2.4 Yeast Cells and Their Degradation Products

Yeasts have long been known to play a number of roles in winemaking, quite apart from their obvious involvement in the primary alcoholic fermentation. The practice of leaving a wine in the presence of the dying and dead yeasts that had previously performed the fermentation is known to modulate the quality of the final product and is an established part of a winemakers' repertoire. That yeast cell mass might also provide a substrate for the adsorption of undesirable compounds has largely been focussed on its use in the removal of ochratoxin A (Caridi 2007; Quintela et al. 2013). A role of cell wall mannoproteins as the binding element had been established leading the way to the trialling of the extracted mannoproteins as removal agents (Bejaoui et al. 2004). Chitin and related compounds from fungal (including yeast) sources have also been found to bind and allow the removal of ochratoxin A as well as toxic heavy metals (Bornet and Teissedre 2008). The role of phenolic compounds in modulating microbial growth has been previously discussed. In this context, yeast mass, and particularly yeast cell walls might affect the availability of relevant phenolic compounds by interacting directly with them (Caridi 2007). The specific mannoproteins present in the cell walls of yeasts are highly variable between species and even strains (Caridi 2007) and as more becomes known about non-*Saccharomyces* wine strains and their role in wine production it is probable that this facet of their nature will open new possibilities.

A further role for yeasts and their degradation products might lie in the substitution of animal proteins in fining operations. Animal proteins, particularly derived from milk and eggs, are of concern due to their status as allergens. Whilst not being appropriate to elaborate on the clinical and legal framework here, it is certainly true that non-animal fining proteins of comparable performance would be greatly received by the winemaking community. Initial studies have shown that protein-rich extracts can effect a protein fining to an acceptable level (Iturmendi et al. 2012). In recent studies the protein component of the extracts was separated from the potentially antagonistic mannoprotein and polysaccharide fractions (Lochbühler et al. 2015).

2.5 Lysozyme

The microbiological stability of wines is mostly assured by the use of SO_2 (Fugelsang and Edwards 2007). SO_2 exhibits good antimicrobial and antioxidant capacities but can cause allergic-type reactions in sensitive individuals, especially asthmatics, and may have negative sensory properties (Vally 2008). Thus, the potential of other antimicrobial compounds, such as lysozyme, as alternatives to SO_2 have been subject of attention from the scientific community (Sonni et al. 2011).

Lysozyme (EC 3.2.1.17) is a widespread enzyme in nature being widely produced commercially from hen's egg white. It causes the degradation of the peptidoglycan cell wall of Gram-positive bacteria by cleaving the β (1-4) bond of *N*-acetylmuramic acid and *N*-acetylglucosamine. Gram-negative bacteria are less susceptible to lysozyme due to the lipopolysaccharide composition of the outer membrane (Nattress and Baker 2003).

Lysozyme is used to control microbial growth in foods such as cheese and wine. In the case of wine production (approved by OIV since 1997, resolution OENO 10/97) it can be used to control the growth of LAB to prevent or restraint the extent of the malolactic fermentation in white and rose wines (Gerbaux et al. 1997). It can also be used in red wines at the end the malolactic fermentation to stop further activity of LAB. The microbiological stability of wines in post-fermentation stages is important to protect the wine against bacterial spoilage activities such as the production of biogenic amines and the increase of volatile acidity (Gerbaux et al. 1997, Gao et al. 2002; Bartowsky 2009; López et al. 2009). Lysozyme has also been studied as a stabilising agent in the biological ageing of specific types of wines such as sherry (Lasanta et al. 2010). The lysozyme treatment was found to successfully control the development of LAB, hence preventing the heterolactic fermentation in these wines. However, Roldán et al. (2012) found that lysozyme may affect the membrane hydrophobicity of the yeasts, inhibiting their aggregation and flotation and influencing the development of the flor velum.

Lysozyme can also be applied in pre-fermentative stages. Gao et al. (2002) found that lysozyme effectively inhibited the growth of spoilage LAB during the alcoholic fermentation of Chardonnay wines and avoided the increase of volatile acidity during stuck/sluggish fermentations. Furthermore, the malolactic fermentation was prevented in these wines. Sonni et al. (2011) studied the effects of the substitution of SO_2 with lysozyme and oenological tannins on the volatile profile of white wines. Those fermented with lysozyme and tannins showed a higher level of esters and a lower total amount of higher alcohols than the wines fermented in the presence of SO_2.

The bacterial sensitivity to lysozyme seems to vary according to species or even at strain level. Delfini et al. (2004) and Azzolini et al. (2010) found a greater antimicrobial activity of lysozyme towards *O. oeni* than *Lactobacillus* and *Pediococcus* species. Some strains were found to be quite resistant (Delfini et al. 2004; Blättel et al. 2009) but the mechanisms underlying this variation are not fully understood. Specific features found in some species or strains may explain their behaviour when exposed to lysozyme. Coulon et al. (2012) demonstrated that the resistance

of *Ped. parvulus* to lysozyme may be due to the presence of the β-glucan that forms around the cell acting as a protective barrier against antibacterial agents. In the presence of β-glucanase, the lysozyme treatment was strongly improved.

Although it could be a valid alternative to SO_2 for controlling LAB in white winemaking, some authors consider that in red wines certain factors might inhibit lysozyme activity. Guzzo et al. (2011) reported that non-flavonoids, flavanols and flavonol compounds, tested individually, did not interfere with the lysozyme activity. However, proanthocyanidins extracted from seed berries, strongly inhibited the lysozyme (Liang et al. 2013). Tirelli and Noni (2008) observed that the native form of the enzyme precipitates with low molecular weight tannins along with other interactions such as sulphonation due to the interaction with SO_2. The scientific data available suggest that wines rich in tannins may not be favourable to the action of lysozyme and that its effectiveness in red wine should be evaluated more rigorously due to enzyme insolubilisation. The combination of lysozyme with must colloids and suspended solids may lead to a significant reduction in the free enzyme (Delfini et al. 2004; Azzolini et al. 2010). Other factors such as pH and ethanol seem not to negatively affect lysozyme activity. In fact, the application of lysozyme may be particularly useful in high pH wines where SO_2 is less effective. A better understanding of the mechanisms involved in the interaction between lysozyme and wine components, especially in red wines, is needed.

It is known that lysozyme can cause allergic reactions (Pérez-Calderón et al. 2007). Weber et al. (2009) provided scientific data about the amounts and risks of lysozyme in wines. They found a strong influence of post-fermentation treatments, such as the application of bentonite as fining agent, on the amount of lysozyme remaining in wine. Kirschner et al. (2009) concluded that wines treated with ovalbumin, lysozyme or casein appear not to present a risk to allergic individuals when filtered according to the standard process. Carstens et al. (2014) applied different filtration and other oenological processes in order to evaluate their potential to deplete lysozyme from wines. Among the oenological procedures tested, only bentonite fining proved to be capable of significantly reducing the allergenic residues. According to these authors, lysozyme may potentially be present in the final product at concentrations that could pose a thread depending on the production technique employed. Good manufacturing practices should be applied to use lysozyme in an effective way and to produce wines free of allergenic residues.

Lysozyme has no detectable effect on wine aroma and taste. However, unwanted side effects have been observed, namely the loss of colour in red wines and formation of haze in whites (Bartowsky et al. 2004).

2.6 Bacteriocins

Bacteriocins are peptides or proteins with antimicrobial activity produced by different groups of bacteria. Several strains of LAB produce bacteriocins with rather broad spectra of inhibition offering potential applications in food preservation

having in mind the reduction of the amount of chemical preservatives (Gálvez et al. 2007). Although the structure, biosynthesis and mode of action of several bacteriocins are quite well known, nisin (produced by *Lactococcus lactis* of non-oenological origin) is currently the only bacteriocin widely used as a food preservative (Cleveland et al. 2001).

The application of nisin to wine has been tackled. Rojo-Bezares et al. (2007) investigated the use of nisin over several wine isolates from different taxa (*Oenococcus, Lactobacillus, Leuconostoc* and *Pediococcus*, acetic acid bacteria and yeast). Significant differences in the sensitivity to nisin were observed, Gram-positive bacteria, especially *O. oeni*, being more sensitive than Gram-negative bacteria. Yeasts, not surprisingly, were found to be the most resistant.

A number of scientific papers have reported on the production of bacteriocins by LAB isolated from wine. Navarro et al. (2000) found antimicrobial activity in strains of *L. plantarum*, capable of inhibiting the growth of around 75 % of the LAB strains studied (four genera). The bacteriocin studied is called plantaricin and the *pln* locus was shown to be widespread among oenological strains (Sáenz et al. 2009).

Other bacteriocins that had demonstrated potential in a number of food applications in terms of food quality and safety were studied against wine microorganisms. García-Ruiz et al. (2013) investigated the use of lacticin 3147, a broad-host-range bacteriocin produced by *Lactococcus lactis* strains isolated originally from dairy products, to control growth of wine LAB. The activity of the bacteriocin was found to be strain-dependent, *O. oeni* strains being the most sensitive and *L. casei* the most resistant.

A genetic screening for bacteriocin-encoding genes was performed in 330 wine LAB strains (Knoll et al. 2008). Eight per cent of the strains, belonging to the species *L. plantarum, L. paracasei, L. hilgardii* and *O. oeni*, showed inhibitory activity towards various wine-related indicator strains. The PCR-based screening performed revealed the presence of plantaricin encoding genes in the *L. plantarum* strains.

Bacteriocin producers were also found among wine pediococci and *Leuconostoc*. Strasser de Saad and Manca de Nadra (1993) isolated *Ped. pentosaceus* strains that produce an inihibitory substance against *Oenococcus, Lactobacillus* and *Pediococcus*. The effect of pediocin PA 1, a bacteriocin produced by *Ped. acidilactici*, on several wine microorganisms was evaluated by Díez et al. (2012). Again, *O. oeni* strains were found to be the most sensitive. Yurdugul and Bozoglu (2002) found an isolate of *Leuconostoc mesenteroides*, subsp. *cremoris* that produced a bacteriocin-like substance.

The combined use of bacteriocins and SO_2 on wine isolates was investigated by several researchers. Interestingly, synergistic effects on bacterial growth inhibition were observed suggesting that appropriate combinations of bacteriocins and metabisulphite could allow a decrease in the levels of SO_2 currently used in the winemaking process (Rojo-Bezares et al. 2007; Díez et al. 2012, García-Ruiz et al. 2013). Synergistic inhibitory effects were also found with ethanol (Díez et al. 2012).

Although the promising results of the activity of bacteriocins against wine spoilage bacteria has generated interest among the scientific community and wine

producers as a means to reduce the use of SO_2 by the wine industry, its use has not yet been approved in winemaking. The precise conditions for an effective application of bacteriocins to wines and musts should be further characterised. Besides the better understanding of the practical application of bacteriocins to wine, recent genomic approaches may be useful for the discovery of new bacteriocins.

2.7 Natural Plant-Derived Products

Plant-derived materials (essential oils and extracts) are naturally rich in phenolic compounds and therefore are strong candidates for this role having both antioxidant and antimicrobial activities.

The antimicrobial activity of essential oils and spices has been known for a long time and has been partially attributed to its phenolic composition. Thymol and carvacrol (from oregano and thyme oil), cinnamaldehyde (from cinnamon) and eugenol (from cloves) are some examples of phenolic compounds which can be found in these natural preservatives and that have a wide spectrum of activity.

The biological activity of essential oils and their application in foods is a very active area of research and has been recently reviewed by some authors (Lang and Buchbauer 2011; Solórzano-Santos and Miranda-Novales 2012). Besides phenolic compounds, essential oils are rich in terpenes, which also contribute to the antimicrobial effect of these extracts.

Recently, the antifungal potential and mode of action of two essential oils mainly obtained from oregano, carvacrol and thymol, were investigated against natural yeast flora of grapes and wine spoilage yeasts (Chavan and Tupe 2014). Growth of spoilage yeasts was found to be inhibited due to membrane damage, leakage of cytoplasmic content and ergosterol depletion.

The use of essential oils in wines seems to be, however, limited due to regulatory constraints and also due to their strong impact on wine sensory characteristics. At present, the only wines where the addition of pine resin is authorised are Greek (Retsina) wines.

More recently, several studies have been published regarding the use of plant natural extracts for controlling the activity of wine microorganisms.

Salaha et al. (2008) evaluated the effect of an alternative antioxidant natural product extracted from black radish on certain red wine quality parameters (colour, phenolic content and antioxidant activity) and were able to produce commercially acceptable dry wines with reduced levels of SO_2. However, the composition of the alternative antioxidant product was not revealed and the antimicrobial activity of this product was not assessed.

Garcia-Ruiz et al. (2012) published an extensive survey of natural products as possible alternatives to reduce or eliminate sulphur dioxide use as antibacterial agent particularly during MLF. This study included products such as spices, flowers, leaves, fruits, legumes, seeds, skins, and agricultural by-products and other natural products. Among the studied natural extracts, the authors found antibacterial

activity in several spices (cinnamon, eucalyptus, thyme), leaves (ginkgo biloba), fruits (pomegranate), beans (soy bean), almond skins, grape seeds, red grape, grape pomace, oak tannins, quebracho tannins and propolis.

A subsequent study by the same research group (García-Ruiz et al. 2013) using some of the natural extracts (eucalyptus leaves and almond skins) indicated that the addition of these extracts might have a slight effect on the volatile composition of the resulting wines and also an increase in the phenolic (flavonol) content, although no sensorial essays were performed in this work. However, another work by González-Rompinelli et al. (2013) in winery conditions has confirmed the effect of the same extracts (eucalyptus leaves and almond skins) regarding microbiological stability and also shown that despite there were some differences in the volatile composition of wines supplemented with these extracts, there were no significant differences in the sensory characteristics of these wines (as assessed by an expert tasting panel) besides confirming the effect of these extracts (eucalyptus leaves and almond skins) regarding microbiological stability.

Recently a collection of oenological woods extracts (cherry and oak wood from different origins and with different degrees of toasting), isolated by pressurised liquid extraction, were tested for their ability to control the microbial spoilage of wines caused by LAB, acetic acid bacteria and *Brettanomyces* yeasts (Alañón et al. 2015). Among the tested microorganisms, acetic acid bacteria were especially sensitive to phenolic inactivation from oenological woods extracts. Cherry wood extract had the broadest spectrum of action of all tested extracts being active against 9 of the 11 tested microorganisms. No correlation was found between the antioxidant and antimicrobial activities, though.

Despite the limited published studies at present the results obtained so far are encouraging and may indicate a promising future regarding the use of natural products as alternatives to reduce the use of SO_2 in wines or to replace it altogether in some wines such as biodynamic or "natural" wines. Further studies are necessary to analyse the antioxidant properties of these natural extracts in winemaking and in vitro and in vivo conditions and to establish if there is a correlation with its antimicrobial effects.

2.8 Other Natural Substances (Chitosan, Peptides)

As said before, there is a great interest in evaluating the potential of compounds with recognised antimicrobial activity as alternatives to sulphites. One of such compounds is chitosan, a linear heteropolysaccharide of *N*-acetyl-2-amino-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose derived from chitin by deacetylation with recognised antimicrobial activity and biocompatibility (Raafat and Sahl 2009). However, the use of chitosan as a potential substitute for sulphites must take into account some of chitosan's limitations, namely the fact that it is only

soluble at acidic conditions achieved by the use of some organic acids (e.g. acetic acid), possesses a high viscosity and coagulates proteins at high pH values.

Gomez-Rivas et al. (2004) studied the antimicrobial action of chitosan against *S. cerevisiae* and the spoilage yeasts *Brettanomyces bruxellensis* and *B. intermedius* in culture medium fermentations. They found that the presence of chitosan above 1 mg/ml resulted in longer lag phases for the *B. bruxellensis* strain assayed. A similar effect was obtained for *B. intermedius* at 0.5 mg/ml and above. The exponential growth phase and the final population densities were not highly affected. Ferreira et al. (2013) showed that chitosan inhibits the growth of *Brettanomyces/Dekkera* at concentrations ranging from 0.2 to 0.5 mg/ml, depending on the molecular weight of the chitosan molecules (the lower the molecular weight, the lower the minimum inhibitory concentration values) and on the assayed strains. Yet, chitosan affected some physicochemical characteristics of wine, particularly the hue and colour intensity. Similar results were obtained by Bağder Elmaci et al. (2015) for *B. bruxellensis.* This species, together with *L. hilgardii* and *O. oeni*, was among the most susceptible wine related microorganisms to chitosan being completely inactivated at 0.2 mg/ml.

The application of chitosan as an antimicrobial agent in the wine industry is relatively recent and the scientific data necessary to support the efficient use of chitosan is still scarce. Further investigation covering a larger number of wine organisms is needed. Also, future work should aim at clarifying the impact of chitosan on wine physicochemical characteristics.

Strategies based on the use of peptides have been proposed to control the activity of wine organisms. Enrique et al. (2007) examined the antimicrobial action of selected short synthetic peptides (sequence-related antifungal hexapeptides and lactoferricin B derived peptides) against wine spoilage yeasts. The antimicrobial action of peptides was found to be dependent both on the food matrix and the target microorganism, *Zygosaccharomyces bailii* and *Z. bisporus* being found to be the most sensitive yeasts. This research group demonstrated that lactoferrin derived peptides were able to inhibit the growth of *Dekkera bruxellensis* and proposed a potential application of peptides in the control of this wine spoilage yeast (Enrique et al. 2008).

The antibacterial activity of peptides against LAB was also studied. Enrique et al. (2009) evaluated the activity of a bovine lactoferrin pepsin hydrolysate and a synthetic peptide derived from bovine lactoferricin. They demonstrated that the peptide activity in fermenting must affected the survival of specific LAB while not affecting the growth rate and alcoholic fermentation of a commercial wine *S. cerevisiae* strain.

Albergaria et al. (2010) and Branco et al. (2014) have shown that certain species of *S. cerevisiae* produce peptides that inhibit the growth of wine-related non-*Saccharomyces* species. These antimicrobial peptides were later found to induce alterations in the intracellular pH, membrane permeability and culturability of *Hanseniaspora guilliermondii* cells (Branco et al. 2015), but further work is needed to fully understand the mode of action of these compounds.

2.9 Conclusions

The search for new solutions for antimicrobial and antioxidant activities from natural, biological sources is certainly not driven exclusively by the need to reduce the SO_2 burden in wines, although this must be considered a major motivation. Increasing numbers of consumers are valuing "natural" attributes and condition their choices in part based on this. The scientific community has responded to this challenge by dedicating considerable resources to seek out new "natural" agents and evaluate their suitability as wine additives, either to substitute SO_2 or to extend the palette of interventions that the winemaker has at his or her disposal. The tools and strategies available to winemakers have always evolved along with the real and perceived needs of the producer and the consumer alike and the technologies discussed in this chapter do little more than illustrate this. However, it is important to record that the type of product that is now presented to the vast majority of consumers, at any price point, is produced by employing tools that the winemaker chooses from all those at his disposal—including SO_2. Any wine on the general commercial market, independent of market segment, must be compared in quality terms with a profile that has been defined by wines produced using SO_2.

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Chapter 3
Applications of Nanotechnology in Wine Production and Quality and Safety Control

Miguel Monge and M. Victoria Moreno-Arribas

3.1 Introduction

In recent years, nanotechnology has advanced rapidly worldwide, providing significant benefits to an increasing number of products from different areas, including electronics, information and communications, energy and environment, transportation, construction, textiles, biotechnology, health, agriculture, and food. (Pérez-López and Merkoci 2011; Tothill 2011). The National Nanotechnology Initiative (NNI) from the EEUU defined nanotechnology as "research and technology development at the atomic, molecular or macromolecular scale leading to the controlled creation and use of structures, devices and systems with a length scale, normally below 100 nanometers (nm)." To give an example of exactly what this means, 1 nm is the length of a chain of five to ten atoms, and a human hair is about 80,000 nm in diameter. Nanomaterials may exhibit different physical and chemical properties compared with the same substances at normal scale, such as increased chemical reactivity due to a greater surface area.

Nanotechnologies enable the management of food ingredients at a molecular level, will have a substantial impact on the food and feed sector in the future in fields such as the treatment of the mechanical and sensorial properties of food—for instance, to achieve changed taste or texture—and modifying nutritional value, potentially offering benefits for both the industry and the consumer. Nanotechnology

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may also be used in food packaging, for example to ensure better protection or to detect how fresh a food is. The specific properties and characteristics of nanomaterials need to be considered for any potential health risks.

The introduction of nanotechnology applications to public health-related areas such as food, cosmetics or drugs will largely depend on how nanoproducts are regulated in the interests of protecting consumers against potential risks posed by using them. In March 2009, the European Food Safety Authority (EFSA)'s Scientific Committee published a scientific opinion on nanoscience and nanotechnologies in relation to food and feed safety. A guidance document on how to assess potential risks related to certain food-related uses of nanotechnology followed in May 2011. It provides practical recommendations on how to assess applications by industry in the use of engineered nanomaterials (ENMs) in food additives, enzymes, flavorings, food contact materials, novel foods, food supplements, feed additives, and pesticides (http://ec.europa.eu/health/nanotechnology/policy/index_en.htm).

The advantages of nanomaterials and nanobiosensors has led to their use in the wine industry processes for different purposes: from raw material preparation, must fermentations and wine manufacture (quality control), and the monitoring of storage conditions to the use of these devices as cost-effective tools for quality and process controls, as well as to ensure food safety. In this chapter, we review the current status of nanotechnology applications in wine, focusing on the use of nanomaterials for wine production, quality and safety control, including nanobiosensors and nanosensors, and we revise and offer an opinion on the future of this technology in enology.

3.2 Nanomaterials for Wine Production and Quality and Safety Control

The use of nanomaterials for the design of new applications in the field of wine production is a very recent research topic. Among all the possible specific applications in this field we have grouped them into three subtopics. A first set of studies has dealt with the design of new nanomaterials for the degradation or removal of pollutants in wine or wine wastewater; a second type of nanomaterial is focused on the immobilization or vectorization of yeast; a third type of nanomaterial comprises those designed as antimicrobial agents. In the following sections, the preparation and mode of action of these nanomaterials, as well as their uses, are described.

3.2.1 Nanomaterials for the Degradation or Removal of Pollutants in Winemaking

Nanomaterials for the degradation or removal of pollutants in wine or wine wastewater are commonly used as degradation or removing agents in other fields of application, such as catalysis or photocatalysis for the degradation of organic pollutants and as

encapsulating agents of small molecules. In the field of catalysis, gold nanoparticles (Au NPs) have been synthesized through an electrochemical approach. These nanoparticles are very active catalysts for the decomposition of aldehyde in 40 % v/v ethanol solutions (Liu et al. 2006). The authors tested the catalytic ability of these Au NPs in wines containing 60 ppm of aldehyde and concluded that this catalytic effect could be extended to commercial wines. In this context, a more recent study (Yu et al. 2010) proposed the synthesis of Au NPs of 10–50 nm size through a combined electrochemical–photochemical approach. This method allows a good control over the nanoparticle size and the obtained nanoparticles are embedded in chitosan biopolymer. The use of 0.05 ppm of Au-Chitosan NPs as a catalyst in commercial white wines permits a degradation of acetaldehyde from 95 to 24 ppm. The experiments were carried out in sealed tubes at room temperature and light-protected. The authors also compared their results with the catalytic degradation of acetaldehyde using commercial Au NPs, obtaining a better catalytic performance when Au NPs were capped with chitosan.

Winery wastewater is seasonally produced and is generated mainly as the result of cleaning practices in winery, such as washing operations during crushing and pressing grapes, rinsing of fermentation tanks, barrels washing, bottling and purges from the cooling process. As a consequence of the working period and winemaking technologies, volumes and pollution loads vary greatly over the year. The wastewater contains high concentrations of phenols, which make them difficult to treat through biological processes due to their phytotoxicity and bacterial toxicity. Several winery wastewater treatments are available, but the development of alternative technologies is essential in order to increase their efficiency and to decrease the investment and exploration costs. A very well-known type of nanomaterial used for the degradation of pollutants is TiO_2-based nanomaterials. This type of nanostructure is able to photocatalyze the degradation of organic pollutants. TiO_2 is an ideal photocatalyst for several reasons. It is relatively cheap, highly stable from a chemical point of view and easily available. Moreover, its photogenerated holes are highly oxidizing, and the photogenerated electrons reduce sufficiently to produce superoxides from dioxygen groups. TiO_2 promotes the ambient temperature oxidation of most indoor air pollutants and does not need any chemical additives. It has also been widely accepted and exploited as an efficient technology for killing bacteria. Thus, the photocatalytic performance of semiconductor TiO_2 nanostructures arises from the formation of photogenerated charge carriers (hole and electron), which takes place when the TiO_2 nanoparticles absorb UV light corresponding to the band gap of the semiconductor. This light absorption leads to the formation of so-called photogenerated holes in the valence band. The formed holes are able to diffuse to the TiO_2 surface and react with adsorbed water molecules, leading to very reactive hydroxyl radicals. The photogenerated holes and the hydroxyl radicals easily oxidize organic molecules at the surface of the TiO_2 nanoparticles. In this context a study by Bacsa and Kiwi reported on the photocatalytic degradation of *p*-coumaric acid, which may be a pollutant of wine wastewater, using nanocrystalline titania as catalyst. The use of tetraisopropyl-orthotitanate molecular precursors for the synthesis of TiO_2 nanoparticles led to the formation of anatase–rutile phase mixtures in the TiO_2 structure, which showed a better performance in its photocatalytic activity

for the degradation of *p*-coumaric acid. The rutile phase induced mesoporosity and larger pore sizes in the TiO_2 nanostructure, which was related to the higher catalytic activity (Bacsa and Kiwi 1998).

Another interesting class of nanomaterials used for wine wastewater treatment is clay–polymer nanocomposites. The use of clays, organoclays (clay minerals treated with organocations), and nanocomposites (clay minerals combined with polymers) in water treatment and environmental remediation is considered an emerging area in applied nanotechnology, and have been widely used for sorption of several organic and inorganic pollutants. Clay minerals constitute a class of hydrous aluminum phyllosilicates with variable amounts of metal cations in their structures. The crystalline structure of clay minerals consists of two-dimensional sheets of $(AlSi)_3O_4$ composition, leading to a stack of layers (platelets) separated by interlayers. The sheet dimensions are around 100 nm–1 μm in breadth and 1 nm thickness. The exfoliation (sheet separation) of clay minerals into polymer matrixes can be carried out when a previous organophilization step has been performed, leading to clay–polymer nanocomposites. Following this idea, Rytwo and coworkers synthesized and developed clay–polymer nanocomposites for their use in the pretreatment of polluted effluents from winery wastewater (Rytwo et al. 2013). Using these nanomaterials a coagoflocculation process, i.e., neutralization and aggregation of suspended colloids into larger particles, was developed. The clay polymer nanocomposites were synthesized by the combination of sepiolite or smectite as clay minerals and poly(diallyldimethylammonium)chloride or chitosan as polymers. Polymer intercalation between clay sheets was observed for smectite-based nanocomposites. All nanocomposites gave rise to good results in the clarification of wine wastewater (Rytwo et al. 2013).

Dendrimers are molecular nanosystems that constitute an interesting class of nanomaterials applied in several fields, especially as encapsulating agents. Thus, dendrimers are nano-sized highly branched macromolecular arrangements that display intrinsic features, such as easy buildup, tunable solubility, high loading capacity, biocompatibility, and localized positioning of functional groups. These unique features make these types of macromolecules ideal for the development of new applications. Among them, dendrimers have been used for the encapsulation and removal of tartaric acid from wines. Together with malic acid, tartaric acid represents more than 90 % of the total titratable acidity of wine and grape musts, whereby tartaric acid is the dominant component in most of the cases. Because grapes cultivated in warm regions tend to have a low content of acidity, must and wine produced from these grapes require acidification. In contrast, vines cultivated under cool climates may produce grapes that are too acidic, and wines obtained from these grapes may need physical, chemical or microbiological deacidification. Moreover, wines would naturally form tartrate salts with potassium or calcium ions that may grow as crystals large enough to represent an esthetic problem. The main processes designed for tartrate removal from wine include cold stabilization (0–4 °C) for long periods, extraction of solvent and further membrane separation and evaporation. An alternative approach recently reported consists of the use of amino-terminated poly(amidoamine) (PAMAM) and poly(propyleneimine) (PPI) nano-sized den-

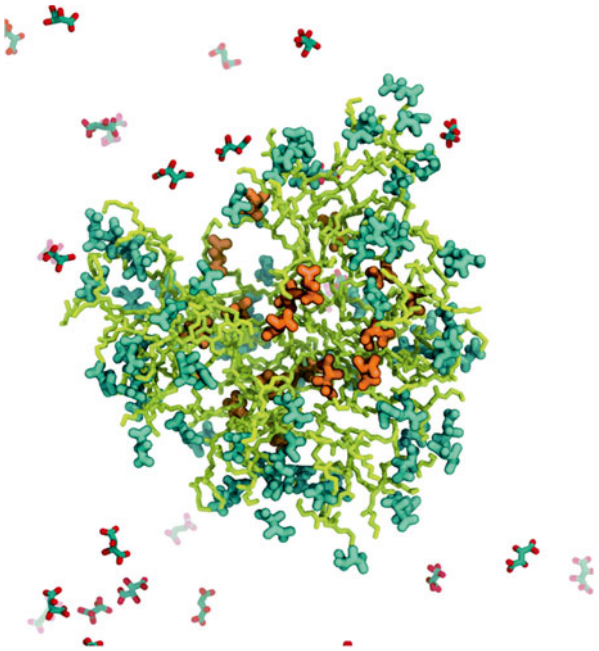

Fig. 3.1 Snapshot of the calculated (molecular dynamics simulation) 100 tartaric acid-1 PAMAM system (reproduced by permission of the Royal Society of Chemistry)

drimers as encapsulating agents of tartaric acid from white and red wines (Schramm et al. 2014). The binding of tartaric acid inside the dendrimer forms a dendrimer-tartaric acid complex that can be separated from wine samples through ultrafiltration or reversed dialysis processes. These systems have been studied through molecular dynamics simulations (see Fig. 3.1).

3.2.2 Nanomaterials for the Immobilization or Vectorization of Wine Yeast

With the rapid development of nanotechnology, nanoparticles have shown great potential applications in many biological fields, including in biomolecules (proteins, peptides, and enzymes) and in microorganisms, such as yeast and bacteria. A second class of nanomaterial used in wine production comprises those related to yeast immobilization or vectorization. In the first case nano-tubular cellulose has shown an interesting promoting activity in bioprocessing. More precisely, nano-tubular cellulose has been reported (Koutinas et al. 2012) as a yeast immobilization support. This nanomaterial was prepared by the delignification of soft wood sawdust with NaOH. The activity of nano-cellulose-supported yeast in the reaction rate and

activation barrier of alcoholic fermentation has been analyzed in detail. The authors conclude that supported yeast diminishes the activation energy and increases the alcoholic fermentation rate.

Another interesting application of nanomaterials in wine production is the magnetic vectorization of yeast using γ-Fe_2O_3 magnetic nanoparticles. The context of application is the removing of yeast biomass from sparkling wine production. Thus, the production of CO_2 in sparkling wine is traditionally obtained by the "*traditional method*." This method consists of a secondary fermentation of a still wine, called a base wine. When the bottle is uncorked, the sparkling wine gives off carbon dioxide that is exclusively produced by this fermentation (endogenous). After this fermentation, the obtained yeast biomass (deposit of yeast lees) has to be removed from the bottle. Traditional separation of the yeast biomass is carried out in two steps: (1) sedimentation and (2) expedition from the bottle using gas pressure or by freezing the deposit of lees by immersing the bottle in a cryogenic bath. This process can be quite lengthy. If incorporated or immobilized yeasts are used or yeasts inside a cartridge designed for this purpose, called Millispark system, are placed in the neck of the bottle, the riddling is avoided and disgorging is facilitated (Martínez-Rodríguez and Pueyo 2009). In a recent report Berovic et al. (2014) proposed that magnetized yeast could be separated from the wine by the use of an external magnetic field. This method includes a previous step in which small (ca. 16 nm) γ-Fe_2O_3 (maghemite) nanoparticles coated with a SiO_2 layer are absorbed in the yeast cell membranes. In order to improve the interaction between the core/shell maghemite/silica nanoparticles and the cell membrane, and to avoid penetration of the nanoparticle inside the cell, a last step of functionalization of the SiO_2 outer shell with 3-(2-aminoethylamino)propylmethyldimethoxysilane (APMS) was carried out. The metabolic activity of magnetized yeast was increased compared to non-magnetized yeast, leading to a speeding up of the fermentation process. The magnetic separation of the yeast biomass is inexpensive and very effective, although its potential for further biotechnological development in winemaking is still unexploited

Also recently, a novel and efficient immobilization of yeast alcohol dehydrogenase (YADH, EC1.1.1.1) from *Saccharomyces cerevisiae* has been developed by using the surface functionalization of chitosan-coated magnetic nanoparticles (Fe_3O_4/KCTS) as support (Li et al. 2010). Alcohol dehydrogenase, which catalyzes the oxidation of alcohols and the reduction of carbonyl compounds such as aldehydes and ketones, has attracted more attention because of its potential applications in preparing various starting materials and intermediates. The surface functionalization of chitosan-coated magnetic (Fe_3O_4/KCTS) nanoparticles was used to immobilize YADH by electrostatic adsorption and covalent binding. Compared to the free enzyme, the immobilized YADH retained 65 % of its original activity and exhibited significant thermal stability and good durability, and could be readily recovered by magnetic separation.

3.2.3 Silver-Based Nanomaterials

A third class of nanomaterial used in wine production and quality and safety control comprises silver nanoparticles (Ag NPs). Silver nanoparticles or silver-based nanomaterials have been used for a long time as very efficient antimicrobial agents and many types of applications in different fields have been designed (García-Barrasa et al. 2011), but it is only now that the necessary studies for microbicidal mechanisms of action are being carried out. Despite the great interest in the applications of these materials in the field of enology, so far studies on the use of silver as an antimicrobial in winemaking have been very scarce. Recently, different studies have been reported with the idea of investigating the potential use of Ag NPs to minimize the use of sulfur dioxide (SO_2) in enology. There is an increasing desire for the development of new alternatives that can replace or complement the antiseptic and antioxidant properties of SO_2, owing to the potential risks of sulfites to human health. Firstly, Izquierdo-Cañas et al. (2012) reported the use of a so-called colloidal silver complex as an alternative to the use of SO_2 in white and red wine making. The silver nanoparticles used in this study consist of a commercial nanomaterial based on Ag NPs of less than 10 nm size, supported on an inorganic inert material of ca. 10 μm size. This nanomaterial is able to control the development of acetic acid and lactic acid bacteria in wines. Later, in another work Garde-Cerdán et al. (2014) have described the antiseptic effect of commercial colloidal silver CAgC. This nanomaterial consists of Ag NPs (1 % in weight) of less than 10 nm supported on kaolin. In this study the use of the nanomaterial alone or in combination with SO_2 was examined in the vinification and storage of wines. The authors concluded that wines elaborated with CAgC showed no microbiological problems. In a recent study (García-Ruiz et al. 2015), the synthesis and characterization of biocompatible Ag NPs and their use for controlling the growth of gram-negative and gram-positive bacteria, such as *Escherichia coli* and *Staphylococcus aureus*, and different wine lactic acid bacteria (*Oenococcus oeni*, *Lactobacillus casei*, *L. plantarum*, and *Pediococcus pentosaceus*) and acetic acid bacteria (*Acetobacter aceti* and *Gluconobacter oxydans*) has been reported. The Ag NPs have been obtained through an organometallic approach, using biocompatible polyethylene glycol (PEG) or glutathione (GSH) as stabilizing agents (see Fig. 3.2). PEG-Ag NPs (2–3 nm size) were more effective against gram-negative strains (*E. coli* and acetic acid bacteria) than against gram-positive strains (*S. aureus* and lactic acid bacteria). GSH-Ag NPs were extraordinarily effective against *O. oeni*, the main species responsible for malolactic fermentation in wines. The results obtained by epifluorescence microscopy suggest damage to the integrity of the membrane after incubation of wine bacteria with Ag NPs (García-Ruiz et al. 2015). These results confirm the potential use of biocompatible silver nanoparticles as an alternative to the use of sulfites in winemaking, although further nano-ecotoxicology studies are necessary to ensure its implementation at the winery.

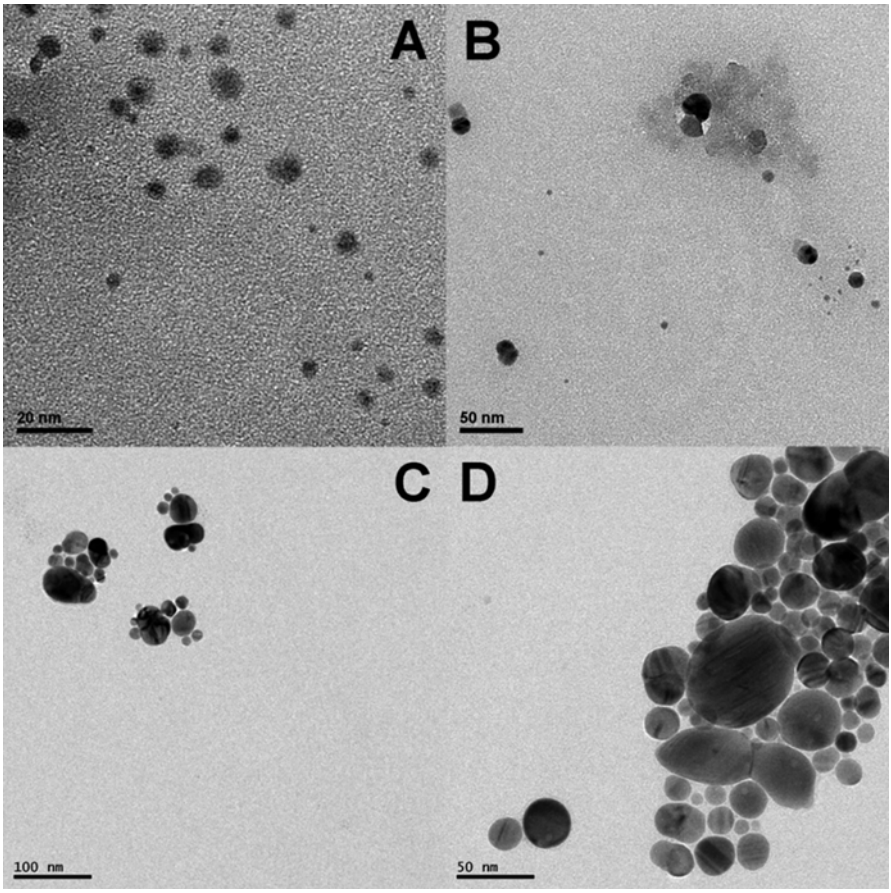

Fig. 3.2 Transmission electron microscopy images of PEG-Ag NPs (**a** and **b**) and GSH-Ag NPs (**c** and **d**) (reproduced by permission of Elsevier)

3.2.4 Nanofibers

Electrospun biodegradable nanofibers are widely used in biomedical applications as nanofibrous scaffolds for tissue generation and tissue engineering, as well as for antibacterial drug delivery. Due to increased awareness of environmental issues, natural biodegradable biopolymers, also popularly called green materials, have been widely studied over the last decade, some of them with applications in viticulture and enology. For example, biodegradable but water-stable nanofiber nonwovens were obtained by the electrospinning of concentrated aqueous dispersions and successfully used for grapevine protection of the European grapevine moth *Lobesia botrana* (Bansal et al. 2012). Most recently, adhesive biodegradable membranes (patches)

Table 3.1 Summary of nanomaterials used for wine production and quality and safety control

Application	Nanomaterial	Substance/microorganism in WINE	Reference
Clarification	Clay–polymer nanomaterials	Winery wastewater	Rytwo et al. (2013)
Immobilization support	Nano-tubular cellulose	Yeast	Koutinas et al. (2012)
Nano-capturing	Dendrimers	Tartaric acid	Schramm et al. (2014)
Magnetized yeast	γ-Fe_2O_3 maghemite NPs	Yeast	Berovic et al. (2014)
Catalyst	Au NPs	Aldehyde	Liu et al. (2006)
Photocatalyst	Nano TiO_2	*p*-coumaric acid	Bacsa and Kiwi (1998)
Catalyst	Chitosan-Au NPs	Acetaldehyde	Yu et al. (2010)
Antimicrobial	Colloidal silver	Acetic and lactic acid bacteria	Izquierdo-Cañas et al. (2012)
Antimicrobial	Colloidal silver	Acetic and lactic acid bacteria	Garde-Cerdán et al. (2014)
Antimicrobial	Ag NPs	Acetic and lactic acid bacteria	García-Ruiz et al. (2015)

for the protection of plant pruning locations from esca fungi attacks were developed using electrospun soy protein/polyvinyl alcohol and soy protein/polycaprolactone nanofibers have been developed Table 3.1 (Sett et al. 2015).

3.3 Nanomaterials for Sensing and Detection

One of the most important applications of nanomaterials to date is their use for the design of sensing devices. Broadly speaking, a *chemical sensor* is a self-contained device that is capable of rendering real-time analytical information about the concentration of chemical species in a given sample, by performing two functions: recognition and transduction. A *biosensor* is a device that consists of a biological and an electronic component to yield a measurable signal. On the other hand, a *nanosensor* is a nanoscopic hybrid assembly, which includes nanoparticles and molecular compounds and it is capable of performing recognition and transduction functions. Taking into account all this, a biosensor in which nanomaterials are implemented can be considered as a *nanobiosensor* (Banica 2012; Malik et al. 2013; Sagadevan and Periasamy 2014).

In the following subsections a classification of nanobiosensors and nanosensors used in the wine research field is grouped in terms of the type of recognition and transduction functions for which they are designed. In addition to the design of specific sensing devices, nanomaterials have also been used for the improvement of separation technique methods, which allow better detection and quantification of wine-related species. The last subsection is devoted to this field.

3.3.1 Nanobiosensors

The most important class of nanomaterial-based sensors used in wine production and quality and safety control is that including bioreceptors as recognition elements, i.e., nanobiosensors. Among them, the most important subcategory is that of enzyme-based sensors, although immunosensor (antibodies as bioreceptors) or microbial sensors (bacteria as bioreceptors) have also been used. The nanomaterials (carbon nanotubes, metal nanoparticles or nanowires, metal oxide nanoparticles, etc.) are used as efficient electrical interfaces.

The analytes of interest include polyphenols, glycerol, ethanol, glucose, L-lactic acid, and sulfite, among others.

Regarding the nanomaterial used, most of the nanobiosensors are built up using multi-walled carbon nanotubes (MWCNTs). Thus, for example, this nanomaterial has been used in combination with different enzymes, such as peroxidase; a mixture of glycerol kinase, creatine kinase, and sarcosine oxidase; pyranose oxidase; alcohol dehydrogenase; glucose oxidase; L-lactate oxidase; tyrosinase; glucose dehydrogenase; or polyphenol oxidase. In many cases, the MWCNTs appear integrated in polymer matrixes in order to build up the corresponding electrodes. For instance, chitosan (Ghica et al. 2009; Lee and Tsai 2009; Monosík et al. 2012a, b, c), poly(vinyl alcohol) (PVA) (Tsai et al. 2007), poly(glucosyl 4-vinylphenylboronate) p(GVPB) or poly(2-hydroxyethyl-methylacrylate) p(HEMA) (Yang et al. 2009), poly(glycidyl methacrylate) p(GMA) (Chung et al. 2012), and poly(acrylic acid) (PAAc) or poly(maleic anhydride) (PMAn) polymers (Kim et al. 2010), have been used with MWCNTs. In other cases, MWCNTs are directly integrated in the carbon paste electrodes (Odaci et al. 2008; Granero et al. 2010) or cast with 1-butyl imidazole bromide ionic liquid and chitosan on indium tin oxide (ITO) glass (Kim et al. 2009).

Also, amperometric biosensors have been developed for real-time monitoring of alcoholic fermentation and malolactic fermentation in wines by Piermarini et al. (2011) and Gamella et al. (2010), respectively. For the fabrication of glucose and ethanol biosensors, graphite screen-printed sensors modified with Prussian blue were coupled with oxidase enzymes, while for the fructose biosensor a bare screen-printed sensor was coated with fructose dehydrogenase and phenazide methansulfate as electrochemical mediator. The biosensor allows the monitoring of alcoholic fermentation by the inoculation of two different strains of *Saccharomyces cerevisiae* (Piermarini et al. 2011). Also, integrated multi-enzyme electrochemical biosensors were developed by the co-immobilization of the enzymes L-malate dehydrogenase (MDH) and diaphorase (DP), or L-lactate oxidase (LOX), together with the redox mediator, and used for the monitoring of the enzyme reactions involved in L-malic and L-lactic acid determination (Gamella et al. 2010).

Gold nanoparticles (Au NPs) alone (Carralero Sanz et al. 2005; Ozdemir et al. 2010; Sánchez-Obrero et al. 2012) or in combination with Fe_3O_4 NPs (Rawal et al. 2012) or MWCNTs (Sartori et al. 2011) are also used as an electrical interface in nanobiosensors coupled with enzyme bioreceptors, such as tyrosinase, pyranose oxidase, sulfite oxidase, or polyphenol oxidase. As an example, Fig. 3.3 depicts the

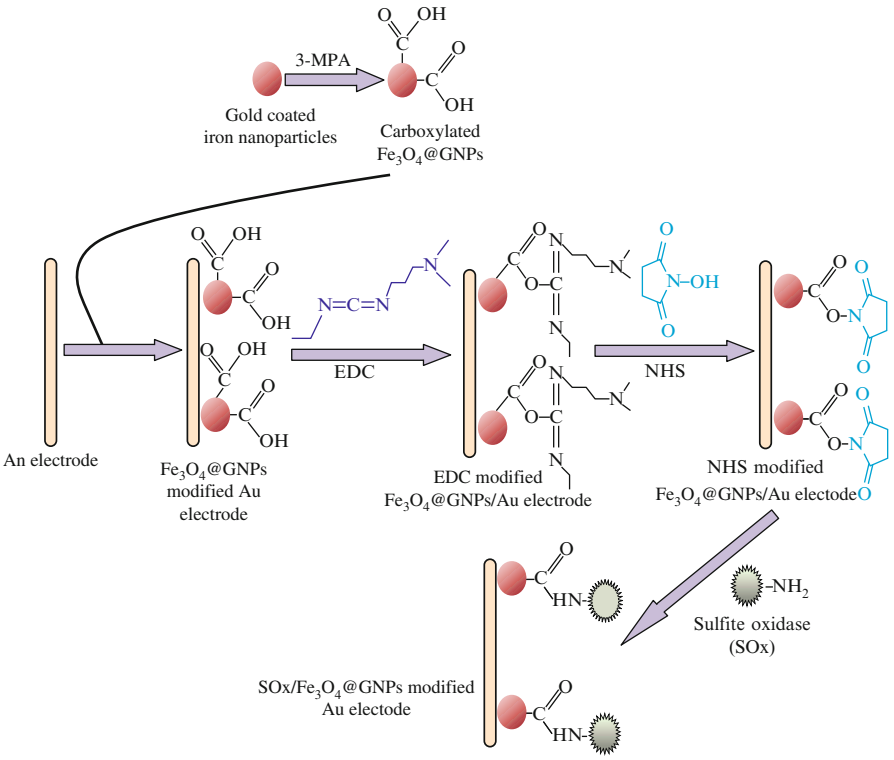

Fig. 3.3 Schematic representation of the SOx/Fe_3O_4@AuNPs/Au electrode nanobiosensor (reproduced by permission of Elsevier)

schematic representation of chemical reactions involved in the fabrication of a nanobiosensor consisting of a gold electrode, Au-coated Fe_3O_4 NPs, and sulfite oxidase (SOx) enzyme. SOx was immobilized onto carboxylated Fe_3O_4@Au NPs through *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC)-*N*-hydroxy succinimide (NHS) chemistry. These nanobiosensors have been used for the detection and quantification of polyphenols, glucose or sulfite in wine samples.

Recently a sensor has been developed that determines the astringency of the wine, a quality that is perceived especially when tasting red wine, which is mainly due to wine tannins. The sensor (which is called "mini-mouth") was used to measure the salivary protein to measure the sensation produced in the mouth by drinking wine (Guerreiro et al. 2014). In particular, the nanosensor that consists of a small plate coated with nanoscale gold particles has great potential not only in the wine sector, but in medical applications, since it measures the amount of proteins and molecules in the mouth both as it is ingesting wine and other matrices/products.

Other nanomaterials used in the design of nanobiosensors used in the wine field include Ag and ZnO NPs in combination with the enzyme laccase, for the detection of phenolic compounds in wine samples (Chawla et al. 2012) or Prussian blue

nanoparticles with sulfite oxidase enzyme (Rawal and Pundir 2012) for the detection of sulfites in wine samples.

The development of label-free immunosensors for pesticide residue analysis (i.e., atrazine) in a complex matrix, such as red wines, has been recently reported. The immunosensor is based on an array of interdigitated electrodes and immunoreagents specifically developed to detect this pesticide. Immunochemical determination of atrazine is possible without the use of any label. An atrazine-haptenized protein was covalently immobilized on the surface of the interdigitated electrodes area (interdigital space) previously activated with (3-glycidoxypropyl)trimethoxysilane (Ramón-Azcón et al. 2008). Subsequently, antibodies (antiIgG) have also been used as bioreceptors with Au NPs as electrical interfaces for the detection of atrazine in wine (Valera et al. 2008, 2010).

Finally, a recent work describes the design of a microbial nanobiosensor for the detection of phenolic compounds in commercial red wines. The device consists of a glassy carbon electrode in which *Acaligenes* sp. (which is known as a potent phenol-removing bacterium) is immobilized on a nanomaterial support formed by the combination of CdS or Cu_2S quantum dots and MWCNTs (Kim et al. 2011).

3.3.2 Nanosensors

Another important class of sensing device including nanomaterials for better performance is that of electrochemical nanosensors. These types of sensing devices are similar to the enzyme-based nanobiosensors described in the previous subsection but without a bioreceptor attached to the nanomaterial electrical interface.

Again, MWCNTs is one of the nanomaterials of choice for the design of electrochemical nanosensors. This nanomaterial provides a very good electrocatalytic activity but also enhanced signal stability and good resistance to passivation. Multi-walled carbon nanotubes have been used for the detection and quantification of polyphenols, glucose, methylglyoxal, tyrosine or gallic acid in wine samples. These MWCNTs can be directly integrated as layers on glassy carbon electrodes (Shenghui et al. 2004; Moreno et al. 2011) or on carbon paste electrodes (Souza et al. 2011). In similar devices, platinum nanoparticles (Pt NPs) were electrodeposited on SWCNTs cast on glassy carbon electrodes (Chatterjee and Chen 2012). In this case, the platinum nanoparticles provide a large surface area for interaction with the analyte and a synergistic electrocatalytic effect with SWCNTs in the reduction of methylglyoxal. In addition, MWCNTs combined with polyvinylpyrrolidone (PVP)-stabilized bimetallic Pt-Ru or Pt-Sn NPs have been used recently for the design of a non-enzymatic nanosensor displaying a high electrocatalytic activity for glucose detection (Kwon et al. 2012).

Gold nanoparticles have also been employed for the design of electrochemical nanosensors with application in the wine field. For example, Au NPs/chitosan nanocomposite films, which combine highly conductive AuNPs with a large number of organic functional groups in the biopolymer, have been used for the determination

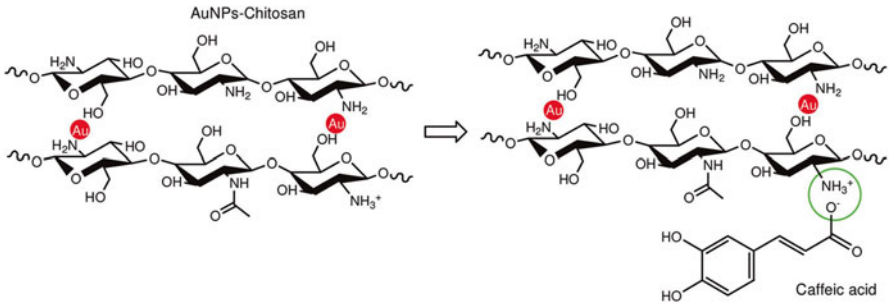

Fig. 3.4 Schematic representation of Au NPs/chitosan films used in the determination of caffeic acid (reproduced by permission of the American Chemical Society)

of polyphenols (Curulli et al. 2012) or the selective determination of caffeic acid (Di Carlo et al. 2012). Figure 3.4 depicts a schematic representation of the Au NPs/chitosan nanocomposite films used in the determination of caffeic acid. These hybrid nanocomposite films combine highly conductive AuNPs with a large number of organic functional groups in the biopolymer that favors the interaction with the functional groups of caffeic acid. In other cases, modified carbon paste electrodes with banana tissue/Au NP or banana tissue/MWCNTs have been used for the determination of catechol (Serdar and Ülkü 2010). Furthermore, the modification of carbon paste electrodes with Au NPs and aluminum titanate nanopowder has been used for glutathione detection (Cubukçu et al. 2012).

Silver nanoparticles have been integrated in poly(thiophene) films in the design of a modified glassy carbon electrode (GCE). This electrode gave rise to a high electrocatalytic activity towards the oxidation of caffeic acid in red wine samples (Karabiberoglu et al. 2013)

Gas nanosensors, which operate through resistive transduction, have also been tested in wine samples. Thus, Au-doped ZnO nanostructures have been synthesized and used in a sensing device for the detection of volatile organic compounds (VOCs), detecting the difference between white and red wines (Wongchoosuk et al. 2009).

A second important class of devices comprises nanosensors using nanomaterials as optical transducers. There are some examples of optical nanosensors used for the determination of analytes in wine. Thus, for example, the evolution of the surface plasmon resonance absorption during the formation process of gold nanoparticles has been used for the detection of endogenous polyphenols in wine samples. The polyphenols are the analyte of interest but also the reducing agent responsible for the reduction of gold(III) salts into Au NPs (Vilela et al. 2012). In a recent study, Santos-Figueroa et al. have reported on the use of hybrid organic–inorganic silica nanoparticles for the chromofluorogenic detection of sulfite (Santos-Figueroa et al. 2013). In this study, the mesoporous MCM-41 inorganic nanoparticles are used as inorganic scaffolds in which red pyrilium stilbene is absorbed, acting as the chromofluorogenic probe. Sulfite anions selectively enhance the fluorescent emission of the organic probe. A third type of nanomaterial used for the design of optical nanosensors is nanostruc-

tured silicon. The device is capable of monitoring the amount of ethyl alcohol in red wines without sample pretreatment, through the study of the red-shift of the resonant peak attributed to the nanoporous silicon that depends on the wine's alcoholic strength (Rocchia et al. 2007). Finally, a luminol-$CoFe_2O_4$ NPs-sulfite chemiluminescent system has been designed for the study of sulfite traces in white wines. Depending on the sulfite concentration the chemiluminescence of the luminol-$CoFe_2O_4$ system could be enhanced or inhibited (Zhang et al. 2013).

3.3.3 Separation Techniques

As is widely known, sample preparation in analytical chemistry has attracted great attention regarding the development of simple and fast analysis. There are various sample preparation methods for wine analysis in which nanomaterials are involved, which address the demand of portable, self-sufficient, easily operated analytical tools.

For example, MWCNTs have been used as solid phase extraction (SPE) sorbents to prepare a packed trapping column for the extraction and cleanup of the sample containing a mixture of *cis*- and *trans*-resveratrol (Lu et al. 2011). The whole separation system was integrated in an ultrafast analytical protocol: SPE-high performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS). The limits of quantification for *cis*- and *trans*-resveratrol were 0.05 and 0.06 ng/mL, making the method valid for trace analysis of this molecule. In another report, an SPE sorbent based on carbon nanotubes was designed by electrochemical polymerization of pyrrole onto a stainless steel frit using ochratoxin A (OTA) as template and carbon nanotubes as nanostructured fibers. After removing the template, the SPE sorbent was coupled online to HPLC, leading to a high selective binding ability for OTA at sub-ppb levels in red wine matrixes (Yu and Lai 2006). Furthermore, immobilized MWCNTs have been used as stationary phase for the determination of melatonin in wine samples through capillary electrochromatography (Stege et al. 2010). In a recent report, a poly(butyl methacrylate-co-ethyleneglyceldimethacrylate) (poly(BMA-EDMA)) monolithic column was prepared and modified with allylamine-β-cyclodextrin (ALA- β-CD) and cuprous oxide (Cu_2O) nanoparticles. This material has been used as a microextraction device for the preconcentration of polychlorinated biphenyls in wine samples, which are determined through a gas chromatography-electron capture detector (Zheng et al. 2014). Polystyrene-coated Fe_3O_4 magnetic nanoparticles have been used for the design of magnetic solid-phase extraction (MSPE) adsorbents. The coupling of MSPE with ultrafast liquid chromatography ultraviolet spectrometry allows the development of a sensitive method for the determination of Sudan dyes in wine samples (Yu et al. 2012). A novel polymer monolith microextraction (PMME) material was prepared by the functionalization of a porous polymer with γ-alumina nanoparticles. The coupling of this PMME material with HPLC permits the determination of Sudan dyes in wine samples (Li et al. 2013).

3.4 Conclusions

From the current state of the art, it is clear that nanotechnology applications are expected to bring a range of benefits to the wine sector with the aim of providing better quality, conservation, and safety. In spite of the recent progress in this area, innovation is taking place at all levels with the use of nanomaterials, including targeting improved detection of wine components and analytes in the raw material and the final product, as well as throughout the whole of the winemaking process. The use of, for example, gold and silver nanoparticles offers new efficient approaches for the sensitive detection and/or elimination of potential safety risks derived from pesticides, mycotoxins, and chemical additives (i.e., SO_2).

In recent years, there has been a growing interest in developing low-cost techniques for the inexpensive and rapid detection of analytes, with special interest in contaminant analysis and safety control. Nanobiosensor technologies based on various transduction modes (e.g., electrochemical, such as amperometric, optical sensors) and assay principles (immunoassays, enzymatic inhibition, etc.) are gaining importance due to rapid screening capabilities. They can be applied in different ways to wine, depending on both the aim and other factors that as a whole would affect the cost.

Until now, most of the developed nanomaterials have been shown to be promising tools for lab processes but they also present some disadvantages, depending of the application. However, due to reproducibility problems as well as interferences, their use in real sample matrices remains limited.

In the meantime, there is still a lack of knowledge about the possible risk to human health and environmental safety posed by the expanding development and use of nanomaterials. The available data indicate that some insoluble nanomaterials can pass through the different protective barriers and the gastrointestinal barrier in particular, be distributed in the body, and accumulate in several organs (Martirosyan and Schneider 2014). There are still large gaps in the understanding of the extent to which one or another type of material can pass through natural biological barriers. Since the biological and toxic effect of nanomaterials are highly dependent on their physicochemical properties (size, shape, charge, coating, solubility, etc.) as well as on dosage, route of administration and duration of exposure, it becomes evident that a clear and precise evaluation of the biological effects of nanotechnology on products should also include a homogeneous exposure classification to ascertain exactly how physicochemical properties correlate with nanotechnology's adverse health effects. The toxicological nature of hazard, exposure levels and risk to consumers from nanotechnology-derived food are at the earliest stage of investigation, and need further investigation in order to unravel the biological outcomes of nanofood consumption.

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Chapter 4
Genetic Improvement and Genetically Modified Microorganisms

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4.1 Introduction

Winemaking stands out as one of the earliest examples of Biotechnology in the history of mankind. Long before the microbiological and biochemical mechanisms involved in alcoholic fermentation were elucidated by Pasteur in 1857, men could already produce wine, as evidenced from the pottery residues found in the Neolithic settlements of Hajji Firuz Tepe and Godin Tepe in the northern Zagros Mountain, Iran (Phillips 2001).

Despite the ancient origins of winemaking, and the values of tradition generally ascribed to it, it could certainly be assured that this industry is currently benefiting from the development of modern Biotechnology. This field experienced a tremendous boost during the second half of the twentieth century due to two key factors: the elucidation of the molecular structure of DNA (Watson and Crick 1953) and the subsequent development of recombinant DNA technology, which allowed the transfer, replication, and expression of genetic material from one organism into another (Cohen et al. 1973). While the use of recombinant microorganisms represented the starting point for the industrial production of a broad diversity of therapeutics, such as the human proteins insulin and growth hormone, its use in the production or modification of any food commodity often raises mistrust and condemnation.

Multiple studies have established that the ascomycetous yeast *Saccharomyces cerevisiae* is the species mainly responsible for conducting wine fermentation. Nevertheless, the relevant contribution of the complex microbial consortia that occur in the different stages of winemaking has been recognized since the middle of the last

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century. During the initial stages of a spontaneous fermentation of grape must, yeast species commonly present on the grape surface and belonging to the genera *Candida*, *Hanseniaspora*, and *Kloeckera* predominate, followed by several species of *Metschnikowia* and *Pichia* during middle stages (2–4 % (v/v) ethanol). Increasing levels of ethanol, combined with anoxic conditions, CO_2, and the presence of antimicrobial agents, such as sulfites, leads to the almost invariable replacement of these species by strains of *Saccharomyces cerevisiae*, far better adapted to oenological conditions.

After alcoholic fermentation, a second fermentative process called malolactic fermentation (MLF) often takes place, especially for red wines. This process is mainly driven by *Oenococcus oeni*, a lactic acid bacterial species especially adapted to this environment, but strains belonging to the genera *Lactobacillus*, *Pediococcus*, or *Leuconostoc* could eventually drive or take part in the process. During MLF malic acid is decarboxylated to lactic acid, which leads to wine deacidification, a general improvement of the organoleptic properties, and increased microbial stability.

Similar to other industrial processes, men have tried first to standardize and then to improve the outcome of wine fermentations. Major advances have been achieved since Emil Christian Hansen, working for Carlsberg Laboratory, pioneered the use of pure yeast cultures for a reliable production of beer in 1875. This key advance in the history of Biotechnology allowed the understanding of the crucial role of proper microbial management for standardizing fermented food products. The introduction of starter cultures and the search for methodologies for strain improvement have been ongoing ever since.

The genome of wine yeast strains has been shaped by centuries of natural selection in a humanized environment, therefore showing specific features. Whole genome based phylogeny shows these strains in a tight cluster with lager strains as the closest relatives to the group (Liti et al. 2009). They are usually diploid, although triploid or allotetraploid strains are often found (Naumov 2000), and can present a high number of aneuploidies (Querol and Bond 2009; Novo et al. 2009; Gibson and Liti 2014). Specific genomic adaptations frequently found in wine yeast strains include a translocation, associated to additional sequence changes and conferring a sulfite resistance phenotype (Pérez-Ortín et al. 2002); interspecific horizontal transfer, and other insertion events involving for example a high-affinity fructose/H^+ symporter, a homolog of the *Saccharomyces pastorianus* gene *FSY1*, and genes coding for oligopeptide transporters or aryl-alcohol dehydrogenases (Borneman et al. 2008; Novo et al. 2009; Damon et al. 2011; Borneman et al. 2013).

Initially, selection of *S. cerevisiae* strains was mainly driven by the search of suitable fermentation kinetics and unnoticeable production of off-odors and flavors. These criteria have nowadays evolved to much more sophisticate ones (Novo et al. 2012). Despite the enormous microbial diversity already available from natural sources, it is often more convenient to genetically improve the features of industrial microorganisms already in use than coming back to screening natural isolates among which the features or combinations of them best suited for specific applications can be extremely rare. Some reasons being (a) the very specific growth conditions micro-

organisms are subject to in the industrial environment, so that it is very unlikely for natural isolates to have been subject to similar selective pressures; (b) the demand for specific metabolic outcomes, also improbable to confer selective advantages under natural conditions; (c) the requirement for specific combinations of phenotypic characters, unlikely to occur together by natural selection.

Improvement of wine yeasts can be addressed by traditional genetic methods, parasexual hybridization, random mutagenesis, or genetic engineering. Sexual hybridization is mainly limited by the low sporulation efficiency and spore viability of many industrial yeast strains, while both random mutagenesis and sexual hybridization are hampered by the complex genomic structure of industrial yeast strains. Additionally, a major drawback of both methods is the high probability of losing desired phenotypic traits by recombination, chromosomal rearrangements, or untargeted mutations in the genome.

On the contrary, the use of genetic engineering allows for a clean and directed manipulation of the genetic information of a given microorganism without the concomitant loss of desired features. In this respect, it is particularly relevant to point out the vast amount of genetic and physiological knowledge available for the wine yeast *S. cerevisiae*. Its complete genome sequence, published almost 20 years ago (Goffeau et al. 1996), represented a milestone in the genomic era as the first genome of a eukaryotic organism publicly available, and opened multiple possibilities for future studies and industrial applications (Nielsen et al. 2013). As a result, numberless examples have proven the usefulness of genetic engineering for obtaining strains with improved oenological traits, capable of satisfying the demanding nature of modern winemaking, improving both wine quality and safety or facilitating specific stages of the production process.

Although genetic engineering allows for a precise modification, insertion or deletion of individual genes, it is well known that most oenological properties are controlled by complex gene, protein, and metabolite interactions. It is therefore necessary to investigate this entangled network of connections by means of whole-cell methodologies, the so-called -omic technologies (i.e., genomics, transcriptomics, proteomics, metabolomics, interactomics, …), and integrate the multiple data generated using bioinformatics and mathematical modelling.

Despite the ongoing development of these cutting-edge techniques and their potential enormous application, strict GMO (genetically modified organism) regulation and consumer demands and preferences, raising issues related to food and environmental safety, have limited their application in the wine industry. This fact has stimulated a revival of alternative non-GMO methods, including adaptive laboratory evolution (Bachmann et al. 2015) that benefits from short generation times and large populations to obtain novel desired phenotypes (and genotypes) by means of a laboratory controlled selective pressure.

The present chapter aims to describe the different techniques for genetic improvement of the two main wine microorganisms, *S. cerevisiae* and *O. oeni*, and highlight the most recent achievements in the field. A summary of the wine yeast genetic improvement examples described in the following sections is shown in Fig. 4.1.

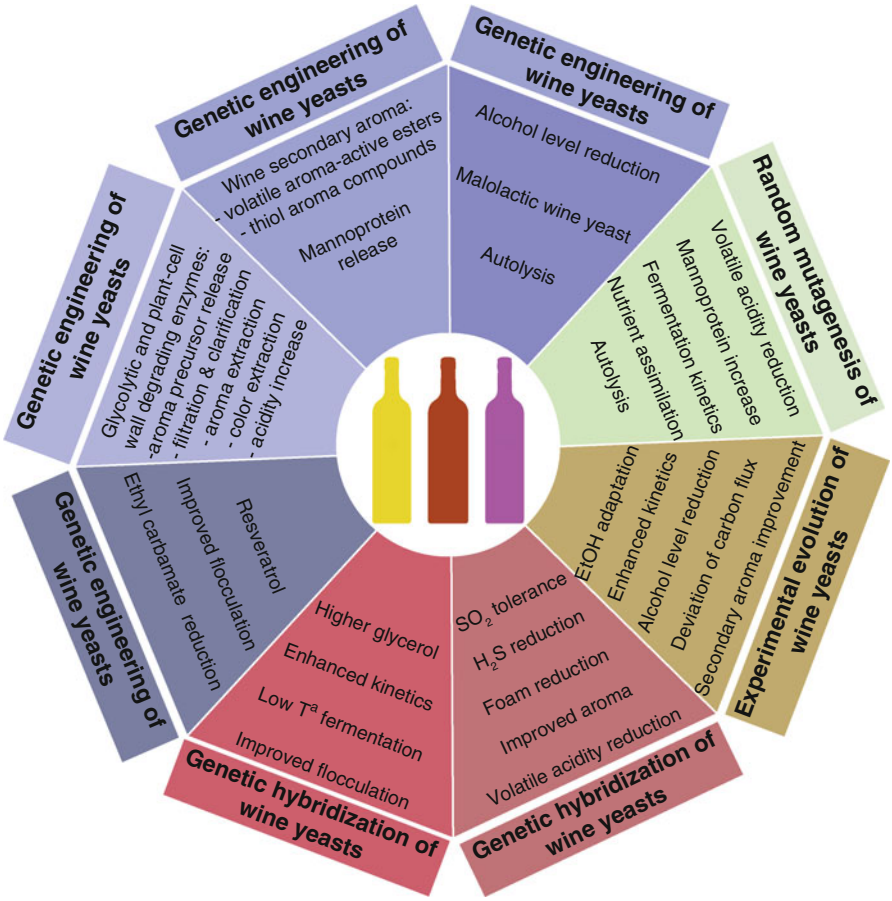

Fig. 4.1 Summary of the different aims historically and currently addressed for the genetic improvement of wine yeast strains, grouped by the methodologies involved

4.2 Genetic Engineering of Wine Yeasts

Genetic engineering allows for a targeted modification of the genome of the microorganism of interest, and is not subject to the species barrier. It demands the development of transformation systems, which in turn require methods to introduce exogenous DNA in the cell, transformation markers, and transformation vectors. Genetic engineering is the genetic improvement method that results in the minimal alteration of the host cell genome. Besides, the outcome of the improvement process is easier to anticipate, especially when combined with powerful analytical and computational tools (metabolic engineering). However, it has limited use when addressing polygenic characters or when the genetic basis is not well understood. Literature

on *S. cerevisiae* genetic engineering includes many publications of proof-of-concept experiments using recombinant laboratory yeast strains. However, this section will only focus on examples of genetic improvement of natural wine yeast strains or direct derivatives (e.g., monosporic cultures). Very few instances of genetic improvement of wine yeasts had been published up to the early 90s. At that time, the degree of development reached by *S. cerevisiae* molecular biology tools contributed to the flourishing of genetically engineered wine yeast strains, initially originating from research groups in Spain, France and South Africa.

Genetic engineering of wine yeasts relies on genetic transformation tools, in turn dependent on the availability of systems to make yeast cells competent (i.e., to accept foreign DNA), selective transformation markers, and replicative or integrative vectors. In addition, in order to drive the expression of heterologous genes, promoters active during the winemaking process are required. Fortunately, the lithium acetate method to induce transformation competence in laboratory strains (Gietz et al. 1992) readily worked for wine yeasts strains (Pérez-González et al. 1993), and has been the method of choice for the construction of most recombinant wine yeast strains published so far (Ramon and Gonzalez 2011). Auxotrophic selection markers, widely used in the transformation of laboratory yeast strains, are not suitable for prototrophic industrial strains. This was overcome by using dominant selection markers, initially antibiotic resistance markers like cycloheximide (semidominant), or G-418 (Pérez-González et al. 1993). Marker rescue systems were used to generate an auxotrophic wine yeast derivative (Puig and Perez-Ortin 1998), in order to improve transformation efficiency as well as to avoid the bad press associated to antibiotic resistance markers. Alternative dominant selection markers were also explored by Cebollero and Gonzalez (2004). Concerning promoters, Puig et al. (1996) performed a systematic study to identify genes expressed during specific phases of wine fermentation, although numerous transcriptomic studies performed afterwards have provided a great deal of useful information for this purpose (Rossignol et al. 2003).

Genetic engineering was originally used in order to introduce killer determinants in wine yeast strains, either as a model system or as a way to increase the potential of modified strains to dominate natural fermentation (Boone et al. 1990). Petering et al. (1991) used a β-glucuronidase expressing recombinant wine yeast strain in order to monitor the fate of the labelled strain during wine fermentation. However, the first wine yeast strains genetically modified in order to improve wine quality were not published until 1993 (Laing and Pretorius 1993; Pérez-González et al. 1993).

4.2.1 Glycolytic and Plant-Cell Wall-Degrading Enzymes

Degradation of plant cell wall polysaccharides is one of the main applications of enzymes in the wine industry. These enzymes help winemakers increase the yield of grape juice, facilitate filtration and clarification steps, and improve the

extraction of aroma precursors and polyphenols from grape skin cells (Ribereau-Gayon et al. 2012). Additional glycolytic enzymes present in commercial preparations help enhance varietal aroma (synergistically with plant-cell-wall-degrading enzymes) of white fresh wines by releasing the aglycone from di-glycoside precursors (Whitaker 1990; Colagrande et al. 1994). Many of the recombinant wine yeast strains developed so far address the heterologous expression of any of these two related groups of enzymes. Genes coding for the target enzymes often come from filamentous fungal species, but they can also originate from other yeast species and bacteria. A necessarily incomplete list of examples would include the heterologous expression of β-(1,4)-endoglucanase from *Trichoderma longibrachiatum* (Pérez-González et al. 1993), two pectin degrading enzymes from *Erwinia sp*. (Laing and Pretorius 1993), pectate lyase from *Fusarium solani* (González-Candelas et al. 1995), α-L-arabinofuranosidase from *Aspergillus niger* (Sánchez-Torres and González-Candelas 1996), β-glucosidase from *Candida molischicana* (Sánchez-Torres et al. 1998), β-(1,4)-enxylanase from *Aspergillus nidulans* (Ganga et al. 1999), α-L-rhamnosidase from *Aspergillus aculeatus* (Manzanares et al. 2003), or β-(1,4)-glucanase from *Butyrivibrio fibrisolvens* and β-(1,4)-xylanase from *Aspergillus niger* (Van Rensburg et al. 2007). Most of these genetically engineered strains gave rise to wines with increased content on varietal aroma compounds, like for example, linalool, as compared to the cognate parental strains; while some of them resulted in improved recovery of phenolic compounds, or facilitated filtration and clarification. In addition, by overexpressing the endogenous *S. cerevisiae PGU1* gene coding for an endopolygalacturonase several authors attained improved wine filterability and yield (Vilanova et al. 2000; Fernández-González et al. 2005).

4.2.2 Wine Secondary Aroma

In addition to the release of varietal aroma compounds from grape derived precursors, yeast metabolism directly contributes to an important fraction of wine aroma. Known as secondary or fermentation aroma, it is mainly constituted by secondary products of yeast metabolism with a special impact of acetate and ethyl esters (Styger et al. 2011). Changing the levels of secondary yeast metabolites has been another target for genetic engineering. For this purpose strains overproducing alcohol acetyl transferase (Lilly et al. 2000; Verstrepen et al. 2003) or containing modified pathways related to the production of thiol aroma compounds by overexpressing either an *E. coli* cysteine-β-lyase coding gene or an endogenous β-lyase (Swiegers et al. 2007; Roncoroni et al. 2011) have been constructed. Some authors have even engineered new isoprenoid biosynthetic pathways in wine yeast strains by expressing the *Clarkia breweri* S-linalool synthase gene in wine yeast (Herrero et al. 2008).

4.2.3 Alcohol Level Reduction

The steady increase in alcohol degree of quality wines observed during the last 20 years is in part a consequence of global climate warming, and in part due to the increasing demand of full body wines, which require longer grape ripening times. However, this increase raises several market, regulatory, and safety issues (Mira de Orduña 2010), and this is the reason why one of the topics that have generated more scientific literature in the field of yeast genetic improvement is the reduction of alcohol levels in wine.

The topic was experimentally reviewed by Varela et al. (2012) who generated a collection of genetically modified *S. cerevisiae* wine yeast strains summarizing strategies previously addressed by other groups. The best results were obtained by diverting carbon flux from ethanol to glycerol biosynthesis by overexpressing the *GPD1* gene, as originally described by Dequin and coworkers (Remize et al. 2000; Cambon et al. 2006; Ehsani et al. 2009). The main drawback of this strategy is the reestablishment of yeast redox homeostasis by diverting carbon flux towards unwanted metabolites like acetate or acetoin. Strategies to overcome these problems have been thoroughly explored by this research group, including deletion of *ALD6* coding for a cytosolic aldehyde dehydrogenase (Remize et al. 2000), or engineering coenzyme specificity of NADH-dependent 2,3-butanediol dehydrogenase (coded by the gene *BDH1*).

4.2.4 Malolactic Wine Yeast

Malolactic fermentation (MLF) is a process naturally carried out by lactic acid bacteria, after alcoholic fermentation, (see below). Malolactic fermentation used to be a source of concern to winemakers due to the unpredictability of the spontaneous process, including the possibility of unacceptable levels of biogenic amines produced, and difficulties often encountered by commercial MLF starters to develop under industrial conditions. The first recombinant yeast strain to get official approval by appropriate food safety authorities (in the USA and Canada) was malolactic wine yeast. Several variants of malolactic wine yeast were engineered including different sources of malolactic enzyme and malate permease, before the commercial strain ML01 was developed. This strain carries the *Schizosaccharomyces pombe* malate permease gene (*mae1*) and the *Oenococcus oeni* malolactic gene (*mleA*). The strain was shown to fully decarboxylate 5.5 g/l of malate in Chardonnay grape must during alcoholic fermentation (Coulon et al. 2006).

4.2.5 Urea and Ethyl Carbamate Reduction

Ethyl carbamate (EC), also known as urethane, is a toxic compound widely found in alcoholic beverages declared as probably carcinogenic to humans (Group 2A) by the International Agency of Research on Cancer (IARC) (Baan et al. 2007, see also

Chap. 1 of this book for further details). The origin of EC in grape wines is a chemical reaction between ethanol and N-carbamoyl compounds such as urea or citrulline. This spontaneous reaction is faster at higher temperatures. Urea, the main precursor of ethyl carbamate in wine, is a side product of incomplete arginine catabolism by yeasts. This compound can be present in different amounts depending on grape must composition, nitrogen supplementation strategies, and the yeast strain used for alcoholic fermentation (Zimmerli and Schlatter 1991; Pozo-Bayón et al. 2012; Zhao et al. 2013).

Under suitable conditions urea can be further metabolized by yeast to ammonium and CO_2. However, under winemaking conditions *DUR1,2*, the gene coding for urea amidolyase, seems to be initially under transcriptional repression (Bussereau et al. 1993), due to the presence of preferred nitrogen sources. In order to avoid urea accumulation Coulon et al. (2006) constitutively expressed the *DUR1,2* gene in an industrial wine yeast strain therefore being no longer subjected to nitrogen catabolite repression. The use of this strain resulted in Chardonnay wines with an ethyl carbamate content reduced by 89.1 % as compared to those fermented by the original wine yeast strain. The commercial version of this strain, ECMo01, is the second of the only two recombinant wine yeast strains that have been commercialized so far (see below).

In order to further enhance urea metabolism during the fermentation of grape must Dahabieh et al. (2009) constitutively expressed the *DUR3* gene, coding for urea permease. This strain performed even better than the previous one in must with high initial urea content.

4.2.6 Flocculation

Flocculation is an important biotechnological character. Efficient wine yeast flocculation after primary alcoholic fermentation leads to the formation of compacted sediments minimizing problems associated with wine clarification (Pretorius and Bauer 2002), and is also a desirable character in the production of sparkling wines by the traditional method, helping in the removal of cells in the final steps of the production process (Soares 2011). Flocculation has a complex genetic basis and strong dependence on environmental factors (Sampermans et al. 2005). Proteins coded by the *FLO* family genes (Flo1p, Flo5p, Flo9p, Flo10p, and Flo11p), known as flocculins, are key for the flocculation features of yeast cells. These glycoproteins are linked via glycosylphosphatidylinositol (GPI) anchor and displayed on the external face of the cell wall (Verstrepen and Klis 2006; Dranginis et al. 2007).

Strategies to enhance the flocculation character of yeast strains include the expression of dominant *FLO* gene from donor *S. cerevisiae* strains (Watari et al. 1991; Wang et al. 2008), or upregulating the expression of endogenous *FLO* genes by a promoter replacement strategy (Verstrepen et al. 2001; Govender et al. 2008). Both strategies would allow for the production of self-cloning yeast strains (see below).

Interestingly, the *HSP30p*-driven expression of *FLO11* in wine industrial strains yielded strong flocculent phenotypes that seem to exclusively occur under red wine fermentation conditions (Govender et al. 2011) but not in standard laboratory media or synthetic must (Govender et al. 2010).

4.2.7 Resveratrol

Resveratrol is a small polyphenol that has been extensively studied for a couple of decades in a large spectrum of therapeutic research areas as described in Part III of this book. Resveratrol levels in wines depend on a number of factors such as grape variety, geographical location, climate conditions, fungal infections of grapes, exposure to UV radiation, and oenological practices (Siemann and Creasy 1992; Jeandet et al. 1995; Romero-Pérez et al. 1996).

González-Candelas et al. (2000) used a recombinant wine yeast strain expressing a *Candida molischiana bgIN* β-glucosidase in white wine fermentation. The use of the engineered strain resulted in increased content of both non-glycosylated and total *cis*- and *trans*-resveratrol content. While the increase in the non-glycosylated forms can be easily explained by this enzymatic activity, the mechanisms underlying increase in total resveratrol content were not completely elucidated.

4.2.8 Autolysis

Traditional sparkling wines are produced by the second fermentation of a base wine followed by a prolonged aging period in contact with yeast cells. During this aging process yeast autolysis takes place, followed by the release of cell components and their breakdown products to the wine. However, this is an extremely slow process. Several genetic engineering strategies have dealt with the modification of the control of the autophagic process, that usually precedes autolysis under normal conditions (Cebollero and Gonzalez 2006), have resulted in the construction of wine yeast strains showing accelerated autolytic features (Cebollero et al. 2005; Tabera et al. 2006).

4.2.9 Mannoprotein Release

Mannoproteins are a class of glycoproteins present as structural components in the outermost layer of yeast cell wall (Klis et al. 2006). They are released to wine during alcoholic fermentation and aging on lees, and positively contribute to wine quality in a number of ways including stabilization against protein haze or crystallization of tartrate salts, retention of aroma compounds, reduction of astringency, enhanced body and mouthfeel, or improved foaming properties of sparkling wines (Waters

1994; Caridi 2006; Núñez et al. 2006; Guadalupe and Ayestarán 2008). Release of mannoproteins can be enhanced by some winemaking practices, like the use of yeast cell wall degrading enzymes or extended aging on lees. Alternatively, mannoprotein overproducing wine yeast strains have been obtained by genetic engineering using two different approaches. Brown et al. (2007) overproduced in yeast two mannoproteins previously characterized as having a positive impact on protein stability, Hpf1p and Hpf2p. On the other side, Gonzalez-Ramos and Gonzalez (2006) identified several yeast genes whose loss of function resulted in a general increase in mannoprotein release during fermentation, and used this strategy con construct several genetically improved yeast strains (Gonzalez-Ramos et al. 2008, 2009).

4.3 Random Mutagenesis of Wine Yeast

Random mutagenesis is based on the use of physical or chemical mutagens in order to increase the rate of appearance of genetic mutations. Usually a large population of cells are treated to relatively low survival rates. Surviving cells can carry from single to tens of mutations, including transitions, transversions, small deletions, or large chromosomal rearrangements (deletions, inversions, or translocations). The rate of each type of mutation will depend both on the type and the intensity of the treatment. In contrast to the simplicity of this first step, the difficult task in genetic improvement by random mutagenesis is the screening phase. A vast majority of the mutant strains obtained after the mutagenic treatment would not be improved for the desired character. They can indeed perform worse than the original strain. The design of powerful selection and detection tools is indeed the main element required for a successful genetic improvement program based on random mutagenesis. This design should be ideally based on a solid knowledge of the biology underlying the desired improved character, as well as imagination and inventive capacity.

While random mutagenesis has played a capital role in the genetic improvement of other industrial microorganisms (e.g., corynebacteria for amino acid production or molds and actinomycetes for beta lactam antibiotic production), examples of the employment of random mutagenesis for the improvement of wine yeast strains are rather scarce. This is probably due to the fact that targets for genetic improvement are quantitative characters, which hinders the design of efficient selections tools. Snow (1983) refers to a series of works published in 1971 by Alikhanyan and coworkers, which used mutagens in order to improve performance of yeast strains for still, sherry and sparkling wines. More recently, random mutagenesis has been employed for the improvement of autolytic behavior of wine yeast strains (Gonzalez et al. 2003; Nunez et al. 2005), the reduction of H_2S production (Cordente et al. 2009), the improvement of mannoprotein release (Gonzalez-Ramos et al. 2010), to minimize volatile acidity production (Cordente et al. 2013), or to improve nitrogen assimilation and fermentation kinetics (Salmon and Barre 1998). In all these examples, design of selection methods was a key element for obtaining the desired mutant strains. Despite only few of them have entered the wine yeast market (Gonzalez-Ramos et al. 2010),

new strains obtained by random mutagenesis will probably become available in the near future, as long as our ever increasing knowledge on wine yeast physiology will allow the design of new and more powerful selection strategies. Quirós et al. (2010) took advantage of the finding that most of the previously constructed recombinant mannoprotein overproducing strains showed tolerance to killer toxin K9 (Gonzalez-Ramos et al. 2009), in order to develop an improved selection system for mannoprotein release enhancement after random mutagenesis.

4.4 Genetic Hybridization of Wine Yeasts

Like animal and plant breeding, some industrial microorganisms are amenable to genetic improvement by sexual hybridization. This classical method allows for the combination, in a single strain, of desirable features from two different parental strains. Breeding programs often target a single feature of one of the parental strains, while looking to keep most of the genetic background of the other strain (because of well tested performance). This can be achieved by successive backcrossing of selected individuals from offspring with one of the parent strains. The use of molecular markers and genome-scale sequence analysis technologies is becoming a good ally of biotechnologists in order to make this process more efficient. Unfortunately, the sexual cycle of many industrially relevant microorganisms is either absent or not sufficiently understood to be used for genetic improvement purposes. Parasexual hybridization can eventually be employed to construct intra or interspecific hybrids, combining interesting features from microbial strains that cannot be crossed by conventional sexual hybridization. Hybrid strains obtained by such methods like protoplast hybridization or rare mating are usually genetically instable, and require of several rounds of cultivation under industrially relevant conditions in order to be stabilized.

A well characterized sexual cycle is one of the many biotechnologically relevant assets of *S. cerevisiae* (Haber 2012). This feature can be exploited for breeding new industrial yeast strains that combine interesting properties from selected parent strains. There are however two main practical limitations to this approach: homotalism, the ability of sexual ascospores to mate with their daughter cells to reconstitute homozygous diploid strains (Thornton and Eschenbruch 1976; Benítez et al. 1983), and lack of genetic markers in order to distinguish and select hybrid cells from those from the original parental strains. The development and recovery of classic genetic methods in order to select auxotrophic or antibiotic resistant strains with almost no genetic manipulation, together with a rational use of killer elements and petit mutants (Ramirez et al. 1998) has greatly facilitated the task of yeast genetic improvement by sexual hybridization (Pérez Través et al. 2012). Intraspecific breeding has been used in order to remove undesirable properties like foam production (Eschenbruch et al. 1982), improve fermentation efficiency and sulfur dioxide tolerance (Thornton 1982), combine flocculation and low H_2S production (Romano et al. 1985), or improve secondary aroma (Shinohara et al. 1994; Steensels et al.

2014). Backcross has been used in order to improve results of breeding programs for the reduction of hydrogen sulfide production, increased temperature tolerance of wine yeasts (Marullo et al. 2007, 2009), or enhanced volatile thiol release (Dufour et al. 2013).

In addition to intraspecific hybrids, species of this genus have been shown to form natural interspecific hybrids. Interspecific *Saccharomyces* hybrid strains have been described in both the winemaking industry and other environments. For example *S. pastorianus* (syn. *Saccharomyces carlsbergensis*) is an interspecific hybrid involving *S. cerevisiae* and *S. eubayanus* (Martini and Kurtzman 1988), and has a long history associated to lager-brewing (Gibson and Liti 2014). The first hybrid strains related with wine-making were described by Masneuf-Pomarède et al. (1998). These strains, isolated from Italian wines, are hybrids between *S. bayanus* var. *uvarum* and *S. cerevisiae*. Similar hybrids have been isolated in other wine producing areas (Demuyter et al. 2004; Antunovics et al. 2005; González et al. 2006; Le Jeune et al. 2007) and include *S. cerevisiae*×*S. kudriavzevii* strains. It was the development of new and powerful molecular methods in strain characterization over the past decade what revealed that many *Saccharomyces* wine strains firstly characterized as *S. cerevisiae* turned out to carry composite genomes of up to three species (Masneuf Pomarède et al. 1998; Lopandic et al. 2007). Some of them were considered as strains of special interest particularly adapted to low temperature fermentation (González et al. 2006). These hybrid strains usually combine the cryotolerant character from one of the parental strains, *S. bayanus* or *S. kudriavzevii*, with the robustness of *S. cerevisiae* under winemaking conditions. In addition, they contribute to specific aromatic profiles (Gamero et al. 2013), one of the features traditionally associated to these strains even before they were identified as hybrid strains (González et al. 2006).

All these findings raised the interest to construct artificial interspecific hybrids in the laboratory, combining features of selected parent strains. Three different hybridization methods can be applied to obtain artificial hybrids, each of them with different advantages and drawbacks: (a) spore to spore mating; (b) protoplast fusion (Curran and Bugeja 1996); and (c) rare-mating (Spencer and Spencer 1996). In order to identify and select hybrid strains it is often useful obtaining auxotrophic derivatives of the parental strains (Pérez Través et al. 2012). Another requirement of these techniques, especially of protoplast fusion and rare mating, where tetraploid strains are typically obtained in first instance, is genomic stabilization. This can be achieved by repeated subculturing, usually under simulated winemaking conditions, followed by individual strain isolation, as well as genotypic and phenotypic characterization (Pérez Través et al. 2012). Mechanisms involved in artificial hybrid stabilization are currently an active research subject (Albertin et al. 2014; Pfliegler et al. 2014; Steensels et al. 2014).

As mentioned above, outbreeding was initially employed to obtain hybrids emulating the cold adaptation of natural ones (Kishimoto 1994). However, increasing attention is being paid to metabolic properties like low volatile acidity production, high glycerol yield, and improved release of aroma compounds (Bellon et al. 2011, 2013).

4.5 Experimental Evolution of Wine Yeasts

Experimental evolution, also known as directed evolution, or adaptive laboratory evolution (ALE), is a simple and powerful tool to genetically improve industrial microbial strains. This method tries to emulate natural selection mechanisms in an accelerated way, by applying a specific selective pressure to a population of the starting microbial strains, over hundreds of generations. Experimental evolution takes advantage of the short generation time of most microorganisms, allowing for hundreds of generations in a relatively short time. As mentioned for random mutagenesis, knowledge of the biology underlying the process of interest and inventive capacity are key factors for a successful genetic improvement program based on ALE. The concept of experimental evolution was first described by Francis and Hansche (1972, 1973) using *S. cerevisiae* as a model organism. Although it was originally developed in a chemostat setup, ALE can also be carried out by repeated batch cultivation. In addition to the experimental evolution of whole microorganisms, the term directed evolution is often employed referring to protein engineering methods that share the same conceptual background but involve a more active intervention of the researcher (Turner 2003). Conceived as a research tool in enzymology or evolutionary biology, its potential to quickly and easily generate microbial strains adapted to specific growth conditions has made this technique very popular during the last few decades.

Wine yeast strains genetically improved by experimental evolution have been developed mainly during the twenty-first century. Improved strains have been obtained by either applying selective pressure directly related with the target improvement, or by designing indirect selection strategies, based on previous knowledge that would connect an easily established selective pressure with the desired metabolic output. Examples of direct selection include, for example, evolution in winelike fermentation conditions, resulting in strains showing faster sugar consumption rates (McBryde et al. 2006), or growth in the presence of ethanol to improve fermentation kinetics in grape must with high ethanol production potential (Novo et al. 2014). Indirect selection for the desired genetic improvement include, for example, partial deviation of carbon flux from ethanol to glycerol production by using sulfite as a selective agent or under hyperosmotic conditions (Tilloy et al. 2014), or the use of gluconate as sole carbon source attempting to reduce ethanol yield (Cadière et al. 2011). By using both direct and indirect selection strategies, a common theme of experimental evolution approaches is that adaptation strategy always involves non-intended metabolic changes. In most of the examples described above, these changes were either irrelevant or conferred an additional advantage to the selected strains (but this might not be always the case). For example, strains selected in gluconate did not show a significant reduction in ethanol yields but in contrast they showed improved fermentative aroma and reduced volatile acidity production (Cadière et al. 2011). This strain was indeed quickly put into the market.

4.6 Genetic Engineering of Wine Lactic Acid Bacteria

Lactic acid bacteria are the second most relevant group of microorganisms in winemaking. They are responsible for the decarboxylation of malic acid to lactic acid in a process known as malolactic fermentation. This process is required and takes place spontaneously for most red wines and some white wines; it makes wines more palatable by reducing the tart taste associated to malic acid, and provides additional advantages as microbial stability and improved aroma complexity. *O. oeni* is the bacterial species more often isolated from spontaneous MLF processes, as well as a widespread starter culture for this purpose (Bartowsky 2005). Some *Lactobacillus* strains and in particular the species *Lactobacillus plantarum* have also been shown to be suitable to drive the process (Du Toit et al. 2011) and there are some commercial MLF starters from this species. Industrial strains have been selected due to their performance for malic acid conversion, no biogenic amine production, and a positive contribution to wine sensory properties. MLF usually takes place after alcoholic fermentation, therefore meaning that lactic acid bacteria must face a harsh environment in which high alcohol content, low concentration of nutrients, low pH and high SO_2 content are the main but not the only hurdles.

As compared to genetic improvement of wine yeast strains, genetic improvement of lactic acid bacteria used or involved in MLF is still taking its first steps. Indeed, to the best of our knowledge, there are not genetically improved lactic acid bacteria in the market for winemaking, either genetically engineered or improved by other means. Considering the challenges usually encountered by winemakers to control MLF, either due to difficult growth of the starter culture in wine, or to biogenic amine production during some spontaneous MLF processes (see Chap. 1), genetic improvement of MLF starters would be of clear interest. Possible targets for improvement would be adaptation to harsh wine conditions, growth at low temperature, or metabolic pathways involved in the production of sensory active compounds (e.g., diacetyl) or biogenic amine production.

Concerning genetic engineering, expression of foreign genes in *O. oeni* is still far to be a routine technique. Several authors have reported successful electroporation (Dicks 1994; Assad García et al. 2008; Eom et al. 2010) and have developed plasmid vectors (Beltramo et al. 2004; Eom et al. 2012), but transformation efficiency is still low. Since several natural plasmids have been identified for this species (Shareck et al. 2004; Favier et al. 2012), generation of vectors from any of these plasmids would eventually help solve some of the bottlenecks in *O. oeni* transformation. A more feasible approach to obtain genetically improved *O. oeni* strains in the short term would be directed evolution, as described above for yeast. However, examples of improved strains of *O. oeni* with this technology are near inexistent. Sumby et al. (2014) cite a work published in a congress (Betteridge et al. 2013) where *O. oeni* adapted to ethanol grows and completes malolactic fermentation faster than the parental strain.

The other bacterial species commercially employed as starter for MLF is *Lactobacillus plantarum* (Du Toit et al. 2011). This species is of interest in diverse fields of food biotechnology (Giraffa et al. 2010) and there are examples of geneti-

cally modified strains of this microorganism to improve its properties as silage inoculant (Rossi et al. 2001) and to be used as microbial cell factory for low calorie sugar production (Ladero et al. 2007). The malic enzyme from *O. oeni* has been cloned in a laboratory strain of *L. plantarum* (Schümann et al. 2012). However, the performance of the transformed strain to degrade malic acid was not improved. The method needs to be improved in regard to vector choice and selective marker. Considering that other *Lactobacillus* species have been improved for acid resistance by adaptive evolution (Zhang et al. 2012), it would be interesting using this approach to improve *L. plantarum* strains.

4.7 Alternatives to Yeast Genetic Improvement

As previously mentioned, even though *S. cerevisiae* is largely recognized as the main agent in grape must fermentation, yeasts belonging to other genera have a relevant contribution to wine quality. Although it presents numerous advantages, the generalized practice of using pure *S. cerevisiae* cultures for must inoculation has been often pointed out as resulting in wines lacking the sensory complexity and variability due to the metabolic activity of these wild yeasts (Lambrechts and Pretorius 2000; Romano et al. 2003). In contrast, an uncontrolled fermentation, mainly driven by wild yeasts and bacteria would often result in must and wine spoilage. Countering this risk was indeed the original advantage behind the commercial success of active dry yeasts. In order to recover in part their positive contributions to wine quality, while keeping fermentation kinetics and consistency under control, wine biotechnologists are postulating co-inoculation or sequential inoculation of *S. cerevisiae* with yeasts belonging to these alternative species, collectively known in this field as non-*Saccharomyces* yeasts. Although aroma improvement (both primary and secondary) has been the main use proposed for non-*Saccharomyces* yeasts until now (Herraiz et al. 1990; Soden et al. 2000; Rojas et al. 2003; Viana et al. 2008, 2011), recent reports describe other possible advantages of specific non-*Saccharomyces* strains, like glycerol production (Ciani and Ferraro 1996), release of mannoproteins (Giovani et al. 2012; Domizio et al. 2014), low volatile acidity (Snow and Gallander 1979; Bely et al. 2008; Rantsiou et al. 2012), or reduction of alcohol content of wines (Gonzalez et al. 2013; Quirós et al. 2014; Morales et al. 2015).

Non-*Saccharomyces* yeasts offer therefore an invaluable source of metabolic diversity, which can be combined with the well-known features of *S. cerevisiae* by designing suitable co-inoculation or sequential inoculation procedures. In contrast to GMO yeast strains, oenological use of these yeasts is subject to almost no restrictions. For example, OIV referring to yeasts to be marketed as active dry yeast simply states: "Yeasts used must be isolated from grapes, musts or wine or cultures originating from the combination of these same yeasts (original mother cultures) which must be stored in genetically stable conditions." This constitutes a clear advantage as compared to, for example, expression on *S. cerevisiae* of metabolic pathways derived

from them. These strains will probably reach a widespread use during the forthcoming years. This will require a more extensive fieldwork, in order to end up with a wider range of isolates from different regions, suitable for different grape must features and winemaking styles. Finally, yeast producers must still solve several problems in order to get an efficient production of these strains as active dry yeast.

4.8 Regulatory and Marketing Issues

Winemaking is an economic activity that takes place in the frame of a regulatory and market network, with some elements being applicable almost worldwide, while others have a local or regional impact. This network includes at least the following:

1. National and international food safety regulations. Wine is a food product and wine production must comply with all the applicable food safety standards. This includes laws concerning the use and trade of products produced from GMOs.
2. International Oenological Codex and International Code of Oenological Practices. Those two publications gather the description of products used in winemaking and practices and processes, including an indication on acceptability and directions and limits of use. They are issued by the OIV (International Organisation of Vine and Wine), and are the main guide for rules governing international wine trade and national regulations concerning this field (at least in countries taking part in the OIV, i.e., almost all relevant wine producing countries apart from the USA and Canada).
3. Appellations of origin and other local associations. In order to benefit from a joint market image winemakers establish their own specific requirements and restrictions on winemaking practices inside these organizations.
4. Market considerations. Wines should compete in a global market. In addition to the design of marketing strategies producers must consider the impact technical decisions can have on brand image, both at home and abroad.

Things to consider would be completely different whether we were dealing with GMO wine yeast strains or those genetically improved by non-GMO methods. There is indeed almost no restriction to the use of genetically improved non-GMO yeasts in winemaking. Strains developed in the laboratory by random mutagenesis, interspecific and intraspecific genetic hybridization, rare mating, or experimental evolution, are readily available for field trials and eventual distribution as fresh or active dry yeast. No legal requirement to demonstrate safety to humans or the environment is applicable for these strains in any country. Yeast producers can distribute these new yeast strains as innovative products, with an added value over more traditional yeast strains. On the other hand, even though some winemakers publicize the use of selected autochthonous yeasts to produce their wines (managing to combine tradition and innovation in a single message), the use of non-GMO genetically improved yeast strains is neither compulsory nor usual. Until a few years ago the wine yeast market almost exclusively relied on natural isolates, and initial breeding

or random mutagenesis experiments remained inside the walls of the laboratories. However, there are an increasing number of genetically improved yeast strains, either publicized as such or not, in the catalogues of wine yeast producing companies, with examples from almost all the techniques described in this chapter.

In contrast, commercialization of food products consisting of, containing, or produced from GMOs is subject to specific regulations. For example, in the EU, they fall under the scope of Directive 1829/2003. According to this regulation authorization by EFSA (European Food Safety Authority) is required to market these products in Europe. Preparing an EFSA application for GMO food is a long and costly process. Only two GMM (genetically modified microorganisms) have been approved so far under this directive, and both are intended just for feed but not for food use. In addition, under this regulation GMO food products must be labelled as containing, consisting, or produced from GMOs. Most other countries have parallel but often less restrictive regulations in force concerning these issues. FDA approval is for example required in the USA, but the process is not so long and labelling is not required.

Not only genetically engineered yeast strains fall under the scope of Directive 1829/2003. Also yeast strains obtained by protoplast fusion would be considered as GMO concerning food applications according to directive 2001/18 of the European Parliament which include the following in the GMO definition: "…live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally." In contrast, this directive unequivocally excludes from its scope "cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods." Interestingly, there is a more recent directive, 2009/41, specific for contained use of GMMs, which is less restrictive, and explicitly excludes protoplast fusion from the description of GMM. The exclusion refers to: "Cell fusion (including protoplast fusion) of cells of any eukaryotic species." The directive opens the possibility of excluding some genetic engineering organisms, proposing a definition for self-cloning whose last sentence reads as follows: "Self-cloning may include the use of recombinant vectors with an extended history of safe use in the particular microorganisms." However this directive is not applicable to food products and it is uncertain whether this will set a trend so that similar definitions would be applied to GMO food in the future.

The widespread attitude of European consumers against GMO food and the requirement for a specific labelling do not encourage access of recombinant wine yeasts to the market, since the expected return would not pay for even the cost of preparing an EFSA application. While the OIV takes an officially neutral position on the use of recombinant yeasts by referring to external food safety regulations, some producing regions have adopted a determined position against GMOs in winemaking by declaring themselves GMO-free zones. Use of GMO wine yeasts has been approved by competent authorities in just three countries (Chambers and Pretorius 2010), and it involves only two recombinant wine yeast strains ML01 and ECMo01 (see above for a description of these two strains). Actual sales information on these strains is not publicly available, but they do not seem to be widely used in these countries, despite they were approved several years ago.

In addition to the public negative perception of GMO food in general, there might be several concurrent reasons for low commercial success of these strains:

1. Wine is a hedonic product, and purchase decisions are influenced by cultural factors (see Part II of this book) some of which might not always suit with images associated to genetic engineering.
2. Tens of different yeast strains are currently sold as active dry yeast, many of them advertised as especially suited for a given winemaking style. Even this genetic diversity is judged insufficient by some authors, and wines made by the use of commercial *S. cerevisiae* starters are considered as suffering from some kind of standardization. Techniques to recover part of the metabolic diversity found in spontaneous fermentations, while keeping the microbiological control of the process are currently developed by most wine yeast producers (Ciani and Comitini 2011). In this context the fact that only two genetically engineered wine yeast strains are currently available (just in a few countries) does not play in favor of GMO yeast strains.
3. The problems targeted by currently available recombinant wine yeast strains do not seem to be perceived as pressing by winemakers. Concerning MLF, currently available bacterial starters, inoculation protocols, and temperature control systems, have greatly improved the control of the process during the last decades. Non-biogenic amine producing starters are also currently available. Finally, EC in wine was apparently a problem associated to specific nitrogen nutrition practices during the fermentation, or to particular wine yeast starters. While it seems to be still a concern in other products like sake, no recent reports exist on problems associated to excess EC in wine (Zhao et al. 2013).

4.9 Conclusions

Maybe it is time to start countering the pessimism and fears of the misuse of the described techniques with the benefits conferred by their successful use. In the meantime, the current development of non-GMO genetic improvement techniques, the success already achieved by some of these strains, and the relatively short time between laboratory work and payback of investment, all suggest an increasing number of genetically improved wine yeast strains will be reaching the market in the forthcoming years, but all them will have been developed by non-GMO techniques. Finally, the use of non-*Saccharomyces* wine yeast strains as a complement to *S. cerevisiae* starters seems to have entered an exponential growth phase, and appears as a good and easy alternative to take advantage of natural yeast metabolic diversity in order to improve wine quality. Concerning *O. oeni*, even though some reports on genetically improved strains have been mentioned in this chapter, we would expect these technologies will take a bit longer to have a significant impact in the wine starters market, according to published activity in this field.

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Chapter 5
Global Climate Change and Wine Safety

Matteo Marangon, Alistair Nesbitt, and Tony Milanowski

5.1 Introduction

The role of climate is critical in determining spatial viticultural and varietal suitability (Fraga et al. 2012; Moriondo et al. 2013; Santos et al. 2012) but climate also plays a significant part in the selection of viticultural and oenological practices used to achieve desired grape quality parameters, viable yields and targeted wine styles. Changes to climate conditions could result in changes to management practices in the vineyard and winery that ultimately affect style and quality of wine. Whilst there is the ability within viticulture and oenology to adapt to climate change through various techniques, adaptive capacity is not infinite for any given variety, location or wine style. This chapter addresses the subject of climate change and wine production with a specific focus on wine safety, an issue that is important to producers and consumers. It includes a review of how climate change may impact viticultural and oenological activities, which in turn, without appropriate management, could affect wine safety. In doing so, potential health and safety risks and recommended strategies to manage those risks are outlined.

5.2 Climate Change

The Intergovernmental Panel on Climate Change (IPCC) is the leading international body for the assessment of climate change. The IPCC has concluded that warming of the world's climate system is unequivocal, with globally averaged combined land

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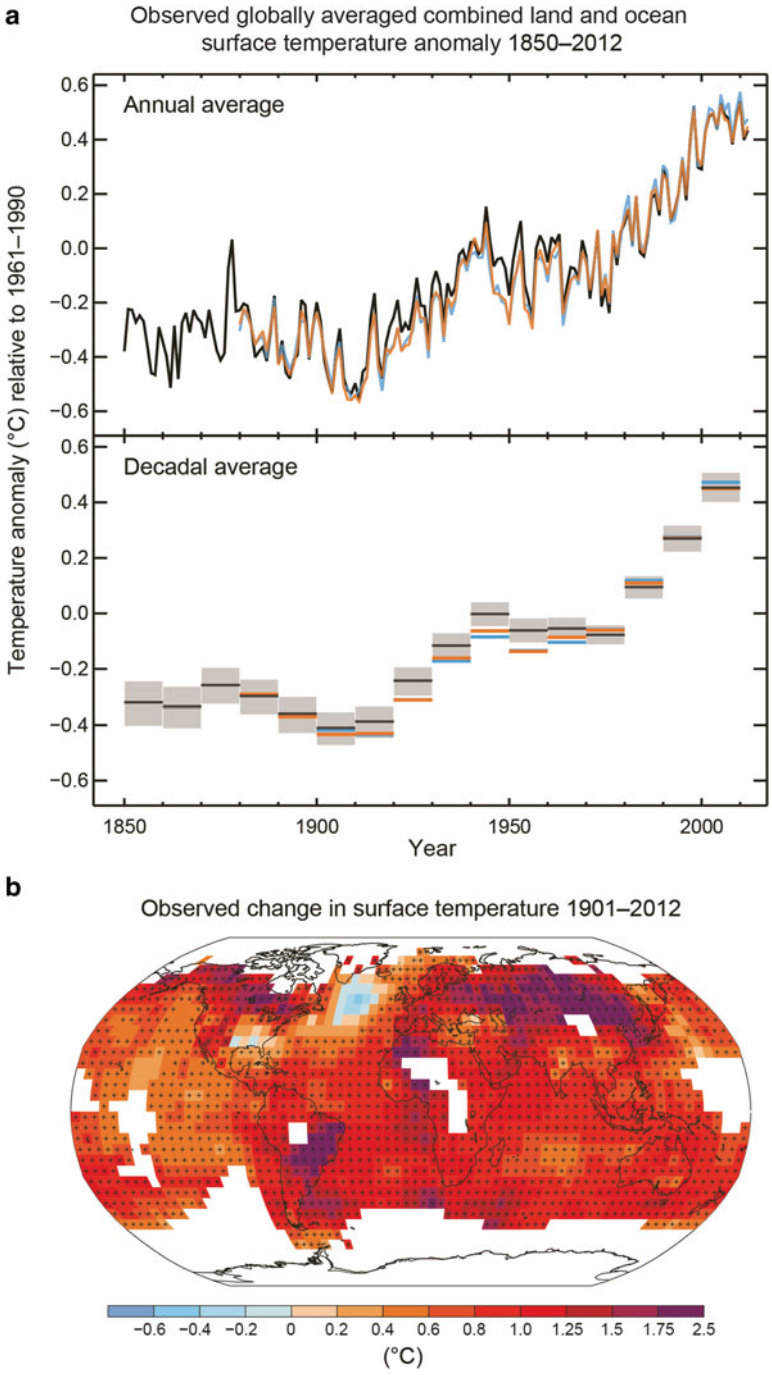
and ocean surface temperature data, calculated by a linear trend, showing a warming of 0.85 °C (0.65–1.06 °C) over the period 1880–2012 (IPCC 2013). The last three decades have been successively warmer than any preceding decade since 1850 (Fig. 5.1a) and between 1901 and 2012, the longest period when calculation of regional trends is sufficiently complete, almost the entire globe experienced surface warming (Fig. 5.1b). Although there is less confidence about changes to precipitation at a global scale, since 1951 precipitation has increased when averaged over the midlatitude land areas of the Northern Hemisphere and the frequency or intensity of heavy precipitation events has likely increased in North America and Europe (IPCC 2013). Changes in extreme weather and climate events have also been observed since about 1950. It is very likely that (1) the number of cold days and nights has decreased; (2) the number of warm days and nights has increased; (3) the frequency of heat waves in large parts of Europe, Asia and Australia has increased and (4) there are likely more land regions where the number of heavy precipitation events has increased than where it has decreased (IPCC 2013).

Human influence has been detected in warming of the atmosphere and ocean, in changes in the global water cycle, in reductions in snow and ice, in global mean sea level rise and in changes in some climate extremes (IPCC 2013). The IPCC concluded that it is extremely likely that human influence has been the dominant cause of the observed warming since the mid-twentieth century. Whilst the causes of climate change are not discussed further in this chapter it is important to note that climate change mitigation practices can be employed by the wine production sector, particularly through energy efficiency, more environmentally sustainable packaging and more efficient distribution methods.

The IPCC uses a series of greenhouse gas representative concentration pathways (RCP) to illustrate future climate conditions. The pathways describe four possible climate futures, which depend on the concentration of greenhouse gases emitted in the future. RCP2.6, RCP4.5, RCP6.0 and RCP8.5 are named after a possible range of radiative forcing values in the year 2100 relative to pre-industrial values (+2.6, +4.5, +6.0 and +8.5 W/m^2, respectively). Global surface temperature change for the end of the twenty-first century is likely to exceed 1.5 °C relative to 1850–1900 for all RCP scenarios except RCP2.6 (Fig. 5.2). Natural internal variability will continue to be a major influence on climate, particularly in the near-term and at the regional scale but inter-annual and decadal variability is unlikely to be regionally uniform (IPCC 2013).

Fig. 5.1 (**a**) Observed global mean combined land and ocean surface temperature anomalies, from 1850 to 2012 from three data sets. *Top panel*: Annual mean values. *Bottom panel*: Decadal mean values including the estimate of uncertainty for one dataset (*black*). Anomalies are relative to the mean of 1961–1990. (**b**) Map of the observed surface temperature change from 1901 to 2012 derived from temperature trends determined by linear regression from one data set (*orange line* in panel **a**). Trends have been calculated where data availability permits a robust estimate (i.e. only for *grid boxes* with greater than 70 % complete records and more than 20 % data availability in the first and last 10 % of the time period). Other areas are *white*. *Grid boxes* where the trend is significant at the 10 % level are indicated by a + sign (IPCC 2013)


a
Observed globally averaged combined land and ocean surface temperature anomaly 1850–2012
Annual average
Decadal average
Temperature anomaly (°C) relative to 1961–1990
0.6
0.4
0.2
0.0
−0.2
−0.4
−0.6
1850
1900
1950
2000
Year
b
Observed change in surface temperature 1901–2012
−0.6 −0.4 −0.2 0 0.2 0.4 0.6 0.8 1.0 1.25 1.5 1.75 2.5
(°C)

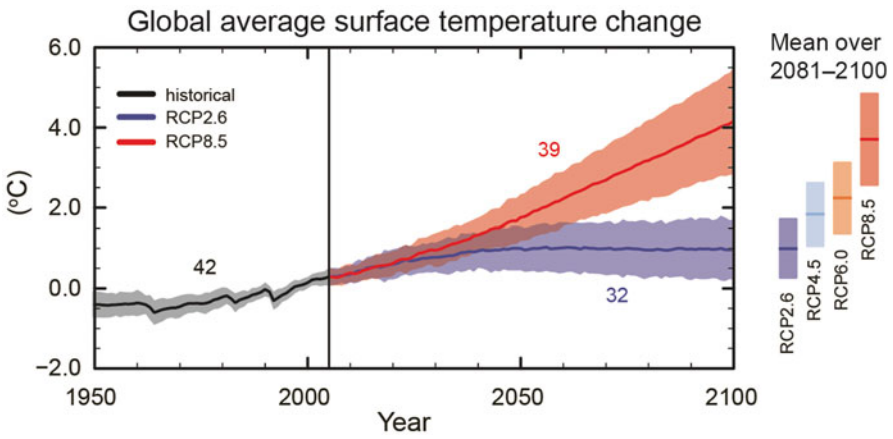


Fig. 5.2 Multi-model (CMIP5) simulated time series from 1950 to 2100 for change in global annual mean surface temperature relative to 1986–2005. Time series of projections and a measure of uncertainty (*shading*) are shown for scenarios RCP2.6 (*blue*) and RCP8.5 (*red*). *Black* (*grey shading*) is the modelled historical evolution using historical reconstructed forcings. The mean and associated uncertainties averaged over 2081–2100 are given for all RCP scenarios as *coloured vertical bars*. The numbers of CMIP5 models used to calculate the multi-model mean are indicated (IPCC 2013)

The IPCC (2013) predicts that the contrast in precipitation between wet and dry regions and seasons will increase, although there may be regional exceptions. The high latitudes and the equatorial Pacific Ocean are likely to experience an increase in annual mean precipitation by the end of this century under the RCP8.5 scenario. In many midlatitude and subtropical dry regions, mean precipitation will likely decrease, while in many midlatitude wet regions, mean precipitation will likely increase by the end of this century under the RCP8.5 scenario (Fig. 5.3).

It is likely that the frequency and duration of warm spells and heat waves, as well as the frequency and intensity of heavy precipitation events, and the intensity and/or duration of drought will increase at a regional or global scale by the end of the twenty-first century.

5.3 Impact of Climate Change on Viticulture and Oenology

Grapes are very sensitive to climate and are predominantly grown in narrow latitudinal bands (30–50°N and 30–40°S) and in specific climatic conditions, characterised by a lack of extreme heat and extreme cold (Schultz and Jones 2010; White et al. 2006). Climate change will have particular significance for grape production where changes or climate-related events occur during the grape-growing season (April–October in the Northern Hemisphere, October–April in the Southern Hemisphere). Increasing temperature trends, a greater frequency of extreme weather, variability, altering precipitation and evapotranspiration trends all have the

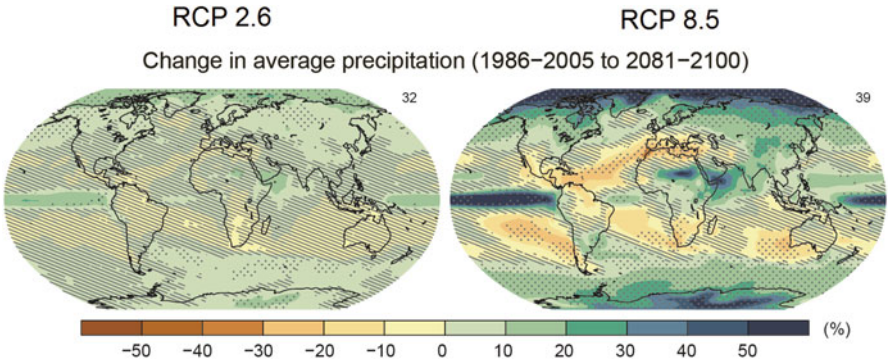


Fig. 5.3 Maps of CMIP5 multi-model mean results for the scenarios RCP2.6 and RCP8.5 in 2081–2100 of average percent change in annual mean precipitation. Changes are shown relative to 1986–2005. The number of CMIP5 models used to calculate the multi-model mean is indicated in the *upper right corner* of each *panel. Hatching* indicates regions where the multi-model mean is small compared to natural internal variability (i.e. less than one standard deviation of natural internal variability in 20-year means). *Stippling* indicates regions where the multi-model mean is large compared to natural internal variability (i.e. greater than two standard deviations of natural internal variability in 20-year means) and where at least 90 % of models agree on the sign of change (IPCC 2013)

potential to impact grape production at annual or longer term time scales (Waters et al. 2005).

Warming conditions will provide potential for new wine-producing regions and will likely lead to a further increase in average growing-season temperatures in existing regions. Recent warming trends and phenological shifts have been found in many wine production regions including Australia, the USA, Canada, New Zealand, South Africa and Europe (Hannah et al. 2013; Jones and Alves 2012; Mira de Orduña 2010). The increase in average temperature during the growing season will impact some key physiological processes, potentially affecting grape berry composition and vineyard productivity.

In addition to changes in average growing-season temperatures, acute weather events such as extreme heat or intense precipitation, and chronic longer term events such as heat waves, drought or prolonged periods of precipitation, will also likely affect grape and wine production above and beyond the impact of conditions that contribute to typical vintage variation. Some existing viticultural regions have already suffered from extreme events, e.g. droughts in South East Australia affecting areas like the Murray Darling Basin, bushfires in Victoria (Australia) leading to smoke taint risk, flooding in the Orange River (South Africa) wine region in 2011 and flooding and hail in central European wine regions in 2013.

These changes and conditions will not affect all production regions uniformly and will likely concern some more than others; indeed rising average temperatures may present opportunities for new production regions. In this chapter the effects of climate conditions on vines and wines are explored because the management of climate and weather events is critical to ensuring production viability, and to safeguarding against potential knock-on effects that could impact wine safety.

Particularly, in subsequent sections, findings related to the impact of main climate changes (temperature increase, precipitation and humidity changes and extreme events) are presented as focussed on viticulture, winemaking and wine safety effects.

5.4 Impact of Temperature Increase

In the context of a warming climate, plant development is mainly affected by driving variables such as temperature, solar radiation changes and CO_2 content (Bindi et al. 1996). Longer and warmer growing seasons with increased average temperatures directly affect the rate at which physiological processes such as photosynthesis and respiration occur in plants (Keller 2010a), and therefore have the potential to increase berry sugar content and pH, lower total acidity, anthocyanin and methoxypyrazine levels (Keller 2010b; Mira de Orduña 2010), and cause modifications to the content of other compounds that could have technological impacts, such as protein levels (Waters et al. 2005).

5.4.1 Effects of Temperature Increase on Viticulture

Grapevines (*Vitis vinifera*) are characterised by a distinct phenological cycle (bud break, flowering, berry growth, veraison, maturation, leaf fall and dormancy) that is predominantly regulated by changes in thermal conditions (Iland et al. 2011). The period between budburst and harvest has advanced and become shorter in some regions and earlier flowering, veraison, ripening and harvests have also been related to warming temperatures (Duchêne and Schneider 2005; Keller 2010c). Research conducted in France found that the phenology of grapes had significantly advanced over a 50-year period with harvest now almost a month earlier (Seguin and de Cortazar 2005). These phenological shifts are likely to cause climatic-related challenges.

Temperature is a major source of seasonal and regional variation in fruit quality traits with direct consequences for wines. Indirectly, the notion that higher temperatures during the growing season yields juice with higher sugar content, higher pH and lower total acidity is well established (Iland et al. 2011; Keller 2010b). It has been recently demonstrated that the effect of temperature on pH and total acidity is varietal specific (Sadras et al. 2013).

Sugar accumulation is directly affected by temperature either via an increase in photosynthetic efficiency during the ripening season especially in cool climates or by indirect sugar concentration due to berry dehydration particularly in warm climates (Keller 2010a).

Increased temperatures also affect grape organic acid content (Iland et al. 2011). The main organic acid in grapes, tartaric acid, is relatively stable during the ripening

process (Melino et al. 2009), while the opposite is true for the second most represented grape acid: malic acid (Mira de Orduña 2010; Sweetman et al. 2009). The decrease in tartaric acid concentration during ripening is mostly due to dilution associated with the increase in berry volume. In pre-veraison malate accumulation is at a maximum between 20 and 25 °C, and decreases at higher temperatures (Keller 2010b). Therefore hot condition pre-veraison, typical of warm growing regions, can significantly impact the accumulation of malate and negatively affect final juice acidity. Post-veraison, the metabolism of sugar starts favouring glucose and fructose accumulation and synthesis rather than catabolism. Hence sugars stop being the major source of carbon for plant growth and are replaced by malic acid, with its content decreasing over time (Sweetman et al. 2009).

Temperature also plays a role in modulating the final content of other compounds in berries essential in determining grape quality, such as phenolics, flavour compounds and proteins. In general higher temperatures lead to riper grapes in which fruity flavours tend to be predominant, rather than the green flavours associated with methoxypyrazine (Harris et al. 2012). Proteins are constitutively produced by the plant during ripening and their concentration is linearly correlated with berry sugar concentration (Pocock et al. 2000).

Global warming has been associated with the migration of diseases poleward at a speed of 2.7 ± 0.8 km/year since 1960 (Bebber et al. 2013). This trend has been observed for several pathogens and vectors affecting vineyards. Increasing temperatures in Europe may increase susceptibility to the European grapevine moth and powdery mildew (Caffarra et al. 2012). Other pests, whose distribution can be considered in part humidity and temperature driven, include the glassy winged sharpshooter which brought Pierce's disease to California, mealy bugs, grass grubs, erinose mites and the Asian lady beetle (Mozell and Thach 2014). Predictions for an important grapevine fungal disease, *Botrytis cinerea*, are unclear. Some authors affirm that high temperatures (>35 °C) could have beneficial effects in protecting plants from several diseases including *Botrytis cinerea*. However, hot conditions associated with high radiation levels are likely to damage grapes due to sunburn, potentially enabling *Botrytis cinerea* infections later in the season (Steel and Greer 2008). Whilst this list is by no means exhaustive it illustrates potential pest problems associated with changing temperature regimes.

5.4.2 *Effects of Temperature Increase on Winemaking*

Under increased growing-season temperatures grapes have the potential to reach maturity quicker. In this context, winemakers are faced with several oenological challenges because producing high-quality wines from grapes with increased sugar levels and lower acidity can seriously affect the production process. High sugar must is problematic as glucose and fructose are primarily metabolised into ethanol, carbon dioxide and heat, and the resulting elevated ethanol levels interfere with different aspects of yeast metabolism, including cellular transport of glucose,

ammonium and amino acids (Leão and van Uden 1982; Leão and van Uden 1984; Leão and Van Uden 1983). High alcohol content can also affect yeast cell fluidity, disrupt plasma membranes and cause cellular damage and cell death (Kubota et al. 2004). In parallel wine accumulates acetyl aldehyde as a result of yeast metabolism, at the same time as ethanol, that can have negative toxic effects on yeast (Bisson 1999). Ultimately high alcohol levels alone, or combined with other stress conditions such as elevated ferment temperatures, low nutrition or the presence of inhibitory compounds like medium-chain fatty acids, can result in fermentations ceasing prematurely (Bell and Henschke 2005). This can lead to the production of wines with residual sugar that in turn are responsible for non-conformance to a desired style and may risk microbial spoilage, from yeasts and bacteria.

When faced with high sugar must winemakers can look to either (1) manage the fermentation process with the aim of producing a high-ethanol wine or (2) reduce the final alcohol concentration. The fermentation process can be better managed by using ethanol-tolerant yeast strains. Yeast ethanol tolerance can also be improved by the presence of sterols, which improve cell membrane fluidity. Sterols can be added by oenological adjuvants, or their production induced by the incorporation of small amounts of air during fermentation. Low-temperature fermentation (<25 °C) can also mitigate the negative impact of high ethanol levels on yeast health (Bauer and Pretorius 2000).

Alcohol reduction can be difficult to achieve in wine. Legal restrictions mean that dilution of initial sugar levels is problematic and generally the addition of water is only allowed when used to prepare oenological additives such as yeast and fining agents. Legal exceptions in some areas do allow the incorporation of water (California) or water-derived from grape concentration processes (Australia).

Ethanol reduction can also be achieved biologically. While ethanol is the primary product of *Saccharomyces* yeast metabolism, yeast can also convert carbohydrates to glycerol, pyruvic acid and other metabolites (Kutyna et al. 2012). There has been considerable research into decreasing ethanol production in favour of the production of glycerol using genetic modification (Varela et al. 2012), directed breeding techniques and adaptive evolution approaches (Kutyna et al. 2012; Tilloy et al. 2014). Although future yeast strains may provide a means of minimising ethanol production (Varela et al. 2012), to date efforts in producing such strains have been unsatisfactory because of diverse problems including the accumulation of high levels of spoilage compounds such as acetic acid (Cordente et al. 2013). Also widespread moratoriums on the use of genetically modified organisms by many wine-producing countries limit the potential for development of customised yeast strains (Borneman et al. 2013).

Ethanol levels can be reduced by winemaking activities during and post-fermentation. With vigorous production of carbon dioxide, the gas flux can entrain ethanol and cause it to be lost to atmosphere (Boulton et al. 1995; Zimmermann et al. 1964). Furthermore, storage of barrels in >70 % relative humidity environment can cause the preferential evaporation of ethanol over water, resulting in a decrease in final alcohol levels (Boulton et al. 1995). Winemakers can also attempt to lower the alcohol content by processes such as nano-filtration, osmosis, osmotic distillation, pervaporation and low-pressure Spinning Cone™ columns (Schmidtke et al. 2012).

However, the uptake of this technology by the industry is limited by legal restriction in some areas, and by their costs and potential sensory implications.

At the same time as increasing sugar levels, acid levels decrease and wine pH increases. Acidity is important for organoleptic reasons because it provides freshness and balance to wine and influences the colour of red wines (Peynaud 1984). It also plays a role in ensuring that wine is less susceptible to oxidation as the extent of polyphenol oxidation decreases at a lower pH (Singleton 1987). Low pH also reduces the risk of microbial spoilage as some spoilage microorganisms, like *Lactobacillus* and *Pediococcus* strains which are unable to grow in wine at a pH below 3.5 (Davis et al. 1986). Finally, the proportion of molecular sulphur dioxide increases at decreasing pH levels, meaning that sulphites are more effective in controlling spoilage (Zoecklein et al. 1995). For all these reasons, acidity and pH are crucial parameters to control.

Wine regions which routinely suffer from musts with high pH and low acidity can often legally acidify wine through tartaric acid additions. Areas with more marginal climates can request a legal dispensation in certain vintage conditions to allow acidification; however this process can be lengthy and authorisation for acidification may be delayed (EC 2008). With climate change this process may require revisions to enable increased or speedier acidification of wines in difficult years.

Acidification methods that do not require addition of acids are also used by winemakers. These include the amelioration of high pH musts with either additions of unripe fruit or blending with varieties naturally high in acidity. Other methods include the preservation of wine acidity by avoidance of malolactic fermentation that reduces malic acid to the relatively weaker lactic acid (Ribéreau-Gayon et al. 2006), or using yeast strains to produce high levels of acids (succinic and lactic) during fermentation as a way of increasing acidity (Su et al. 2014). An alternative to increasing acidity is to mitigate the possible negative effects of both oxidative and spoilage risk of high pH through the use of reductive handling techniques, such as inert gas blanketing and sparging, or through increasing sulphite levels (Oliveira et al. 2011).

Grapes at increased maturity will exhibit higher levels of proteins, anthocyanins and polyphenols. During the winemaking process these may be extracted to higher than desired levels, effecting wine style and organoleptic character. Winemakers may need to modify processes such as skin maceration, grape pressing or temperature to modulate the extraction levels. Alternatively they can look to remove excessive levels of these compounds through the use of fining agents (bentonite, gelatin, ovalbumin or wheat) (Maury et al. 2003; Pocock et al. 2011). These operations can impact the final quality and safety of wines in different ways.

5.4.3 Effects of Temperature Increase on Product Safety

One of the wine safety concerns associated with increased ripeness is increased sugar and consequently higher alcohol levels. Harmful consumption of alcohol has been highlighted by the World Health Organisation (WHO) as having a significant

impact on human health with approximately 5 % of total world deaths and diseases attributable to harmful use of alcohol (World Health Organisation 2010). A recent global alcohol strategy targets a 10 % reduction in harmful alcohol use by 2025 (World Health Organization 2014) and one means of achieving this aim is to reduce alcohol content in alcoholic drinks, including wine (World Health Organization 2010). As a consequence, potential increasing wine alcohol content, caused by a changing climate, is in conflict with the WHO aims.

Wines that are susceptible to microbiological spoilage because of their high pH and low levels of molecular sulphite are a potential wine safety risk. When spoilage occurs in a wine, unknown microorganism can grow, thus producing metabolites that can represent a safety concern. Major risks include the potential for lactic acid bacteria to produce biogenic amines such histamine, tyramine and putrescine in sufficient quantities to cause concerns for vulnerable consumers (Landete et al. 2007). In addition, some microorganisms use amino acids such as arginine in their metabolism producing high levels of urea and citrulline, which in turn reacts with ethanol to form ethyl carbamate which is classified as a probable human carcinogen (IARC 2010; Mira de Orduña et al. 2000).

As way of combatting an increased risk of microbial spoilage, winemakers may be forced to employ higher levels of sulphite to protect wines. However sulphites are a known wine safety concern and total levels allowed are regulated by all wine-producing countries (OIV 2014). The WHO found that in countries with significant wine consumption patterns, wine can be a major contributor to the total dietary sulphite intake and may cause recommended adult daily intake of 0.7 mg/kg body mass to be exceeded (FAO/WHO 2009).

The process of increased wine fining can also cause wine safety concerns. Some proteinaceous fining agents may persist in wine after use and can cause allergic reaction in consumers. Studies have shown the potential for gluten, milk, egg and fish material to remain in the wine and result in positive enzyme-linked immunosorbent assay (ELISA) or skin-prick testing (Vassilopoulou et al. 2011). To minimise safety concerns for at-risk consumers, winemakers can adopt different strategies: (1) quantify potential allergen residues, (2) reduce quantities via bentonite fining or filtration (3) or label wines with appropriate warning guidelines (Deckwart et al. 2014). While labelling for potential allergens is required in many locations, including the EU, Australia and New Zealand, increasing fining agent residues poses potential risks in countries where additions are not strictly regulated (Deckwart et al. 2014).

Riper grapes have also higher quantities of unstable proteins that need to be removed through bentonite additions (Waters et al. 2005). Under climate change conditions there are two potential risks. Higher protein content may require a higher degree of bentonite fining to remove them, a process that can alter the metal ion content in wines with potential increase of the aluminium, iron and arsenic levels leading to potential health or legal concerns (Catarino et al. 2008; Tariba 2011). The second risk is that some wine proteins are known allergens including endochitinase, lipid transfer proteins and thaumatin-like proteins (Marangon et al. 2009) that have the potential to cause allergic reactions in some consumers (Pastorello et al. 2003).

5.5 Impact of Precipitation and Humidity Changes

In some regions periods of drought and water stress may become more common under climate change conditions, thus increasing the need for irrigation where able. Conversely, other regions could see an increase in rainfall or in extreme precipitation events, with possible consequences such as increases in erosion, changes in the spatial distribution of pests and diseases that affect grapes and vines and increases in disease pressure, particularly associated with warmer conditions (Chakraborty et al. 2000).

5.5.1 Effects of Precipitation and Humidity Changes on Viticulture

The extent and distribution of precipitation over the vegetative period are critical to viticulture, as water stress and excessive humidity can lead to a wide range of positive or negative effects (Austin and Bondari 1988). Whilst a critical reduction in water availability for vines can be managed through irrigation, the costs and required water availability can make irrigation unviable. Reduced water availability during the growing season can have different effects at different phenological stages, causing poor shoot growth, low leaf area and hence limited photosynthesis, poor flower cluster development, flower abortion and poor berry fruit set (Fraga et al. 2012, 2013; Iland et al. 2011). On the other hand, excessive precipitation and humidity, particularly during the early stages of development, can promote excessive vegetative growth, resulting in denser canopies and a higher likelihood of disease problems (Fraga et al. 2012), as well as sugar dilution and delayed berry ripening (Fraga et al. 2013).

Given that changing climatic conditions may alter the frequency, intensity, distribution and type of pests and diseases present in vineyards, viticulturists will be faced with a dynamic pest population environment and therefore will need suitable pest control strategies that they can apply readily. Preventing the spread of diseases through additional applications of plant protection products could increase costs (economical and environmental) and risks, including those to humans caused by residues and toxins.

5.5.2 Effects of Precipitation and Humidity Changes on Winemaking

With changing precipitation and humidity levels in vineyards there is the risk of increased occurrence of fungal diseases such as *Botrytis cinerea* (grey rot), *Aspergillus* spp., *Plasmopara viticola* (downy mildew) and *Erysiphe necator* (powdery mildew) (Barata et al. 2012). These diseases and the direct effects of heavy

precipitation or hail (referred to in Sect. 5.6) can damage berry skin integrity, leading to an increased risk of microbial spoilage in wines (Barata et al. 2012). Damaged fruit requires extra care in the winery to minimise possible quality losses from accelerated oxidation or organoleptic taints such as mouldy and earthy attributes (Steel et al. 2013). One option is to remove diseased fruit before it enters the winery, by harvest selection or sorting (Evans 2013). If grapes cannot be sorted prior to processing then winemakers need to try to minimise the impact of fungal contamination by increasing the use of sulphites, minimising the exposure of must to oxygen, using gentler processing techniques, minimising the contact between grape juice and solids and using fining agents (Steel et al. 2013).

An increase in the use of plant protection products to mitigate or minimise the risk of infection in the vineyard comes with the risk of inappropriate use (e.g. too high rates, too close to harvest date) that can leave residual spray on the fruit (Vaquero-Fernández et al. 2013) which may be problematic during the wine production process.

5.5.3 *Effects of Precipitation and Humidity Changes on Product Safety*

Fungal-contaminated grapes can contain mycotoxins such as ochratoxin A (OTA), a nephrotoxic, teratogenic and immunotoxic fungal metabolite found in many food and beverage products including wine (Shephard et al. 2003). Ochratoxin A (OTA) is classified as possible human carcinogen by the International Agency for Research on Cancer (IARC 1993), and its occurrence is predominantly associated with contamination by *Aspergillus carbonarius* and to a lesser extent *Aspergillus niger*, *Aspergillus aculeatus* and *Aspergillus tubingensis* (Medina et al. 2005). The incidence of OTA in wines is related to climatic conditions. In warm growing regions like Southern Europe and South Africa, OTA is the principal mycotoxin found in wines, and its occurrence is related to the presence of *Aspergillus* species (Anli and Bayram 2009), while wines from cooler regions can also contain OTA, but the fungi responsible are likely from the *Penicillium* species (Battilani et al. 2003; Varga and Kozakiewicz 2006). In the first resolution of 2005, the International Organisation for Vine and Wine (OIV) indicated that processing fruit gently and minimising extraction from grape solids are the best practices to reduce OTA in wines (OIV 2005). In addition, removal of skins and racking reduces the risk of fungal contaminants, particularly when coupled with the use of yeast hulls and fining agents such as activated charcoal (OIV 2005; Del Prete et al. 2007). Some strains of lactic acid bacteria have also been shown to reduce OTA contamination, and these species should be used in preferential inoculum of wines undergoing malolactic fermentation (Del Prete et al. 2007; Shetty and Jespersen 2006).

Another mycotoxin, aflatoxin B1 from *Aspergillus flavus*, one of the most carcinogenic natural materials, has been detected in vineyards (Inoue et al. 2013; El Khoury et al. 2008) and represents a risk to winemaking because if it is present in

high levels in grapes it can be transferred to the resulting must (El Khoury et al. 2008). The content of aflatoxin B1 is reduced during fermentation through the action of yeast adsorption, but further steps similar to the precautions taken for OTA may also be employed (Csutorás et al. 2014; Inoue et al. 2013).

Residual plant protection products found on harvested grapes can affect fermentation and other biochemical process, depending on specific residue types and species of microbiota present (Caboni and Cabras 2010; Calhelha et al. 2006). They also have the potential to persist in the winemaking process if not properly managed, and can be detected in finished wines (Pesticide Action Network Europe 2008). As plant protection products include a diverse range of functional chemicals including heavy metals (Tariba 2011), their removal in winemaking needs to include strategies that target different compounds. For instance, residues of fenamidone, pyraclostrobin and trifloxystrobin can be adsorbed by wine solids such as grape skins and yeast lees that can subsequently be removed through racking (Garau et al. 2009). Other products such as valifenalate, iprovalicarb, fenhexamid, metalaxyl and procymidone are more persistent and leave greater residues in wines (Čuš et al. 2010; González-Rodríguez et al. 2011). More persistent products can be removed or reduced through additional steps such as (1) employing carbonic maceration techniques to reduce cyprodinil (Fernández et al. 2005); (2) fining with activated charcoal and casein to reduce agents such as fludioxonil, pyrimethanil, penconazole, imazalil and tetradifon (Sen et al. 2012) and (3) fining with bentonite to remove carbendazim (Ruediger et al. 2004).

5.6 Impact of Climatic Variability and Extreme Events

Climate change may result in increasing variability and extreme events in some areas. Seasonal variability can be attributable to an increased occurrence of extreme events such as unusual periods of high temperatures, heavy storms, hail, frost drought and bushfires (Mira de Orduña 2010; Olesen and Bindi 2002).

5.6.1 Effects of Climatic Variability and Extreme Events on Viticulture

Heavy precipitation and hail can have devastating effects on the current season's crop and on the following years' harvest. They can cause severe plant defoliation before adequate reserves have been accumulated, thus negatively impacting development the following spring, and leading to a decrease in bud fruitfulness and production (Iland et al. 2011; Olesen and Bindi 2002).

Cool-climate wine-producing regions are particularly exposed to the risk of early frost events due to the advancement of budburst, driven by increased air temperature in the spring period (Molitor et al. 2014). Frost events can limit the fruitfulness and

yield of vines as frost-damaged shoots originating from the primary buds are replaced by secondary buds that are less fruitful (Iland et al. 2011). Several methods to protect vines from frost are being trialled or are available, including hydrophobic particle films and acrylic polymers (Fuller et al. 2003), application of plant growth regulators and oils to delay budbreak and the use of sprinklers, wind machines, heaters, helicopters and several chemical sprays (Poling 2008).

Extended periods of high temperatures, possibly accompanied by drought, can result in severe vine damage due to water stress, protein denaturation caused by extreme heat, damage to plant tissues and in extreme situations bushfires. This latter issue has been observed, more recently so, in several wine-growing regions: Australia, California, British Columbia, Southern Europe and South America. Bushfires not only impact the ecology of environments and the safety of populations, but have the potential to damage vines and vineyard infrastructures (Scarlett et al. 2011). By looking at the effect of smoke on the physiology of grapevine leaves, Bell et al. (2013) reported that for most varieties, short-term exposure to smoke had no effect, or a transient effect, on leaf physiology. All varieties examined recovered to pre-smoke functioning within 48 h. Major damage to plants is associated with a level of heat at which severe defoliation occurs (Scarlett et al. 2011). In turn defoliation could have negative effects on yield, floral initiation and accumulation of plant reserves (Iland et al. 2011). Grapevines exposed to smoke during sensitive periods of growth produce wines that can contain smoke-related aromas and flavours making them unpalatable. This most likely causes a decline in product quality and potential financial losses (Mayr et al. 2014).

5.6.2 Effects of Climatic Variability and Extreme Events on Winemaking

Climatic variability and extreme events may predominantly influence yields, but will also necessitate winemaking adaptation. In particular, winemakers will have to deal with grapes with undesired levels of key parameters such as ripeness, acidity and flavour profiles as discussed previously. In the case of smoke-tainted grapes, winemakers need to adapt their processing to avoid extraction of volatile phenols and guaiacol glycoconjugates), in the main by reducing skin contact time (Kelly et al. 2014).

5.6.3 Effects of Climatic Variability and Extreme Events on Product Safety

The potential safety concerns associated with changes in grape composition and residuals caused by extreme events and climatic variability are similar to those highlighted in the previous sections. Notably, winemakers will need to be vigilant

against rising alcohol levels, the formation or introduction of dangerous metabolites and the occurrence of wine spoilage. Winemakers will also have to manage potential residues from fining agents, metals or incorrect employment of plant protection products. Steps outlined previously will need to be employed to ensure production of safe and high-quality products.

5.7 Conclusions

The relationship between climate change and wine production is complex and not uniform across different wine-producing regions. In this context, the experiences and knowledge of any given region can often assist in managing 'new' situations in another. Vineyard managers and winemakers are familiar with managing some degree of climatic or weather variability, usually represented through vintage variation. Where conditions exceed these accepted 'norms' of variance, additional management practices may need to be employed to ensure product quality, consistency and safety. Variability and extreme conditions in some respects present greater threats than changing averages as their acute nature places producers in unfamiliar conditions that they must quickly adapt to and manage.

This chapter has identified three core aspects of climate change that are likely to affect viticulture presently and under future scenarios: temperature, precipitation and extreme events. The extent of changes will vary between regions, temporally and under different RCP scenarios. Many of the potential viticultural impacts identified in literature relate more to issues of yield, wine style and commercial viability than to wine safety.

Key wine safety risks identified in this chapter include high-potential alcohol levels caused by increasing or extreme temperatures; toxic metabolites including biogenic amines formed through wine spoilage as an indirect result of climatic conditions and increased sulphite levels added to prevent spoilage. Viticultural and winemaking activities employed to cope with climatic conditions can also lead to the increased presence of residues. These can be from practices such as plant protection product applications and/or the use of fining materials that potentially introduce allergens.

All of these risks can be managed through production processes critical to ensuring that desirable and safe products are produced. However, the ability of these processes to be implemented and adhered to in all wine-producing and prospective wine-producing regions remains uncertain and consequently a possible source of risk. In both existing and emerging regions there is a need for adequate guidance, regulation and legislation to ensure that management practices, employed under specific climatic conditions, are legal and result in a product that is safe. Having in place adequate quality assurance processes in conjunction with appropriate monitoring by stakeholders should mean that wine products can be produced under climate change conditions without the risk to product or consumer safety, as long as conditions allow sustainable business viability.

Above all, due diligence and product quality assurance are increasingly important in protecting consumer safety. Under climate change conditions knowledge transfer, viticultural and oenological expertise and the ability to quickly adapt to manage different situations are critical to product viability and safety. What is possibly harder to ensure is the maintenance of a given wine style or organoleptic character.

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Part II
Wine Consumer Preferences

Chapter 6
Wine Quality Perception: A Sensory Point of View

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6.1 Introduction

Wine is a complex product, which had moved from a nutritional food to a hedonic beverage in consumer representation. This explains why the pleasure generated when drinking a bottle of wine, which is linked to perceived quality (Lawless et al. 1997), is now one of the main motivations of consumers when consuming the product. This fact has increased the demand of quality products in the market. In this context, it is of high interest for wine producers to understand the underlying indicators of consumers' quality perception as well as the relative importance they attach to these cues when inferring quality in wine. This is a valuable knowledge that

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enables producers to increase quality perception of their products in the market and to reach the alignment of consumers' expectations, needs and wants. Notwithstanding, at the present time little has been published examining consumers' wine sensory preferences or quality perception most probably due to the high cost of consumers' research or confidentiality issues (Lockshin and Corsi 2012).

This chapter is aimed at giving an overview of the current state of knowledge of the indicators of wine quality perception from a sensory point of view. For this purpose, the factors driving perceived quality of wine as a product, the characteristics of the consumer and the consumption situation are firstly reported. Then, the methodologies most usually employed to evaluate hedonic perception of consumers are discussed. Finally, wine properties linked to quality perception are reviewed.

6.2 Factors Driving Perceived Quality

Given the multidimensional character of wine, understanding its quality construct entails to disclose the factors linked to (1) the product itself, (2) the characteristics of consumers and (3) the consumption situation (i.e. physical surrounding and occasion).

6.2.1 Factors of Quality Perception Linked to Wine as a Product

Jover et al. (2004), Charters and Pettigrew (2007) and Veale and Quester (2009) among others suggested that the construction of wine quality perception is based on both intrinsic and extrinsic factors of the product. Intrinsic cues are those related to the wine itself (physically part of it) and cannot be modified once the product is bottled. These properties are linked to its organoleptic properties such as flavour, colour or mouthfeel. Extrinsic cues refer to properties which are not physically part of wine (Olson and Jacoby 1972) such as price (Mueller et al. 2010b), country of origin of wine (Veale and Quester 2008, 2009; Sáenz-Navajas et al. 2014), bottle weight (Piqueras-Fiszman and Spence 2012), the type of company that bottled the wine (winemaker, wine merchant, cooperative) (Sáenz-Navajas et al. 2013b), type of wine or appellation (Martinez et al. 2006), back label information (Mueller et al. 2010a), label aesthetic (Chrea et al. 2011; Rocchi and Stefani 2006) or the presence/absence of awards (Chrea et al. 2011; Lockshin et al. 2006).

Consumers rely on both extrinsic and intrinsic cues when inferring wine quality. Extrinsic cues appear to be important in both expected (before being consumed) and experienced (after being consumed) quality perception (Veale and Quester 2009), especially when consumers experiment difficulty in the evaluation of intrinsic quality (D'Alessandro and Pecotich 2013). The importance of extrinsic cues on the representation of wine quality also lies in the fact that in most wine purchase situations

consumers cannot taste wine and thus evaluate intrinsic factors, what forces them to rely on wine's extrinsic cues. However, the intrinsic characteristics of previously experienced wines play a major role in repurchase situations.

6.2.2 *Factors of Quality Perception Linked to Consumer's Characteristics*

Wine quality perception and the importance given to both extrinsic and intrinsic factors cannot be understood unless the characteristics of the consumer judging the product are taken into account. The quality perception of wine varies widely among wine consumers and is dependent on diverse factors such as their age (Kallas et al. 2013; Ginon et al. 2014), gender (Bruwer et al. 2011), household incomes or level of education (Bindon et al. 2014), wine knowledge (King et al. 2010; D'Alessandro and Pecotich 2013; Hopfer and Heymann 2014) or cultural origin (Prescott 1998; Torri et al. 2012; Sáenz-Navajas et al. 2014). This is the reason why there is an increasing interest in carrying out consumer studies able to uncover wine preferences based on consumer's differences and aimed at characterising segments of consumers with common features (Carbonell et al. 2008). However, in most cases, the identification of consumer segments based on socio-demographic variables such as age, gender, household incomes or level of education is not possible since they do not seem to be the main drivers defining consumers' wine preference (Bindon et al. 2014). Consumers' wine knowledge and culture/nationality seem to be predictors of quality perception.

6.2.2.1 Consumers' Wine Knowledge

Wine knowledge results from two interrelated mechanisms: expertise and familiarity (Chocarro et al. 2009).

Familiarity is linked to mere exposure, which refers to the accumulated number of product-related experiences (Alba and Hutchinson 1987) and encompasses consumers' frequency of purchase and consumption of a product (Chocarro et al. 2009). The relative importance given by consumers to extrinsic and intrinsic cues varies depending on consumers' familiarity with wine and with their quality cues (Rao and Olson 1990). In the absence of wine familiarity, consumers base their quality evaluation on simplified heuristic (experience-based) cues (Banovic et al. 2012). Consumers moderately and highly familiar with a product tend to use intrinsic cues to a greater degree than consumers less familiar with the product who mainly use extrinsic cues (Rao and Olson 1990). According to Banovic et al. (2012) an increase in familiarity with the quality cues facilitates the access to product information stored in semantic memory, developing stronger correlations between known cues and expected quality. This could explain why experts, even if they mainly base their quality judgements on intrinsic cues (D'Alessandro and Pecotich

2013), also rely on extrinsic cues such as wine origin (D'Alessandro and Pecotich 2013) or variety (Corduas et al. 2013) as they can recall memory of sensory characteristics (intrinsic factors) of previously experienced wines which lead them to their final quality evaluation (Rao and Olson 1990). This familiarity effect, which is product specific (Park 1981), has been shown to influence the extent to which consumers interpret information for developing wine quality judgments (Sáenz-Navajas et al. 2014).

Expertise is the skill and/or knowledge in a particular area. The level of expertise is linked to the speed of acquisition of knowledge, organisation and processing, and memory (Hughson and Boakes 2002). The superior performance of experts in comparison with consumers when describing wines has been attributed more to a cognitive than perceptual process (Hughson and Boakes 2001). In line with this, Castriota-Scanderbeg et al. (2005) showed that different brain areas were activated when consumers with different levels of expertise (sommeliers vs. naïve consumers) tasted a wine. More experienced consumers (sommeliers) showed activation of areas implicated in gustatory/olfactory integration in primates, involving the left insula and adjoining orbitofrontal cortex. Moreover, a bilateral activation in the dorsolateral prefrontal cortex was observed. These activations are involved in higher cognitive functions, such as memory, as well as the ability of integrating several sensory modalities. Differently, naïve consumers showed activations in the primary gustatory cortex and brain areas, including the amygdala which is related to a more emotional and global experience. The memory representations of novices (nonexperts) seem to be episodic since they are mainly based on a few exemplars of each type of wine and more associated to the emotions generated by the context in which they tasted the wines than the taste of the wines by itself. As a consequence novice responses tend to be related to their personal experience of consuming resulting in personalised and subjective responses (Parr et al. 2011). This results in a broad range of preferences and quality patterns among consumers that can be in some cases opposed to experts' perception (Machado et al. 2011; Sáenz-Navajas et al. 2013a; Hopfer and Heymann 2014).

Contrary to consumers, experts regularly attend formal wine tasting sessions, in which they often have information about the wines they taste, which leads to a lower variability and higher consistency in responses compared to novices (Urdapilleta et al. 2011). This higher consistency of experts is attributed to the building of shared semantic sensory memory representations of wine (Urdapilleta et al. 2011) especially for experts belonging to the same wine culture (Ballester et al. 2008; Sáenz-Navajas et al. 2013a).

6.2.2.2 Consumers' Culture

Although preferences and perceived quality vary widely among consumers, some consensus emerges within cultures (Prescott 1998; Williamson et al. 2012). This cultural effect has been attributed to pronounced differences in diet habits, beliefs, norms and practices among cultures. Differences in food preference between

cultures have been demonstrated (Jaeger et al. 1998; Antmann et al. 2011; Chung et al. 2012), even among culturally close countries such as countries within the European Union (Sachet et al. 1995). Most studies on cultural effects converge in concluding that an increase in familiarity translates to an increase in preference and quality perception (Schnettler et al. 2008). This link between familiarity and preference explains the fact that consumers tend to like best products from their own country. Yet, some studies carried out with wine show that higher preference or quality perception is not always linked to domestic products (Torri et al. 2012; D'Alessandro and Pecotich 2013; Sáenz-Navajas et al. 2013a). This absence of preference for wines of consumers' country has been attributed either to the presence of unpleasant attributes such as leather in local wines (Torri et al. 2012) or to the influence of regional familiarity and the locality in the quality judgements (Sáenz-Navajas et al. 2013a; D'Alessandro and Pecotich 2013).

Other studies even failed to show an effect of culture on quality perception (Verbeke and Ward 2006). This absence of cultural difference is attributed to either the lack of knowledge about origin cues (Grunert 2005) or the fact that consumers make use of quality indicators which are pretty much the same in most countries (Aurifeille et al. 2002). These discrepancies and the evident globalization of the market stress the importance of carrying out cross-cultural studies to increase the understanding of preferences and perceived quality of wine consumers.

6.2.3 Factors of Quality Perception Linked to the Consumption Situation

The physical surrounding and the specific occasion defining the consumption situation have been shown to strongly influence food preference and quality perception (Jaeger et al. 2010).

Concerning the *physical surrounding*, different studies have demonstrated that food served in different places results in different quality ratings (Meiselman et al. 2000; King et al. 2007). These changes in hedonic perception seem to be affected by consumers' expectations about the eating location (Meiselman et al. 2000). Together with the specific location, the ambience of the environment defining the physical surrounding has been shown to influence food assessments. For instance, Sester et al. (2013) demonstrated that beer perception was different according to the ambience evaluated by means of an immersive approach. The authors focused their experiment on elements of the overall setting such as music, furniture or projection of different clips for evoking different ambiences. Similarly, Stroebele and De Castro (2004) demonstrated an effect of colours, light, smell and temperature of the location on food judgements and Tempesta et al. (2010) highlighted the importance of landscape in wine quality perception.

Likewise, the *occasion of consumption* and its appropriateness have been shown to influence quality perception of the product (Giacalone et al. 2015). More specifically, the appropriateness of the occasion of consumption affects consumers' per-

ception and acceptance ratings towards the product. The same product is not suitable for every occasion (Piqueras-Fiszman and Jaeger 2014). Giacalone et al. (2015) showed that the situational appropriateness of beers is related to the familiarity of consumers with the product. More familiar beers were shown to be more appropriate for most occasions. On the contrary, unfamiliar ones were more associated with fewer specific occasions such as "make a gift" or "as an alternative to wine for a dinner". This may be the result of the atypical appearance of the studied unfamiliar beers, which was linked to prestige and exclusiveness. Thus, the level of appropriateness, which is linked to familiarity with the product, is suggested to be an important factor employed when inferring quality. Notwithstanding, the fact of being unfamiliar does not automatically yield a higher quality perception, but there is an important trade-off between the different extrinsic and intrinsic factors of the product. Piqueras-Fiszman and Jaeger (2014) demonstrated that the appropriateness of the product was dependent on the occasion of consumption (breakfast, lunch, afternoon snack, dinner). This different perceived appropriateness, which has an important effect on consumers' preference and quality perception, was successfully related to consumers' emotional responses to food. However, concerning wine, there is an evident lack of scientific results in this area, which would undoubtedly further increase the knowledge in understanding consumers' quality and preference constructs.

6.3 Methodologies Used to Evaluate Wine Quality Perception

The evaluation of consumers' hedonic ratings (either preference or quality perception) of wine is performed mainly according to two methodologies: (1) consumer surveys and (2) experiments with access to the product.

6.3.1 Consumer Surveys

This first approach aims at understanding consumption behaviour and attitudes of consumers towards wine by collecting declarative statements based on previous experience. This methodology is characterised by the important number of consumers involved, which can range from around 100 (Ginon et al. 2014) up to 400 (or more) consumers (Kallas et al. 2013). Highly structured questionnaires (with close- or open-ended questions) are usually employed (Corduas et al. 2013), which include direct ratings of quality perception and/or preference, as well as the rating of the importance of both extrinsic and/or intrinsic wine cues (usually in 7- or 9-point Likert scales with 1=dislike extremely and 7/9=like extremely), socio-demographic (age, gender, household incomes, level of education) and wine purchase/consumption behaviour information. As an alternative to rating scales, Ginon et al. (2014) have proposed a free listing task. Consumers were asked to list all criteria

underlying the purchase of a bottle of wine. Participants could quote as many expressions or terms that came spontaneously to their mind. This approach estimates the importance of factors by the percentage of consumers who mention it but also by the order in which it is mentioned in the list.

Regardless of the type of method employed for evaluating wine quality/preference or disclosing the importance of wine factors on hedonic ratings, cluster analysis is performed to identify consumer segments with different key factors driving quality/preference perception. Then, differences in the socio-demographic or wine purchase/consumption behaviour characteristics among clusters are calculated to characterise consumer segments.

Surveys are carried out in different settings such as in the street (Martinez et al. 2006), in local wine/liquor shops (Lockshin et al. 1997; Kallas et al. 2013), in neutral locations (Guerrero et al. 2000), in tasting rooms (Ginon et al. 2014), at home (Fotopoulos et al. 2003), by posting (Jaeger et al. 2009) or e-mailing (Jover et al. 2004) questionnaires, using self-administrated questionnaires or face-to-face interviews.

Surveys have evident drawbacks, especially because actual choice experiences respond to intuitive thinking and are rarely guided by rational considerations recorded by surveys. This might explain the fact that wine intrinsic cues are usually declared to be important factors by consumers as a determinant of their wine choice and consumption (Jaeger et al. 2009; Corduas et al. 2013). However, results derived from experiments in which consumers are confronted to real wine bottles and have the opportunity to taste the product indicate that intrinsic cues seem to be less relevant (Mueller and Szolnoki 2010). In line with these results, Koster (2009) claimed that questionnaires should be limited to observational facts or even abandoned in favour of more indirect behavioural and observational methods.

6.3.2 Experiments with Access to the Product

The second group of methodologies are related to experiments, in which consumers have access to the product. Consumers can have access either simultaneously or separately to intrinsic (they can taste wine) and extrinsic factors (they are informed about the wine they taste and can manipulate the bottle).

6.3.2.1 Simultaneous Access to Intrinsic and Extrinsic Properties

D'Alessandro and Pecotich (2013) carried out an experiment for understanding quality judgements of both novices (nonexperts) and experts by using a controlled set of hypothetical wine samples. The joint effect of two extrinsic factors (country of origin of wine and brand) and intrinsic characteristics on wine quality perception was studied. Consumers were asked to score the perceived quality of different wine

samples in the presence of varying cues: intrinsic quality (2 levels), country of origin (5 levels) and brand information (2 levels). Results showed that nonexperts experienced difficulty in evaluating intrinsic quality and even when detecting sensory differences between the two wines with different expected intrinsic quality, they were unable to assign a meaning to these differences. This difficulty in judging wine intrinsic quality could have led them to rely on basically extrinsic cues such as the image of the country of origin of wines, and to a lesser extent the brand name, when inferring quality as shown by Lange et al. (2002). Differently, experts based their quality judgements mainly on intrinsic cues, although the extrinsic factor, country of origin of wine, was also an important factor influencing their perception. This could be explained by the fact that experts base their judgements on their previous experience (intrinsic cues) and knowledge (extrinsic cues) of wines, which can influence their quality judgements.

Alternatively, Mueller et al. (2010b) performed a two-stage experiment designed to mimic the process of a consumer choosing a bottle of wine from the shelf (exclusive access to extrinsic cues), tasting the product (access to extrinsic and intrinsic cues) and making a repurchase decision (access to extrinsic and intrinsic cues). A first online discrete choice experiment with a controlled hypothetical set of Australian Shiraz red wines was carried out, in which consumers had access exclusively to extrinsic factors. Participants were asked to choose the wine they would most and least likely buy for a dinner at home with friends or family. Then, a separate informed sensory hedonic test was carried out with commercial samples (Australian Shiraz wines), in which both extrinsic and intrinsic cues were available. Consumers rated each wine for overall liking on a 9-point hedonic scale (‘dislike extremely’ to ‘like extremely’) relative to a reference standard wine. Finally, consumers had to indicate if they would repurchase (binary response: yes/no) the wine. In parallel, the same sample set of wines was characterised by a trained panel and further correlated to liking scores.

Another approach is that designed by Mueller and Szolnoki (2010), which involved a three-stage procedure for obtaining hedonic ratings (rated in structured 7-point scales from ‘dislike not at all’ to ‘like very much’) of wines evaluated in the presence of the following information: (1) exclusively intrinsic cues (blind tasting), (2) exclusively extrinsic cues (no tasting) and (3) combination of extrinsic and intrinsic cues (informed tasting). Besides hedonic ratings, consumers rated in structured 7-point scales their acceptance of a given price (3.99€) per bottle (from ‘too expensive’ to ‘too cheap’) and their purchase intent (from ‘very unlikely’ to ‘very likely’). In general, two extrinsic factors (label style and brand evaluation) were the strongest drivers for informed liking followed by intrinsic quality evaluated under blind tasting (with no access to extrinsic cues). Besides this general effect, three clusters of consumers with different liking drivers were identified. They differed according to consumers’ age and wine expertise. The limitation of the study was that only one wine (dry Riesling from the German Rheingau region, vintage 2004) was evaluated blind and then in different informed conditions (bottles with different extrinsic factors: brand, grape variety, origin and packaging). This could have led to underestimating the weight of intrinsic characteristics and inflating the impact of extrinsic factor in the rate

of consumers' liking. The authors suggest that selecting more polarising wines, covering a wide range of liking rates (from dislike extremely to like extremely), would have resulted in a higher impact of intrinsic over extrinsic cues than that observed.

These informed tasting methodologies have the advantage of confronting consumers with more realistic situations in which they have simultaneous access to both extrinsic and intrinsic cues. This also allows understanding the interplay of both intrinsic and extrinsic wine attributes. However the reported studies have certain limitations due mainly to the hypothetical nature of wine samples, each being a combination of several a priori-fixed intrinsic and extrinsic factors. On the other hand, using real wines rather than hypothetical wines would lead to a fatigue problem as tasting more than 6–8 wines would be the maximum for regular consumers.

6.3.2.2 Separate Access to Intrinsic and Extrinsic Properties

Extrinsic Properties

The evaluation of the extrinsic cues driving consumers' hedonic ratings is usually carried out either by controlled laboratory approaches or simulated choice experiments. The formers deal with controlled-choice experiments in unrealistic scenarios. Consumers are forced to trade-off between levels of the studied variables, thereby revealing their preferences for the product. Stimuli are commonly hypothetical sample sets (Lockshin et al. 2006; Mueller et al. 2010b; Mtimet and Albisu 2006; Raz et al. 2008; Tempesta et al. 2010) although real commercial wine samples have also been employed (Veale and Quester 2008). This approach has the advantage of being able to tap implicit determinants of food choice which are not accessible via simple surveys. However it has the disadvantage of following a 'reductionist' approach (Koster 2009) searching for behavioural mechanisms by varying single variables under the exclusion of all other extraneous and disturbing influences and thus creating a scenario quite different from actual choice situations.

As an alternative, methodologies based on the idea of unconscious decision-making by simulating choice situations have been developed. For instance, Chrea et al. (2011) studied the influence of region, grape, price, vintage and awards on Australian consumer choice at a central testing location with real commercial wine samples. Consumers were told to pick 3 bottles out of 16 to take for a dinner party at home with friends and then to create a choice ranking among these 3 bottles. Results of this real choice experiment were not consistent with those obtained by either online survey or discrete choice experiment (with hypothetical wine samples), showing differences in the relative importance of the extrinsic attributes (wine label) analysed by the three methods. Another simulated choice experiment was carried out for measuring the impact of extrinsic cues in the acceptability of partially dealcoholised wines by manipulating the information on the label (Meillon et al. 2010). The real-life setting was a home use test (HUT). French consumers received a set of three bottles and two questionnaires at home and they had to evaluate their expectations and overall liking before and after tasting on

continuous hedonic scales. The results obtained from the more natural situation, the HUT approach, were compared with those obtained in a controlled laboratory situation (CLT) yielding similar hedonic rankings but with higher scores in HUT than in the CLT.

Similarly, in an attempt to create a realistic context, Sáenz-Navajas et al. (2013b) simulated a wine shop by using typical decorating elements such as bottles, wine glasses, pictures or a cheque-out desk. Consumers were asked to choose individually five (out of 24) wine bottles according to their perceived quality. Participants were told that they will keep one of the bottles selected as an incentive for their participation in the study and granting their choice real consequences. This approach was compared with a laboratory approach (categorisation task). In the categorisation task the same 24 real and commercial wine bottles were presented on a table and participants had to group bottles in three categories according to their quality perception (low, average and high quality). Then, they were asked to explain the criteria they used for creating wine quality groups. The simulated shop and the laboratory-based categorisation task showed similar results. Besides, even if the quality judgements of consumers in the simulated wine shop were closer to those taking in real purchase situations, the categorisation task emerged as an interesting and less costly (in terms of money and space) approach. The authors underlined that an important advantage of the categorisation task is to provide quality judgements for all bottles, not just for the highest quality wine as the purchase approach. Recently the categorisation task carried out with real commercial wine samples followed by a segmentation-based strategy has demonstrated to be an effective tool for assessing consumers' representation of quality in different countries (Sáenz-Navajas et al. 2014). The biggest advantage of this methodology is that it is easy to understand by consumers, regardless their culture, age or level of education.

Intrinsic Properties

In the wine industry, quality judgments of products are traditionally carried out by experts or winemakers, which are mainly based on wine intrinsic properties and primarily driven by the absence of negative attributes or faults and then by the combination of positive sensory attributes (aroma, taste and in-mouth sensations) assembled to reach harmony and balance rather than a predominant note in wine (King et al. 2010; Sáenz-Navajas et al. 2012). These judgments are based on experts' technological and wine production experience (oenological and viticulture processes). However, there is a wide range of evidence that experts' judgments cannot predict consumers' quality perception and/or liking and guarantee the success of wines in the market (Corduas et al. 2013; Hopfer and Heymann 2014). Eves (1994) two decades ago already suggested that the acceptability of wines in the market should be measured using consumers rather than exclusively experts.

To further disclose the sensory drivers of consumers' preferences or quality perception, ratings of consumers are usually correlated to aroma, taste and in-mouth

sensory profiles obtained from trained panels by means of multivariate statistical tools such as partial least square regression (PLS) (King et al. 2010; Bindon et al. 2014) and external (Torri et al. 2012; Sáenz-Navajas et al. 2012) or internal (Hopfer and Heymann 2014; Lesschaeve and Bruwer 2012) preference mapping. Most reported consumer studies evaluate either liking or quality using hedonic ratings by means of Likert scales of 7 or 9 points (1 = dislike extremely and 7/9 = like extremely). Alternatively, categorisation tasks have recently emerged as an interesting approach for studying consumers' perception towards wine sensory intrinsic properties. Participants are asked to sort wine samples according to their global intrinsic quality perception in different quality categories or levels going from very low to very high quality. This approach has successfully been applied (Sáenz-Navajas et al. 2013a; Hopfer and Heymann 2014) to evaluate the wine quality representations of consumers from different cultures (California, France, Spain) and with different levels of expertise (experts vs. nonexperts).

6.4 Wine Properties Linked to Quality Perception

6.4.1 Extrinsic Properties

Price has repeatedly been found to be a key driver of wine choice (Lockshin et al. 2006; Mueller et al. 2010a) and this effect is particularly significant when intrinsic properties are not available in product choice. Higher prices are related to higher quality wines. The type of company that bottled the wine (winemaker, wine merchant, cooperative) has been shown to be an important extrinsic cue involved in quality assessment (Sáenz-Navajas et al. 2013b). Thus, being bottled by a cooperative seems to decrease wine quality perception, probably due to the historical pejorative bound linked to cooperatives in France (Humbert and Jacket 2010).

The label aesthetic also seems to be an important factor when judging quality. A study carried out in Australia (Chrea et al. 2011), a 'new-world' wine producer, revealed that liking for 'classic labels' was associated with some psychodemographic characteristics including more highly involved consumers with higher subjective knowledge and purchasing more bottles of red wine. In line with these results, another research carried out in Italy (Rocchi and Stefani 2006), a 'traditional' and 'old-world' production country, pointed out the level of consumer involvement as a determining factor for the value given to either traditional or modern features. Thus, in some cases tradition could be interpreted as a sign of reliability, and in others as a sign of lack of innovation.

The presence of awards in bottles seems also to be an important quality indicator for Australian (Chrea et al. 2011; Lockshin et al. 2006), French (Sáenz-Navajas et al. 2013b, 2014) and Spanish (Sáenz-Navajas et al. 2014) consumers among others. Lockshin et al. (2006) related the importance consumers give to awards to their level of involvement: low involved consumers tend to use wine awards to a larger extent than high involved ones. Besides, these authors suggested that although the

presence of awards is able to increase the likelihood of choosing a wine, this only holds true for lower and middle priced samples.

Mueller et al. (2010a) have shown in a wine choice experiment, limited to back label presentation, that back label information may have a positive effect on consumer choice, except for chemical wine ingredients which caused strong adverse reactions for some consumers. More recently, Piqueras-Fiszman and Spence (2012) have shown a consumer trend (mainly Spanish consumers) towards associating the weight of the bottle, the price of the wine and its quality. Thus, higher quality wines are expected to come in heavier bottles.

Even if many other extrinsic factors (such as brand, vintage or bottle form among others) affect quality inference the relative importance of each cue has shown to be dependent on consumers' culture (consumers' region of origin) and wine knowledge (Sáenz-Navajas et al. 2014). To illustrate this, the quality categories attached to a set of 24 wine samples (half Spanish and half French wine bottles) and their relationship with the extrinsic factors for Spanish consumers are shown in Fig. 6.1 (more knowledgeable consumers) and Fig. 6.2 (less knowledgeable). Globally, both groups of consumers (with higher and lower wine knowledge) use similar extrinsic cues to infer quality such as type of wine (*joven*, *crianza* and *reserva* for Spanish wines or *AOC regional* or *AOC Village* for French wines), vintage year, label design (modern or classical), bottling place (cooperative, merchant or estate bottled), awards (absence/presence), bottle weight (light or heavy) and/or back label information (production information, meal pairing or aroma expectations). However the importance consumers attach to extrinsic cues varies according to their wine knowledge. Especially, the country of origin of wine (Spanish vs. French wines) arises as the most important extrinsic cue for less knowledgeable consumers, while consumers with higher knowledge in wine are able to interpret and use a wider range of cues.

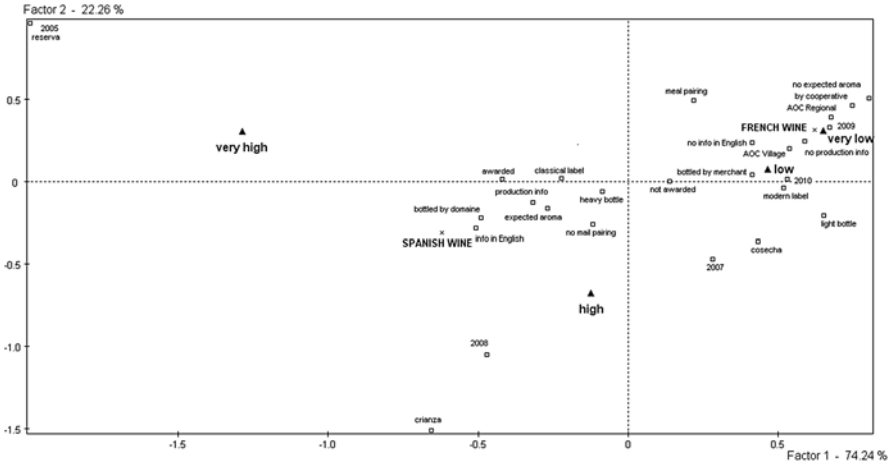

Fig. 6.1 Projection of bottle extrinsic factors/attributes on the bi-dimensional CA map yielded from a categorisation task based on quality perception (categories: very low, low, high or very high quality) of Spanish consumers with less wine knowledgeable

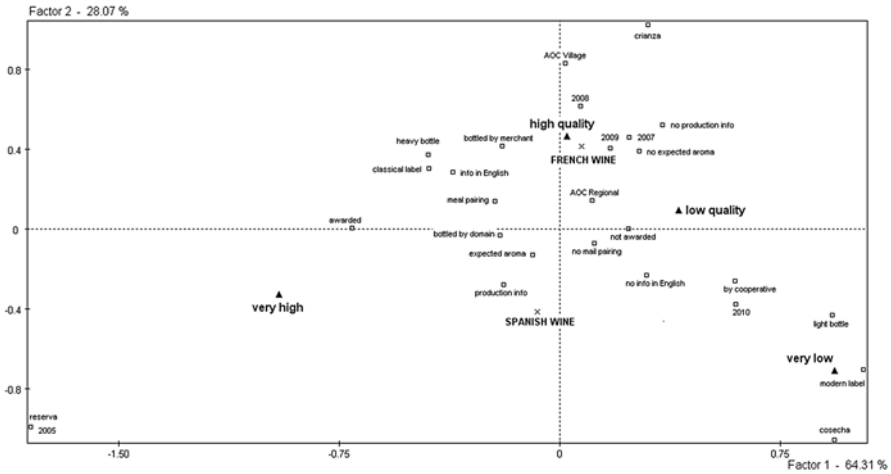

Fig. 6.2 Projection of bottle extrinsic factors/attributes on the bi-dimensional CA map yielded from a categorisation task based on quality perception (categories: very low, low, high or very high quality) of Spanish consumers with higher wine knowledgeable

6.4.2 Intrinsic Properties

Among the available literature, research works dealing with assessments carried out by experts from different countries or highly involved wine consumers coincide in positively correlating fruity and woody aromas as well as the general mouthfeel sensation of astringency to high-quality red wines from Australia (Lattey et al. 2010; Mueller et al. 2010b), Spain (Sáenz-Navajas et al. 2012; Saenz-Navajas et al. 2011), France (Sáenz-Navajas et al. 2013a) or Uruguay (Varela and Gambaro 2006). On the contrary, animal, vegetal or undergrowth aromas are linked to negative quality perception and thus lower liking according to experts. Mueller et al. (2010b) showed that sweetness and fresh fruit aromas had a significant positive influence on informed liking of commercial Australian Shiraz red wines. On the contrary, sherry, medicinal, eggy-reduced and earthy-vegetal attributes were negatively linked to consumers' evaluations, regardless their price.

One important observation is that the fruity category can be roughly separated into two different subcategories: dried or fresh fruit. While the fresh fruity note holds a positive relationship with quality, the role played by the dried fruit note seems to be dependent on wine categories (mainly linked to the segment of price to which wines belong). For example, in Spanish Premium (Sáenz-Navajas et al. 2012) and Uruguayan Tannat (Varela and Gambaro 2006) wines the dried fruit attribute positively correlates with quality, while in high- and low-standard Spanish (Sáenz-Navajas et al. 2012) wines it holds a negative correlation. This suggests the existence of different attributes evoking either positive or negative expert quality judgements depending on the different wine categories, belonging to different price segments.

Concerning nonexperts, it has been generally observed that their preferences for white wines are related to fruity sweet attributes and are opposed to wines displaying oaky and burning attributes. This holds for consumers from different cultures such as Korean (Yoo et al. 2008), Canadian (Lesschaeve and Bruwer 2012) or New Zealanders (Lund et al. 2009). Notwithstanding, unlike experts, which have generally an aligned quality concept and preferences based on their technological experience, consumers/nonexperts do not share a common quality representation and present high variability in their hedonic ratings (Ballester et al. 2008; Hopfer and Heymann 2014). Thus, different segments of consumers are usually identified in consumer studies by means of cluster analysis. Lund et al. (2009) segmented liking ratings of New Zealanders for Sauvignon Blanc wines in two clusters. The first cluster, mainly formed by young consumers (<35 years) and Sauvignon blanc drinkers, preferred aromas such as stone fruit, passion fruit, capsicum, sweet sweaty passion fruit, fresh asparagus and boxwood/cat urine. On the contrary, the second cluster, mainly formed by red-wine drinkers and women, mostly appreciated bourbon and flinty/mineral character in wines.

King et al. (2010) evaluated the preference for Sauvignon wines inoculated with different fermentation yeasts and identified two clusters of consumers according to their preferences: More experienced consumers preferred wines high in aromas such as box hedge, and intermediate aroma intensity, and a less experienced cluster of consumers appreciated mainly strong and predominating aromas of bruised apple and cooked flavours but wines presenting medium aroma intensities were punished. In another study investigating Australian consumers' preferences towards Sauvignon Blanc wines, King et al. (2011) found three segments. The first group preferred green, solvent and citrus aromas, the second and most numerous (43 % of consumers) appreciated fresh and cooked green attributes, while among the third cluster of consumers overall fruit, tropical and confectionary aromas were preferred. Interestingly, wine preference was best predicted by a quadratic model, revealing an optimal level for cat urine/sweat attribute.

With regard to red wines, Frost and Noble (2002) and Torri et al. (2012), who investigated preferences among American and Italian consumers, consistently showed a link between low preference and the leather character. Sáenz-Navajas et al. (2015) have recently reported that French and Spanish experts and consumers' quality perception of red wines were driven by different sensory attributes. While experts agree in relating red fruity (such as cherry) character to quality wines, nonexperts found samples with attributes such as dried apricot, fresh wood and leather higher in quality. Interestingly, wines with specific animal nuances such as perspiration and cat urine were negatively related to both consumers' and experts' quality perception (Fig. 6.3). The fact that the specific animal-like leather character has been found to be negatively correlated to consumers' likings but positively to quality perception in the above-mentioned publications could be the result of either that preference and quality representations are not correlated for nonexperts (Hopfer and Heymann 2014) or it could be explained in terms of familiarity of consumers with the leather aroma. Thus, consumers more familiarised with this attribute in wines would find this nuance a positive aroma related to higher quality samples.

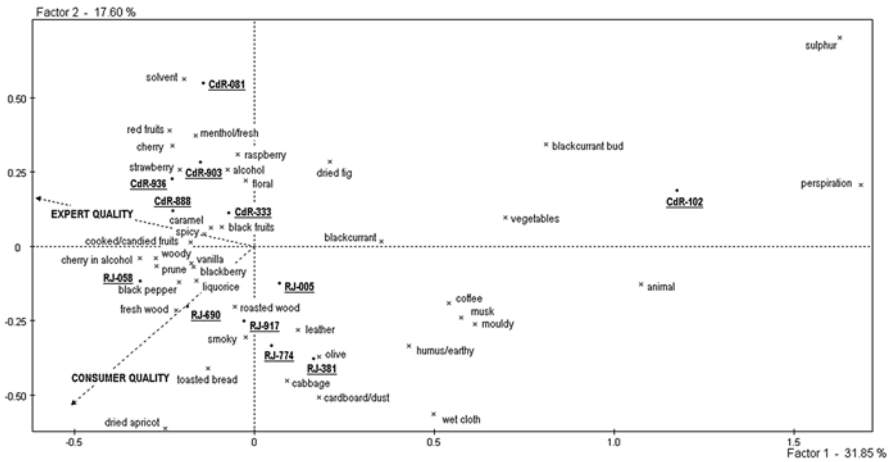

Fig. 6.3 Projection of intrinsic factors/attributes on the bi-dimensional CA map yielded from a categorisation task based on quality perception (categories: very low, low, high or very high quality) of consumers with different level of expertise (experts vs. nonexperts/consumers)

Bindon et al. (2014) evaluated consumer preference for five red Cabernet Sauvignon wines elaborated with grapes with different level of ripeness. It was observed that wines with the lowest alcohol content (12 % v/v) were the least preferred, while wines ranging from 13 to 15.5 % were equally liked by consumers. Besides, three clusters of consumers were identified. The first cluster, being the most numerous (44 %), preferred wines characterised with terms such as overall fruit, vanilla and purple colour, while sour wines presenting cooked vegetable and sewage/drain aromas were the least preferred. The second cluster showed preference for wines high in overall fruit, dark fruit, bitterness and hot mouthfeel, while red fruit and fresh green aromas were not appreciated. The last cluster showed preference for sour wines with red fruit and fresh green aromas.

As far as in-mouth properties are concerned, Spanish consumers associate astringent red wines to lower quality exemplars (Sáenz-Navajas et al. 2013a), contrary to what has been found for Spanish (Saenz-Navajas et al. 2011) and Australian (Lattey et al. 2010) experts or highly involved Uruguayan consumers evaluating Tannat wines (Varela and Gambaro 2006).

6.5 Conclusions

Taken together, wine perceived quality and preference of consumers cannot be understood unless the interplay of the factors linked to the product itself (*What?*), the characteristics of consumers (*Who?*) and the consumption situation (*How?* and *Where?*) are globally considered. A broader perspective on how the consumer

experiences the product should be taken into account, which undeniably implies associating consumers' emotional responses to wine.

The wine industry has to value that this knowledge is vital for increasing the success of their wines in the market. In this field, the discipline of sensory analysis, more especially consumer science, can largely contribute.

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Chapter 7
Wine Preference and Wine Aroma Perception

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7.1 Introduction

An odor (or scent) is the sensation that results when the olfactory receptors in the nose are stimulated by specific chemical compounds in gaseous form (volatile compounds). Broadly speaking, in English language there are many terms that one can use to refer the sense of smell and different types of smells (aroma, fragrance, perfume, odor, scent). However, there are some subtle differences among them. While the term odor means a clearly recognizable smell, normally issuing from a single source, that can be both pleasant and unpleasant, the terms aroma and fragrance are used primarily by the food and cosmetic industry to describe a pleasant odor, and are sometimes used to refer to perfumes. In the scientific literature, odor and aroma are indistinctively used and during this chapter both terms will be used as synonyms.

Wine is a special complex matrix, which contains a wide array of inorganic and organic constituents which contribute to its unique aromas, tastes, and oral sensations. Already in the 1990s, it was acknowledged that wine contains on the order of 600–800 volatile aroma compounds (Rapp 1990). It is also recognized that aroma is the major contributor to overall flavor perception (Polaskova et al. 2008) and it is one of the most important intrinsic factors that influence wine quality and consumer preferences (King et al. 2010). Thus, it is not strange that the characterization of wine aroma compounds, the elucidation of their odorant characteristics, but also the understanding of the impact of different viticultural and enological practices on the wine aroma profile have been the aim of a large piece of research recently reviewed (Robinson et al. 2014b).

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The complex aroma of wine is derived from many sources, including the grape, yeast, and microbial fermentations and post-fermentation treatments such as oak storage and bottle aging. The origin of these compounds allows us to classify wine aroma compounds in (a) primary or varietal aroma coming from the grape, (b) the secondary aroma produced during alcoholic or malolactic fermentation, and (c) the tertiary aroma or bouquet, which results from the transformation of the aroma during aging (Rapp and Mandery 1986). There are several excellent revisions providing accurate information about the chemical components involved in wine flavor (Ebeler 2001; Etievant et al. 1986; Polaskova et al. 2008; Rapp and Mandery 1986). These volatile compounds are highly heterogeneous and include alcohols, esters, aldehydes, ketones, acids, terpenes, phenols, and sulfur compounds present in variable concentrations from milligram to nanogram per liter. The total content of aroma compounds in wine is approximately 0.8 to 1.2 g L^{-1} (Rapp 1990; Rapp and Mandery 1986). However, there are many differences in the amount and type of wine aroma compounds among wines depending on both viticultural (climate, soil, water, cultivar, grape-growing practices) and enological (condition of grapes, fermentation, post-fermentation treatments) factors.

The great development of analytical techniques and instruments has allowed to advance from the first studies focused in the analysis of major volatile compounds to the analysis of compounds present in very low concentrations (even at levels below of ng L^{-1}) but with very low odor thresholds. Due to the great complexity of the wine matrix, the analysis of some minor, but *key* aroma compounds might require pre-concentration steps, the use of stable isotopic dilution analysis, and multidimensional gas chromatography coupled to the most modern powerful detectors such as time-of-flight mass spectrometers to obtain reliable results. Several authors have published interesting revisions on advances in the aroma extraction, concentration, separation, and detection methods applied for wine volatile analysis (Ebeler 2001; Munoz-Gonzalez et al. 2011; Robinson et al. 2014a, b).

These important studies on wine aroma composition have highlighted the complexity of the wine volatile fraction, but we already know that not all of these compounds have sensory relevance for wine aroma, or in other words they might not have an impact on wine aroma perception. To try to elucidate the sensory relevance of wine volatiles, gas chromatography-olfactometry (GC-O) studies have been then performed. In the last years several reviews in food flavor analysis have been published about the olfactometry technique (d'Acampora Zellner et al. 2008; Ferreira et al. 2009; Plutowska and Wardencki 2007). All of these GC-O methodologies have in common the combination of instrumental and descriptive sensory techniques to determine the odor activity (compounds present at concentration below or above the sensory detection threshold) and description (smell) as well as the time of odor activity and the intensity of the odor of volatile compounds. Following the GC-O screening for impact odorants, the odor activity value (OAV) of each compound can be calculated by dividing the concentration of a compound by its odor threshold. In these studies, compounds with OAVs >1 are considered of sensory relevance for wine aroma.

Nonetheless, few wine research studies have included the sensory validation of GC-O data and/or OAVs to determine the real impact of key odorants, taking into consideration synergistic, enhancement, and suppression effects of different odorants as well as other wine matrix components (Villamor and Ross 2013) (Villamor and Ross 2013). These studies are currently known as omission-reconstitution tests and involve the preparation of a recombinant aroma by addition of the target aroma compounds selected on the basis of their OAVs or dilution factors (DFs) to a synthetic wine. The aroma models are then compared with the original wine for similarity or difference using triangle or duo tests (Aznar et al. 2001; Pineau et al. 2009). Other studies include omission or addition experiments to evaluate the aroma models when one compound is eliminated (Ferreira et al. 2002a; Guth 1997) or added (Escudero et al. 2004) to the model.

But in spite of these necessary studies, this information is still not enough to completely understand the flavor of a wine, and wine consumer preferences. Interactions among odorants, interactions between sense modalities, and matrix effects can all impact odorant volatility, aroma release, and the overall perceived flavor (or aroma) intensity and quality. Besides this, the effect of human physiology and specifically oral physiology on wine aroma release during wine consumption and its relationship with wine aroma perception will open a new and challenging topic of research for wine flavor scientists in the following years. An overview on all of these aspects is provided in the following sections.

7.2 Wine Aroma Perception

Aroma perception from foods and beverages is a sequential process that starts when we smell the food and the volatiles travel through the nose to the olfactory epithelium where they are perceived (orthonasal route). However, during eating and drinking odorant compounds are also released into the mouth during oral food processing and travel through the nasopharynx route to reach the olfactory epithelium. This route is usually called retronasal route (Fig. 7.1). Whichever their route, orthonasal or retronasal, volatile molecules released from foods or beverages interact with the olfactory epithelium. Here, there are sensory cells with receptors to which odorant molecules can bind reversibly as a first step towards the generation of an electric signal. Sensory cells are neurons. A receptor neuron has a dendritic pole bearing fine cilia immersed in the nasal mucus. The ciliary membrane hosts receptor macromolecules. The cells possess an axon that projects the olfactory bulb and conveys electrical signals elicited by receptor activation. In the olfactory bulb, axons synapse with second-order neurons that in turn project to the primary olfactory cortex. From there, the olfactory message is sent to many other areas in the brain for a complex processing (Holley 2006).

In the 1980s, Rozin suggested that olfaction can be seen as two functionally distinct senses: one sense for identifying objects at a distance (orthonasal percep-

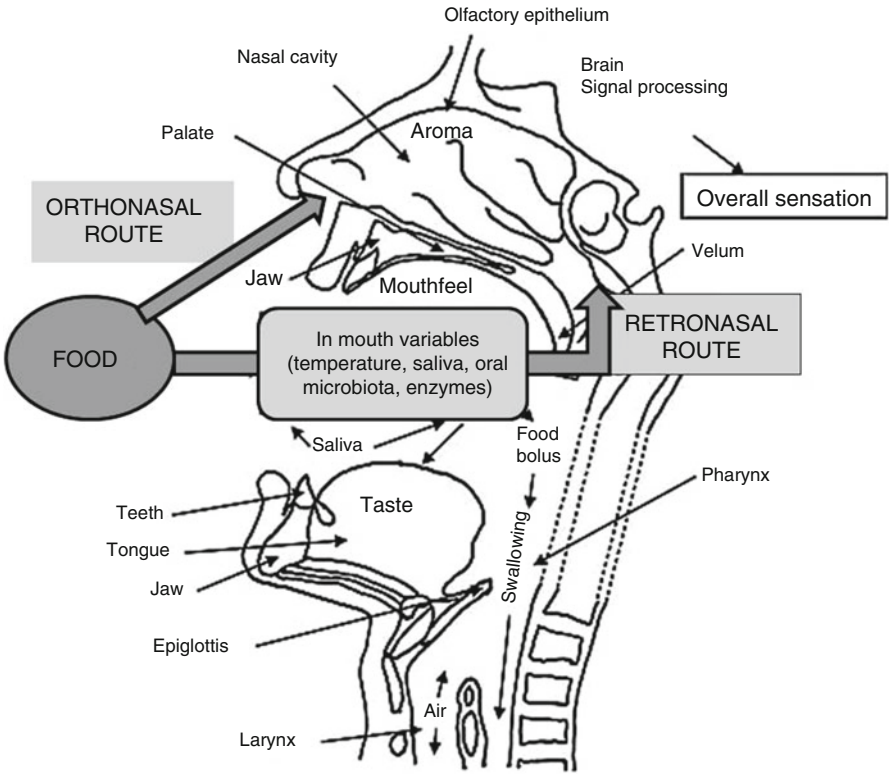

Fig. 7.1 Mechanism of release and perception of sensory stimuli during food consumption. Modified from Gierczynski and co-workers (Gierczynski et al. 2011)

tion) and another sense that contributes to flavor and hence food identification in the mouth (retronasal perception) (Rozin 1982). While these "two senses" physiologically differ perhaps only in the efficiency of delivery of odors to the olfactory epithelium (Voirol and Daget 1986) the information delivered by each may differ in its cognitive impact (Prescott 1999). Thus, it has been postulated that the identification of foods is the combination of the food's qualities (taste and retronasal odors) into a unitary perception. In agreement with Prescott, consumer's initial response to foods suggests that this is how sensory properties are perceived. Although we perceive through multiple senses, sensory information is commonly integrated to produce a whole percept. In fact, when food is in the mouth, taste, olfactory, chemesthetic, and tactile senses are concurrently stimulated (Prescott 1999).

This multisensory perception of food has brought the concept of flavor as the most appropriate to define the sensorial experience perceived during drinking or eating. The olfactory components of a food (such as wine), together with tastes and other sensory properties, identify the foods located in the mouth. Flavor could be considered as a distinct sense which is cognitively constructed from the integration of distinct physiologically defined sensory systems (mainly olfaction and taste).

There is scientific evidence that shows that tastes and odors are encoded in the brain as part of a unique perceptual system, in the form of distinct flavor entities (Prescott 1999).

7.3 Perceptual Aroma Interactions

The processing of complex stimuli by the olfactory system is a central issue in the understanding of odor perception in natural conditions because the odors we perceive come mostly from complex mixtures of odorants. The perception of single odorants and mixtures is a product of both interactions at the level of olfactory receptors and interactions during neural processing of olfactory information. In the case of a mixture of odorants, competition may occur at the olfactory receptor level as well as inhibitory interactions at the neural level. Therefore, the perception of an odorant mixture is not a simple sum of the percepts of the unmixed components (Laing and Jinks 2001). The impact of perceptual interactions on perceived flavor has been summarized in excellent revisions (Auvray and Spence 2008; Delwiche 2004; Stevenson et al. 1995).

As already stated (Barkat et al. 2012), most of the studies concerning odor mixture processing have been conducted in animal models (Coureaud et al. 2008; Derby et al. 1996). From them, it has been demonstrated that a binary mixture can be perceived in at least two ways. First, each component of the mixture remains separate and identifiable. This type of perceptual processing has been called dissociative, analytical, or elemental (Derby et al. 1996). In the second type of perceptual processing, the mixture is perceived as an entity, conveying a unique quality not present in its single components. This phenomenon has been called associative, synthetic, or configural processing (Derby et al. 1996). It has been shown that compared with the olfactory systems of naive subjects, the specific training and exposure to odors experienced by expert subjects (flavorists, perfumists, oenologists) lead the olfactory system to engage more readily an elemental processing of odor mixtures (Barkat et al. 2012).

In the specific case of wine, the presence of perceptual odor interactions is favored because of the simultaneous presence of many different odorants (chemical compounds) provoking that the final perception will be the result of a complex brain processing in which some odors are integrated into a single perception. In wines, some odorant compounds might act in competitive or even destructive way (Atanasova et al. 2004) while others interact to form a new and different perception. As previously stated (Ferreira and Cacho 2009), the presence in the wine of whole sets or aroma chemical members of a chemical homologous series displaying similar odors makes that the role of some chemicals should be considered as a part of a combination and the final role of each of them can only be determined via different sensory experiments (such as omission-reconstitution tests).

In an early work, it was shown that when the woody character of a wine increases, the flavor complexity decreases, and the intensity of fruity and floral notes is also

reduced. This suggested an interaction between the fruity and woody notes of wine (Moio et al. 1993). In a later study, Atanasova and co-workers showed this effect in more detail using three binary mixtures of wine aroma compounds (Atanasova et al. 2004). The two first mixtures involved whisky lactone (woody note) that was mixed separately with two esters (fruity note), ethyl butyrate and isoamyl acetate. For the third mixture, guaiacol was mixed with ethyl butyrate (fruity note). The results of this study confirmed the presence of perceptual quantitative interactions between fruity and woody odorants. In fact, they also suggested that quantitative mixture interactions observed at low but suprathreshold intensity levels might be different from those observed at higher intensity levels. Moreover, they stated that hyper-addition could occur when mixing low iso-intense fruity and woody odors.

Besides the abovementioned aroma compounds, perceptual interactions among wine aroma compounds have been described for furanones (furaneol and homofuraneol), C13 norisoprenoids such as β-damascenone, sulfur compounds such as dimethyl sulfide or diacetyl, and acetoin, acetic acid, and γ-butyrolactone, which might indirectly contribute to fruity expression in red wines (Lytra et al. 2013). These examples emphasize the importance of perceptive interactions on the intensity and quality of red wines' fruity aromas. Pineau and co-workers demonstrated that in some complex mixtures in dearomatized red wines, very small variations in the concentrations of some ethyl esters were perceived even at concentrations far below their individual olfactory thresholds and affected their red and blackcurrant aromas (Pineau et al. 2009). They demonstrated that ethyl propanoate, ethyl-2-methylpropanoate, and ethyl-2-methylbutanoate were involved in blackberry aromas, whereas ethyl butanoate, ethyl hexanoate, ethyl octanoate, and ethyl 3-hydroxybutanoate impacted red berry aromas.

More recently Lytra and co-workers investigated the role of 12 esters present in a mixture at the average concentration usually found in red wines, especially on fruity character (Lytra et al. 2013). They performed omission tests in the aromatic reconstitutions that were prepared in hydroalcoholic solutions and they investigated the occurrence and nature of interactions and their origins from chemical, physicochemical, and psychophysical points of view. Their results revealed the indirect impact of ethyl propanoate, ethyl 3-hydroxybutanoate, butyl acetate, and 2-ethylpropyl acetate present at subthreshold concentrations, on fruity aroma expression (red and blackberry fruit aromas). The presence of ethyl-3-hydroxybutanoate and 2-methylpropyl acetate in the mixture led to a significant decrease in the olfactory threshold of the fruity pool demonstrating their synergistic effect in increasing the overall intensity. Employing sensory tests they showed that besides ethyl-3-hydroxybutanoate, the omission of each of these compounds had a significant attenuating effect on blackberry and fresh fruit aroma intensity. The compounds with similar chemical structures participate, both quantitatively and qualitatively, in modulating fruity aromas and, specifically, naturally enhancing blackberry and fresh-fruity aromas.

Besides the existence of odor-odor interactions, there are some works in the literature that have shown interactions between taste and some typical wine aroma compounds (Dufour and Bayonove 1999b; Welge-Lussen et al. 2005). These interactions have been proven to affect an important quality of wines as it is the astrin-

gency. In an interesting work, Saenz-Navajas and collaborators showed that the addition of a white wine aroma extract (mainly described as fruity) to a reconstituted and dearomatized red wine determined a decrease in astringency and bitterness and an increase in sweet perception, which is mainly produced because of the inverse relationship between astringency and bitterness to fruity aroma (Saenz-Navajas et al. 2010b).

7.4 Wine Matrix-Aroma Interactions

One of the most important factors that can limit the rate of release of aroma compounds during wine consumption could be the interaction between aroma and nonvolatile matrix components. Aroma compounds can physically or chemically interact with wine matrix components such as polyphenols, glycoproteins, and polysaccharides. This can change the distribution of aroma compounds between the aqueous solution and the vapor phase (partition coefficient), therefore altering the odorant volatility, and might influence headspace partitioning of volatiles producing two opposite effects: a retention effect, therefore decreasing the amount of aroma in the headspace, or a "salting out" effect, provoking an increase in the headspace concentration of a volatile compound because of the increase in the ionic strength of the solution (Jouquand et al. 2004). Interesting reviews on the impact of wine matrix composition on wine aroma release have been recently published (Munoz-Gonzalez et al. 2011; Pozo-Bayón and Reineccius 2009; Villamor and Ross 2013).

The extent of odorant-matrix interactions can be measured by analyzing the concentration of the analyte in the headspace above the solution, typically by using gas chromatography procedures. As it has been indicated in some revisions on this topic (Polaskova et al. 2008; Pozo-Bayón and Reineccius 2009), in general, much more work has focused on studying aroma release under equilibrium conditions as opposite to dynamic conditions. Other methodologies such as the equilibrium dialysis (Lubbers et al. 1994) or spectroscopy methods such as RMN have also been used to evidence these interactions (Dufour and Bayonove 1999b; Jung and Ebeler 2003). The interactions between aroma compounds and wine matrix components produce different effect on wine aroma and they have been summarized in Table 7.1.

One important aspect to consider in these studies is that in most of them the effect of wine matrix has been studied by using one or several aroma compounds and a much reduced number of wine matrix components. Although very valuable, these works do not consider the whole complexity of the nonvolatile wine matrix. In one of these scarce studies, Robinson and co-workers (Robinson et al. 2009) carried out a factorial design to determine the role of some important wine matrix components (ethanol, glucose, glycerol, catechin, and proline) on 20 representative wine aroma compounds. Their results showed an important effect of ethanol followed by glucose and a very small effect of catechin, glycerine, and proline. More recently, Villamor and co-workers studied the combined effect of ethanol, tannin, and fructose through the use of HS-SPME-GCMS (Villamor and Ross 2011).

Table 7.1 Major wine matrix compounds and their effects on wine aroma determined in different studies using different analytical approaches

Wine matrix compound	Effect on wine aroma	Tested aromas	Analytical approach	References
Ethanol	– Contributes to wine aroma "per se" – Increases wine viscosity – Masking/enhancing aroma perception – Increases aroma solubility (decreasing aroma release) – "Marangoni effect" (improves aroma transfer from the bulk to the surface of the hydroalcoholic solution)	Alcohols, esters, pyrazines, terpenes, ketones, aldehydes, C13 norisoprenoids, acids	Sensory analysis GC-O Static HS (HS-SPME; APCI-MS) Dynamic HS (APCI-MS, Purge &Trap)	Jones et al. (2008), Le Berre et al. (2007), Petrozziello et al. (2014) Pineau et al. (2007) Ferreira et al. (2002b), Guth and Grosch (1998), Villamor and Ross (2011) Athes et al. (2004), Aznar et al. (2004), Camara et al. (2006), Conner et al. (1998), Hartmann et al. (2002), Le Berre et al. (2007), Petrozziello et al. (2014), Robinson et al. (2009), Villamor and Ross (2013), Whiton and Zoecklein (2000) Aprea et al. (2007), Le Berre et al. (2007), Tsachaki et al. (2005), Tsachaki et al. (2009)
Glycerol	– Contributes to wine sweetness and viscosity – Does not contribute to aroma volatility	Alcohols, esters	– Sensory analysis Static HS (HS-SPME)) Dynamic HS(Purge & Trap)	Jones et al. (2008), Lubbers et al. (2001), Nurgel and Pickering (2005) Robinson et al. (2009) Lubbers et al. (2001)
Phenolic compounds	– Hydrophobic or π–π interaction with aroma compounds reducing or increasing compound volatility – Effect depending on the polyphenol and aroma compound structure and polarity and on the concentration essayed	Aldehydes, pyrazines, esters, terpenes, mixture of many different chemical classes	Sensory analysis GC-O Static HS (HS-SPME, LC-SH) Dynamic HS (exponential dilution, HS-SPME) RMN	Aronson and Ebeler (2004), Goldner and Zamora (2010), Lorrain et al. (2013), Petrozziello et al. (2014) Villamor and Ross (2013) Escalona et al. (2001), Goldner and Zamora (2010), Mitropoulou et al. (2011), Petrozziello et al. (2014), Robinson et al. (2009), Rodriguez-Bencomo et al. (2011), Villamor et al. (2012), Villamor and Ross 2011) Aronson and Ebeler (2004), Dufour and Bayonove (1999b), Jung and Ebeler (2003) Dufour and Bayonove (1999b), Jung et al. (2000)

Polysaccharides	– Different effects depending on the type of polysaccharide and aroma compound, concentration and conformational status – Decreasing aroma release due to hydrophobic interactions of some volatile compounds with mannoproteins	Esters, alcohols, terpenes, acids, aldehydes, ketones, mixture of many different classes	Sensory analysis GC-O Static HS (HS-SPME, LC-SH) Dynamic HS (exponential dilution, equilibrium dialysis) RMN	Comuzzo et al. (2006), Chalier et al. (2007), Jones et al. (2008) Comuzzo et al. (2006) Comuzzo et al. (2006), Chalier et al. (2007), Mitropoulou et al. (2011), Rodriguez-Bencomo et al. (2011) Chalier et al. (2007), Dufour and Bayonove (1999b), Langourieux and Crouzet (1997), Lubbers et al. (1994) Dufour and Bayonove (1999a)

GC-O gas chromatography-olfactometry, *HS-SPME* headspace SPME, *LC-SH* liquid calibration-static headspace

Although most of these studies have monitored changes in wine aroma composition by using analytical approaches (GC-MS analysis), the importance of these interaction in the sensory characteristics of wine has been highlighted in the work of Pineau and co-workers. They showed that the detection threshold of an important wine aroma compound, such as β-damascenone, was 1000 times higher in a reconstituted red wine than in a hydroalcoholic solution (Pineau et al. 2007). Based on these findings, authors suggested the revision of the odor activity values (OAV) calculated for different types of wine aroma compounds. In fact, a later and wider study was carried out by Rodriguez-Bencomo and collaborators. In this work, authors used an aroma mixture composed of 40 representative wine aroma compounds to aromatize at different levels of concentration five types of dearomatized and reconstituted wines (white, sparkling, sweet, red, and aged red wines) (Rodriguez-Bencomo et al. 2011). They showed significant differences in the headspace concentration of aroma compounds when compared with reconstituted and synthetic wines (hydroalcoholic solution, pH = 3), the latter without matrix effect. This study revealed a great "matrix effect" that in general provoked the retention of most of the aroma compounds essayed, reducing their release from wine. This effect might be able to produce a sensory impact on wine aroma perception, as it has also been shown (Saenz-Navajas et al. 2010a).

Nonetheless, most of these studies have been performed in static conditions, which although very valuable to determine the chemical nature of these interactions do not represent the retronasal delivery of volatiles during a real wine consumption situation. To overcome this drawback, very recently Muñoz and co-workers have evaluated the role of wine matrix composition on the in vivo aroma release during the consumption of different types of wines (Munoz-Gonzalez et al. 2014b). For this study authors used a retronasal aroma-trapping device (RATD) that was previously optimized and validated (Muñoz-González et al. 2014c). The system incorporated a tenax polymer to entrap the exhaled breath of the panelists during the consumption of a total of 100 mL of wine that was further desorbed and analyzed by GC-MS. During this work five wines of different wine-making technology (young and aged red wines, sweet wine, white wine, and a sparkling wine) were employed. All of them were adjusted to the same ethanol level (except the sweet wine) and aromatized with a mixture of four target aroma compounds at the same aroma concentration. Results showed that the aroma released during wine intake was different depending on the type of wine consumed. It was found that red wines released higher amount of aroma compared to white and sweet wines. A further correlation analysis using many wine compositional parameters showed a direct relationship between wine polyphenols and aroma release. It is worth noting that a complementary study with the same wine types but following an in vitro approach using an artificial mouth coupled on line with a PTR-ToF-MS also confirmed differences in the real-time aroma profiles depending on wine matrix composition (Muñoz-González et al. 2015b). In agreement with the in vivo study, red wines showed higher AUC and Imax values after 30 s of monitoring time (Muñoz-González et al. 2015b). The higher aroma release determined during the real or simulated red wine intake could be related to the formation of complexes between

human saliva proteins (in the surface of the throat or in the saliva added to the wine in the in vivo or in vitro experiments, respectively) and polyphenols (more abundant in red wines) as might also occur in in vitro conditions (Mitropoulou et al. 2011).

7.5 Physiological Interactions and Aroma Perception

Once the food or beverage is introduced into the oral cavity, it will be submitted to an oral processing more or less intense depending on the type of food material. During this process, different physiological factors such as the breathing flows, the temperature of the oral cavity, the saliva flows and composition, the adsorption of odorants into the oral mucosa, and the impact of oral microorganisms might determine differences in the aroma release pattern which in turn might affect wine aroma perception. The role of physiology factors and more specifically the impact of oral physiology on wine aroma release is a scarcely studied aspect and only some recent works have dealt with it to explain wine aroma perception and consumer preferences.

7.5.1 Respiratory Flows

The exhaled air during wine consumption sweeps the volatiles retained in the mucosa layer of the throat and mouth helping aroma compounds to be transported till the aroma receptors in the olfactory epithelium (Buettner and Beauchamp 2010). In an early work, Voirol and co-workers showed how the aroma perception was affected by the air volume that reaches the olfactory receptors (Voirol and Daget 1986). In the case of liquid foods such as wine, the highest amount of aroma is released as a unique pulse after swallowing causing the so-called exhalation breath. Once the highest pulse of aroma released has been produced, very little amount of aroma is released in the subsequent expiration episodes (Rabe et al. 2004). Therefore, in this case, the breath capacity could be an important parameter limiting aroma release. However, different studies have found different results. For example, it has been suggested that a greater respiratory rate could contribute to bring more volatiles to the upper airways, and consequently more volatiles could be present in the expired air of the panelists (Hanaoka et al. 2001; Pionnier et al. 2004). Nonetheless, in another study performed in vivo and in vitro using an artificial throat, Weel and co-workers found that an increase in the flow rate resulted in a decrease in aroma release due to a dilution effect (Weel et al. 2004). Recently, Muñoz-González and co-workers showed that individuals with higher breathing capacity (estimated as forced vital capacity and vital capacity) released higher amount of aroma during wine consumption compared to individuals with lower breathing capacity (Fig. 7.2) (Munoz-Gonzalez et al. 2014b). These results seem to support the idea that the higher the breathing flow, the higher the amount of aroma available to reach the olfactory system.

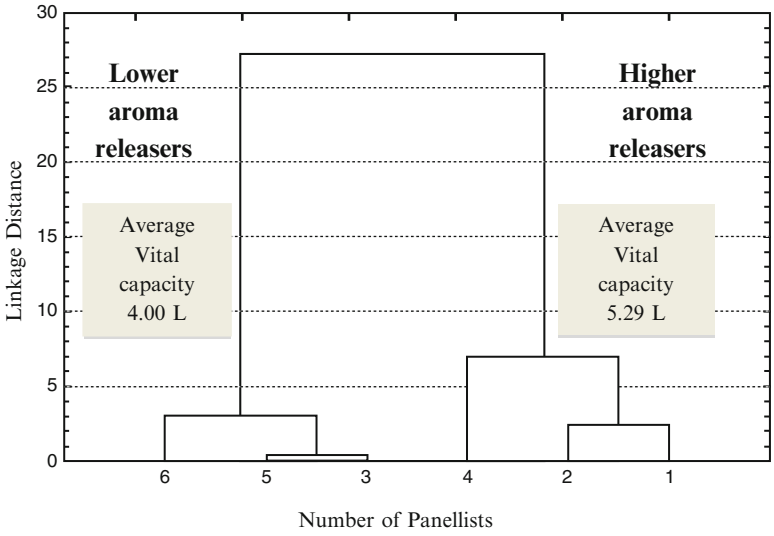

Fig. 7.2 Dendrogram showing the clustering of panelists in two groups: lower and higher aroma releasers obtained with the total aroma release data after the consumption of 100 mL of five types of wines using a retronasal aroma-trapping device. For more details see reference (Muñoz-González et al. 2014b)

7.5.2 Oral Temperature

The average body oral temperature is 37 °C, and in the mouth above 36.8 °C ± 0.4 °C is being considered as stable without much differences among individuals. However, oral temperature might vary during the consumption of different foods (e.g., ice cream vs. soup). This fact will affect the partition of volatiles between the gas and liquid phase. In fact, it has been shown that for many volatile compounds aroma release increases with an increase in food temperature (Linforth et al. 2002) which could be due to the higher mass transfer of volatiles into the gas phase as a result of an increase in the partition coefficients of the volatile compounds and a major matrix viscosity (Lubbers and Butler 2010).

From a sensory point of view, a possible sensorial consequence of the food/liquid heating within the mouth could be a higher aroma intensity because of the release of higher amount of a specific molecule, or even the detection of some compounds that occurred in the sample at concentration below their threshold (Delwiche 2004). However, some studies have proven that an increase in sample temperature (20, 40, 60 °C) influenced the orthonasal ratings of beef-type flavorings, but not retronasal ratings (Voirol and Daget 1986). Other studies on odor-temperature interactions in sweetened fruit beverages failed to find a temperature influence when aroma was presented retronasally (Cliff and Noble 1990; Noble et al. 1991). This apparent contradiction might be explained by the fact that once a liquid is placed in the mouth, it is rapidly brought to body temperature. Then, temperature differences in such stim-

uli would be rapidly nullified, making differences in odor intensity fleeting. Nonetheless, in the case of solid foods (such as beef steak), flavor ratings increase with temperature, suggesting that it could be due to the less rapid change in temperature of solids that would occur in the mouth (Delwiche 2004).

Although in the case of wine consumption the effect of wine temperature on aroma release has not been studied so far, in the work of Muñoz and co-workers using in vitro conditions simulating the dynamic conditions accounting for during drinking, an important effect of "in-mouth" temperature on wine aroma release was shown (Munoz-Gonzalez et al. 2014a). In this work authors used a saliva bioreactor cell that allowed the incorporation of a gas flow and saliva with a digital temperature control. Aroma release was monitored by means of HS-SPME at two sampling points. A first sampling corresponded to the introduction of the wine in the mouth (18 °C) in the sampling vessel with saliva. In this case, a first aroma extraction that accounted for 2 min, started at 25.5 °C and finished at 32.3 °C, was performed. These conditions might better represent the oral phase when the wine is introduced in the mouth and the temperature of the wine: saliva mixture is lower than physiological temperature. During the same experiment the sampling was also performed 10 min later when the temperature was already stable (36 °C), which better matched a postoral phase after swallowing which might be important to explain aroma release from the wine depots remaining in the mouth. With this experiment authors proved that a relatively small change in "in-mouth" temperature as a consequence of the introduction of the wine (cooler than the mouth) dramatically affected the release of most of the tested aromas (45 aroma compounds), increasing their release.

7.5.3 Saliva

Saliva is a complex dilute aqueous solution with different compositions depending on the respective physiological status, types of food consumed, oral hygiene, etc. (Neyraud et al. 2012). Saliva contains numerous inorganic salts (sodium, calcium, potassium, chloride, phosphate, and bicarbonate) and organic components such as enzymes (amylase, lipases, proteases, etc.) (Buettner 2002a; Buettner 2002b; Neyraud et al. 2012) and proteins (mucins, proline-rich proteins, histidine-rich proteins, etc.) (McRae and Kennedy 2011; Salles et al. 2011).

Previous studies have shown that saliva might exert an important role on aroma release through different physicochemical (dilution of aroma due to the aqueous phase of saliva, changes in the pH of the food, hydration of the food which favors aroma release, interaction with salts causing a salting out effect, interaction with proteins), chemical (degradation of odorants), biochemical (degradation of odorant or release from aroma precursors), or even physiological effects (impact on velum-tongue seal formation and swallowing performance), which form part of many previous works performed on this topic (Buettner 2002a, b; Friel and Taylor 2001; van Ruth and Buhr 2003; vanRuth et al. 1996).

In the case of wine, the effect of saliva has been mainly studied because of its involvement in wine astringency (Cala et al. 2012; de Freitas and Mateus 2012; Mateus et al. 2004; Rinaldi et al. 2012) among others. However, there are very few studies focused on the role of saliva on wine aroma release (Genovese et al. 2009; Mitropoulou et al. 2011). One could objectively think that the relatively short intraoral period of consumption of liquid foods seems to support the idea of a limited action of saliva on wine aroma release during wine consumption. However, the formation of an intraoral (and pharyngeal) aroma reservoir (Buettner et al. 2001) and the fact that natural swallowing of saliva is continuously performed make the idea that saliva might exert an important role in the perception of wine aroma during consumption perfectly viable. Indeed, it could be possible that saliva composition and flows could affect the persistence of aroma perception during the postoral phase of wine consumption. Very recently, using in vivo conditions, it was shown that enzymatic degradation of palm wine odorants due to saliva was not noticeable among pyrazines, pyrrolines, and most alcohols but was quite pronounced among aldehydes, esters, and thiols (Lasekan 2013).

Nonetheless, there are very few studies concerning the effect of saliva on wine aroma release and results are also contradictory. In the work of Genovese and collaborators, saliva induced, in general, a decrease on aroma release for most of the wine volatiles, and this effect seemed to be more important in white than in red wines (Genovese et al. 2009). On the contrary, Mitropoulou and co-workers observed an enhancement on the release of hydrophobic compounds from model wines and a decrease in the release of the most hydrophilic compounds in the presence of saliva, although this effect was dependent on the concentration of tannins and polysaccharides (Mitropoulou et al. 2011). Both works were, however, performed in very different conditions: by using dynamic conditions in the work by Genovese et al. (2009), and by using a static headspace approach in the work of Mitropoulou et al. (2011). The dynamic conditions are advisable to achieve more realistic conditions to that accounting for during food consumption; however, the static conditions have been shown to be better suited for the study of interacting effects that otherwise might be underestimated with the first approach (Fabre et al. 2002; Friel and Taylor 2001). More recently, Muñoz-Gonzalez and co-workers carried out a large systematic study in order to elucidate the influence of saliva on wine aroma release by using static and dynamic headspace SPME conditions (Munoz-Gonzalez et al. 2014a). Reconstituted wines (previously dearomatized and lyophilized) with different nonvolatile wine matrix composition (red and white) and a synthetic wine (without matrix effect) were used. All the wines were aromatized with a mixture of 45 volatiles representative of the wine aroma profile and adjusted to same ethanol level. In addition, two types of saliva (human and artificial) and control samples (with water) were used to better understand the different mechanisms that saliva might induce on the release of aroma compounds from wine. Results of this work showed that in static conditions most of the aroma compounds were equally affected by the type of saliva and matrix composition. The addition of saliva (artificial or human) provoked a significant decrease of aroma release for most of the tested compounds. However, the extent of this effect was not just depen-

dent on the wine type (red or white), but also on the aroma chemical class (higher retention and lower release for the most hydrophobic compounds). For instance, it was observed that red wines with human saliva showed the lowest values of aroma release. Authors suggested that the higher amount of polyphenols and neutral polysaccharides in red wines might favor the formation of complexes involving salivary proteins (e.g., PRPs) and wine polysaccharides, which could "encapsulate" hydrophobic compounds resulting in a reduction on aroma release (Mitropoulou et al. 2011). However, in dynamic conditions a minor effect of saliva compared to wine matrix composition was evidenced, which could be linked to the limitation of the dynamics conditions (displacement of the equilibrium), which might reduce the retention effect produced by proteins (Fabre et al. 2002) or by other wine matrix components (e.g., polyphenols, polysaccharides).

7.5.4 Oral Mucosa

As previously stated, during the consumption of liquid foods as a wine, the major part of aroma compounds reaches the olfactory receptors after swallowing like a pulse of aroma usually called "swallowing breath" (Buettner and Schieberle 2000). This is due to the formation of a thin layer of the liquid sample on the surface of the pharynx acting as an aroma reservoir ready to be released by the expiration flows. The existence of this liquid layer after liquid (or semisolid) food consumption has been visualized employing physioanalytical techniques such as videofluoroscopy (Buettner 2002a). However, additional aroma peaks could be perceived by further actions of saliva since a proportion of the aroma containing liquid remains in the mouth and pharynx as a film coating. This, indeed, provides insights that two modes of aroma release and perception following food and beverage intake can be distinguished: the immediate aroma impression when liquid food is just swallowed and the prolonged retronasal aroma perception after swallowing, often called after-odor (Buettner 2004). This type of aroma perception resulting in the long-lasting aroma perception of some odors following wine intake is a wine feature of special importance during wine tasting and it is an important characteristic to assess wine quality. In spite of that, the number of scientific works focused on the chemistry behind this phenomenon is largely scarce, and these studies have been carried out from a sensory point of view. For example Goodstein and co-workers performed a time-intensity study and they observed differences in the persistence of some aromatic notes in model white wine (Goodstein et al. 2014). They observed that fruity notes are less persistent than coconut, mushrooms, or floral notes, which is also in agreement with other recent works (Baker and Ross 2014a, b).

The chemistry behind the aroma persistence phenomena has been very little explored. In some of these works, the persistence of aroma compounds after the intake of two types of wines or from a palm wine was measured and these data were compared with the intraoral aroma release (Buettner 2004; Lasekan 2013). In these works authors showed differences on aroma release depending on the type of aroma

compound (physicochemical properties and on the type of studied wine). Recently, Muñoz-González and co-workers have suggested that wine red intake produced higher aroma release compared to white wine intake due to differences on the non-volatile wine matrix composition, and specifically on the amount of total polyphenols in the wines (Munoz-Gonzalez et al. 2014b). The explanation they gave is related to the formation of polyphenol-aroma complexes that might bond to oral (and throat) mucosa layer, which favors aroma release. Although this hypothesis needs to be confirmed, there are scientific evidence that supports the idea that saliva can increase the "stickiness" to oral surface of polyphenols and their prolonged retention in the oral cavity (Ginsburg et al. 2012). This could be explained by the interaction of these compounds with proteins forming part of the mucosal pellicle that covers the oral (and throat) surfaces. The mucosal pellicle is a protein-rich bacteria-free adsorbed film that assembles on all surfaces within the oral cavity and it is formed by the selective adsorption of salivary proteins derived from whole saliva (Ash et al. 2013). Ginsburg and co-workers showed that polyphenols in beverages held in the mouth for short period of times (30 s) might also be retained in the oral cavity for long periods despite a constant saliva flow (Ginsburg et al. 2012). Even if the existence of these mechanisms is the origin of this aroma release dynamics, the role of aroma compound properties and the molecular mechanisms involved need to be established.

7.5.5 Oral Microbiota

Oral microbiota is one of the most complex bacterial communities associated with the human body and it is formed by more than 700 bacterial species (Tian et al. 2010). The different microenvironments in the oral cavity (cheeks, palate, tongue, tooth surface, gingival areas, and saliva) have their own microbiota (Requena et al. 2010). Therefore, oral microbiota varies in composition on distinct surfaces (e.g., tooth, mucosa), and at sites on a specific surface (e.g., fissures, gingival crevice), which shows the adaptation capacity of these microorganisms. However, most of them belong to genera *Gemella, Granulicatella,* and *Streptococcus* y *Veillonella* (Aas et al. 2005). In addition other factors such as diet, age, and type of diet might affect the bacterial diversity.

These microorganisms can adhere to oral surfaces and form an organized multispecies community known as biofilms (Kuramitsu et al. 2007). The main sources of nutrients for oral microbiota include saliva, crevicular fluid, and host diet. Although saliva is the main nutrient source, due to its chemical composition and continuous production, food is rich in a wide variety of components which could be used by the microbiota to generate secondary products. Initial adhesion invariably involves the interaction of bacterial surfaces with the acquired pellicle derived from salivary constituents adsorbed onto the surfaces of the oral cavity, which serves as a substratum for the adhesion of the so-called early colonizers (*Streptococcus, Actinomyces, Veillonella* y *Neisseria*) (Aas et al. 2005). The anaer-

obic conditions produced by the early colonizers favor the adhesion of secondary colonizers such as *Fusobacterium spp*.

The metabolic impact of oral microbiota on typical wine aroma compounds such as polyphenols has been previously described (Kamonpatana et al. 2012; Walle et al. 2005). Even it has been suggested that in liquid and semisolid foods the role of oral microbiota could be even higher since the absence of a solid food matrix might facilitate the release of these compounds and the action of oral microbiota (Walle et al. 2005).

Besides polyphenolic compounds, Starkenmann and co-workers showed the ability of some oral anaerobic bacteria to hydrolyze odorless cysteine-S-conjugates from onion, bell pepper, and grapes into their corresponding odorant thiols (Starkenmann et al. 2008), which might be related to a delay in aroma perception, as was already observed by Peynaud and collaborators after the consumption of Golden Sauvignon grapes (Peynaud and Jacques 1996). More recently, in vivo degradation of phenolic volatile precursors has been found, which are associated to unpleasant aromatic nuances such as "toasted" and "burnt", in which they suggested that oral microbial could be involved (Mayr et al. 2014).

In addition to these studies, it has also been shown that oral microbiota can hydrolyze odorless glycosidic aroma precursors into odorant aglycones. For this study, Muñoz-González and co-workers followed two methodological approaches involving the use of representative oral bacteria (*Streptococcus sanguinis, S. oralis, S. mutans, Actinomyces naeslundii, Veillonella dispar, Fusobacterium. nucleatum, Staphylococcus aureus, Enterococcus faecalis*) or the whole oral microbiota isolated from human saliva. In the latter, fresh saliva was incubated in aerobic and anaerobic conditions in the presence of the grape glycosidic aroma precursor (Muñoz-González et al. 2015a). In addition, fresh saliva was submitted to different thermal treatments in order to obtain sterile (without microorganisms or enzymes) and nonenzymatic (without enzymes) saliva samples. Odorant aglycones released in the culture broths were isolated and analyzed by HS-SPME-GC/MS.

Results from this study showed the ability of all the oral bacteria tested to hydrolyze grape aroma releasing different types of aglycones (terpenes, benzenic derivatives, and C6-alcohols). This capacity was dependent on the type of bacteria, *A. naeslundii* being the highest aroma producer. In the second approach, using the total microbiota isolated from human saliva, two experiments were performed. In the first one, a pooled saliva sample was submitted to different growing conditions in the presence of the grape glycosidic extract, and linalool release was monitored. Interestingly, this compound was only detected in the saliva samples growing in anaerobic or aerobic conditions, but not in the sterile and nonenzymatic saliva. In a second experiment, the saliva from the three individuals was independently incubated in aerobic and anaerobic conditions. In this case, large interindividual differences that were not related to quantitative differences in oral microbiota were observed; thus authors suggested that they could be due to differences in oral bacteria composition.

Although the large incubation time employed in this study (till 48 h) is far from wine consumption conditions, this work has provided valuable information about the

capacity of oral microbiota to hydrolyze grape glycosides, which are important odorant compounds on the basis of their low odor threshold and in general pleasant aroma nuances. Another consideration that could be pointed out from the abovementioned study is the relatively short residence time of wine within the oral cavity, which might suggest a limited effect of oral microbiota on wine aroma perception. However, as previously stated, results from recent research suggest a possible interaction of some wine matrix nonvolatile compounds with oral and pharyngeal mucosa which might increase the residence time of aroma precursors and free aroma compounds in the oral/pharyngeal cavities, thus increasing their susceptibility to oral parameters (saliva, oral microbiota, etc.) (Munoz-Gonzalez et al. 2014b). Anyway, these types of works invite us to think in the role of oral microbiota on wine aroma generation and they pointed out the necessity of new studies in order to determine the meaning of this effect on retronasal aroma perception during wine consumption.

7.6 Conclusions

It is clear that aroma is a main actor when one tries to explain wine consumer preference. It is because of this that a great amount of work has been focused on the chemical characterization of wine aroma compounds and on trying to determine their sensory meaning. In spite of this, the correlation between sensory and analytical studies is far to be understood. This will require complementary human-centered approaches, in order to monitor what happens with wine aroma compounds once they interact with the human body during consumption. The incorporation of oral physiology factors in this scenario is necessary to understand the aroma transformation of the original wine aroma composition ("wine in the glass") into the "active" aroma delivered to the olfactory receptors. This task will require a multidisciplinary approach combining analytical, physio-analytical, and sensory techniques and new methodologies using in vivo experiments or the development of in vitro representative physiological setups. Therefore, in the following years we will attend to this new scenario, in which human physiology will be taken into consideration to better understand wine aroma perception and consumer preferences and wine choices.

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Chapter 8
Dealcoholised Wines and Low-Alcohol Wines

Fernando Zamora

8.1 Introduction

In recent years, wines have gradually increased in alcohol content (Godden and Muhlack 2010), probably because winemakers are looking for grapes with a high phenolic and/or aromatic maturity (Kontoudakis et al. 2010, 2011a). Moreover, climate change is generally considered to be increasing this tendency (Jones et al. 2005; Mira de Orduña 2010). If the temperature during ripening is higher than the optimum, the grape pulp matures faster, and the pH and sugar concentration become too high. The period between veraison and industrial maturity therefore decreases, bringing the date of the harvest forward. This makes it more difficult to pinpoint proper aromatic and phenolic maturity, and leads to unbalanced wines (Zamora 2014).

In this situation, there are only two possibilities. Grapes can be harvested when the potential alcohol value is appropriate or when complete maturity has been reached. In the former case, it must be assumed that complete phenolic and/or aromatic maturity has not been reached (Gil et al. 2013b). In the latter case, the ethanol content of the grapes would probably be excessive. Neither of these situations is conducive to obtaining high-quality wines, and winemakers are obviously concerned by this problem. Since current trends in the wine market are oriented towards the production of very ripe wines, winemakers tend to wait for full maturity, which generally leads to very high alcoholic content. This phenomenon is even more common in the case of red wines, because deep-coloured and full-bodied wines are usually preferred by consumers.

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The Australian Wine Research Institute (AWRI) reported an increase in the mean from 12.4 to 14.4 % for red wines and from 12.2 to 13.2 % for white wines between 1984 and 2008 (Godden and Muhlack 2010). In another example, the alcohol level of Alsace wines increased from 9 to 12 % between 1970 and 2005 (Duchêne and Schneider 2005). This trend has also been observed in many other wine-producing countries (Schultz and Jones 2010). These data are clearly worrying, and affect wine production and the market. In fact, an excess of ethanol content creates several drawbacks associated with the wine's sensory perception, its commercialization and its winemaking process that are listed below.

Drawbacks of high ethanol content associated with the wine's sensory perception:

- Increase in the perception of warmth or hotness (Wilkinson and Jiranek 2013).
- Increase in the perception of astringency, bitterness and sourness (Martin and Pangborn 1970; Fischer and Noble 2004).
- Some wine aroma and flavour attributes are masked by the excess of alcohol (Le Berre et al. 2007; Robinson et al. 2009).
- In general, unbalanced wines, especially when the service temperature is high (Guth and Sies 2001; Gil et al. 2013b).

Drawbacks of high ethanol content associated with wine commercialisation:

- Excess alcohol consumption has negative effects on human health (Grønbæk 2009; Evans 2013).
- The presence of a high alcohol content on the label often discourages some potential consumers who prefer drinking light and responsibly (Meillon et al. 2010b; Saliba et al. 2013).
- Some countries apply higher taxes when the wines have a high alcohol level (de Barros-Lopes et al. 2003; Contreras et al. 2014).

Drawbacks of high ethanol content associated with the winemaking process:

- The complete consumption of sugar by yeast during alcoholic fermentation is sometimes complicated due to excessive alcohol content (Bisson 1999; Bisson and Butzke 2000).
- The subsequent development of malolactic fermentation is also difficult due to excessive alcohol content (Lonvaud-Funel et al. 1988; Capucho and San Romao 1994).
- When there are difficulties with the alcoholic fermentation, the wines often reach excessive volatile acidity (Zamora 2009; Costantini et al. 2009).
- The application of techniques to reduce ethanol content is time consuming and costly and may affect the quality of the wine (Gil et al. 2013b; Meillon et al. 2013).

The modern wine industry is very concerned with all these issues, and especially with health prevention policies, and is therefore interested in wines with a moderate alcohol level.

The minimum ethanol content of wine is usually regulated by law, but this minimum value can vary depending on the country. The International Organisation of Vine and Wine (OIV) stipulates a unique minimum actual alcoholic strength of 8.5 % vol, with the flexibility to be reduced 7.0 % vol (Ruf 2013). Beverages with a lower alcoholic strength cannot be considered as wine, even if they are strictly and exclusively made from grapes. However, there is a waiver allowing 4.5 % vol for certain geographical indications in the European Union, and recently Australia has reduced the limit from 8.0 % vol to 4.5 % vol. In Argentina and China, the limit is 7.0 % vol, whereas some other countries have not specified any minimum value.

Due to the market's interest in low-alcohol beverages of vitivinicultural origin, the 10th General Assembly of the OIV, which met on 22 June 2012 in Izmir (Turkey), authorised some separation techniques that can be used either to dealcoholise wines or to correct the alcohol content of wines (Resolutions OIV-OENO 394A-2012 and OIV-OENO 394B-2012). These resolutions make a clear distinction between a correction of the wine's alcohol content and its dealcoholisation. Specifically, the correction of a wine's alcohol is limited to 20 % of the initial alcohol content, in order to reduce an excessive level of ethanol to improve its taste balance. Beverages obtained by this means are included in the category of "wine" as long as their alcoholic strength exceeds the established minimum. Otherwise, if the alcohol content is reduced by more than 20 %, it cannot be included in the "wine" category.

For this reason, OIV has also adopted two resolutions (OIV-ECO 432-2012 and OIV-ECO 433-2012), including two new product definitions:

- "Beverage obtained by dealcoholisation of wine" for products with an alcohol content below 0.5 % vol (generally alcohol-free wines).
- "Beverage obtained by partial dealcoholisation of wine" for products with an alcohol content between the required minimum for wines and 0.5 % vol (generally low-alcohol wines).

The OIV is currently working on new resolutions to define products which do not fall into any of the three defined categories. Specifically, these would cover beverages with an alcohol reduction greater than 20 % but which still comply with the minimum alcohol level established for wines (Ruf 2013).

There is clearly great interest in curbing the excess alcoholic strength of some wines, and also in producing low-alcohol wines and/or alcohol-free wines that suit market requirements, while maintaining their sensory quality in all cases.

A wide variety of possibilities for controlling or reducing alcohol content in wines have been proposed (Schmidtke et al. 2012; Saha et al. 2013; Gil et al. 2013a, b; Zamora 2014), using a wide range of strategies that are described below. Some of these strategies are aimed at reducing sugar content in grapes or in grape juice, while others are aimed at directly reducing alcohol in wines.

8.2 Selection of Varieties and Clones That Ripen Later

The ability of the different *Vitis vinifera* varieties to accumulate sugars varies greatly depending on the variety and even the clone (Duchêne et al. 2012). The rootstock also exerts an influence on the accumulation of sugars (Harbertson and Keller 2012). Over the last 100 years, the selection of nurseries for *Vitis vinifera* has aimed at obtaining varieties, clones and rootstocks that ripen faster to obtain wines with high alcohol content. The reason was simply that the wine was priced according to its alcohol content. However, the problem nowadays is exactly the opposite, and it is necessary to restart a new selection in search of varieties, clones and rootstocks better adapted to the new climate conditions. This solution is probably the best, but it requires long and laborious studies and a considerable financial investment in order to replant most of the vineyards. This is one of the new challenges for modern viticulture (Jakab et al. 2013).

8.3 Adaption of Viticultural Practices to the New Climate Conditions

The development of viticultural practices has also been historically aimed at encouraging the accumulation of sugars in the berries (Jackson and Lombard 1993). However, current trends in wine production and the problems caused by climate change mentioned above have led to many common practices being completely reconsidered (Novello and de Palma 2013) in order to obtain grapes with an adequate phenolic and/or aromatic maturity, but without an excessive sugar content (Clingeleffer 2007).

To that end, various strategies have been suggested: increasing the crop load (Kliewer and Dokoozlian 2005), shading bunches (Chorti et al. 2010), choosing proper irrigation techniques (Clingeleffer 2007; Fernández et al. 2013), modulating source-sink relationships by removing leaves (Palliotti et al. 2013) or topping shoots (Stoll et al. 2010) and applying anti-transpirant resins to leaves (Tittmann et al. 2013) or plant growth regulators to grapes (Han and Lee 2004). All these practices have proven to be promising, although the results have not always been conclusive (Novello and de Palma 2013) probably because of the high degree of heterogeneity inherent in different soils, climates, varieties, rootstocks, etc. Further studies are needed in this regard to clarify many of the actual discrepancies. Nevertheless, this approach is today probably the most easily acceptable by traditional winemakers and consumers.

8.4 Selection of Yeasts with Lower Sugar/Ethanol Transformation Ratio

Alcoholic fermentation is the anaerobic transformation of sugars—mainly glucose and fructose—into ethanol and carbon dioxide. This process, which is carried out by yeast, can be synthesised in this overall reaction:

$$\underset{\text{Hexoses}}{C_6H_{12}O_6} \longrightarrow 2\underset{\text{Ethanol}}{CH_3CH_2OH} + 2\underset{\text{Carbondioxide}}{CO_2}$$

According to the stoichiometry of the process, 15.45 g/L of sugars are theoretically required to obtain 1 % vol alcohol. However, at the same time as this overall reaction many other biochemical processes are taking place, making it possible to turn the grape juice into wine. Besides ethanol, several other compounds are produced throughout alcoholic fermentation, such as higher alcohols, esters, glycerol, succinic acid, diacetyl, acetoin and 2,3-butanediol (Zamora 2009). Without the production of these other substances, wine would have little organoleptic interest. Moreover, some of the sugars present in the grape juice are used by yeasts to increase their biomass.

Furthermore, under real winery conditions alcoholic fermentation is not exclusively carried out by a monoculture of one yeast strain. At the start of the winemaking process, several species of yeasts may be present in the grape juice, even when sulphur dioxide is present and selected yeasts are inoculated (Constantí et al. 1998; Beltran et al. 2002). Various non-*Saccharomyces* yeasts are usually present during the early stages of alcoholic fermentation. Afterwards, *Saccharomyces cerevisiae* is the predominant yeast during the latter stages of fermentation, because of its greater resistance to high ethanol concentration (Fleet and Heard 1993). Nowadays, most wineries inoculate selected dry yeasts and this greatly increases the initial population of *S. cerevisiae*. The use of sulphur dioxide also favours its imposition. However, yeasts other than *S. cerevisiae* and even some bacteria are clearly able to grow in grape juice and metabolise sugars, creating products other than ethanol. All these factors mean that the real sugar/ethanol transformation ratio by yeast is significantly higher than the theoretical stoichiometric value.

Another factor to take into account is that ethanol is a volatile compound and consequently it evaporates to a greater or lesser extent depending on the rate carbon dioxide is released, the temperature and the dimensions of the tank. Moreover, some winemaking operations such as pumping over, racking off and "délestage" can also favour ethanol evaporation.

It is consequently difficult to determine what the real sugar/ethanol transformation ratio is during alcoholic fermentation. For this reason, the term "potential" or "probable" alcohol content is usually used to predict the ethanol content of a wine from the sugar content of the grape juice. OIV considers an average transformation ratio of 16.83 g/L. However, winemakers usually use a transformation ratio of 16.00 g/L for white wines and 17.00 g/L for red wines. The difference is mainly because white wines are generally fermented at low temperatures and without aera-

tion in order to conserve all the flavours and prevent oxidation. In contrast, red wines are fermented at high temperatures and with some operations involving aeration such as pumping over, to favour the colour and polyphenol extraction from skins and seeds.

From the above, it follows that a possible means of reducing the alcohol content of wine would be to decrease the sugar/ethanol transformation ratio of yeasts. However, this is not easy because natural strains of *S. cerevisiae* have a similar ethanol yield (Michnick et al. 1997; Malherbe et al. 2003). For this reason, this strategy involves redirecting a great amount of sugars from grape juice towards by-products other than ethanol. Many researchers have been working in this field for more than 20 years, trying to find new carbon sinks in *S. cerevisiae* (Bauer et al. 2013; Tilloy et al. 2013). The subject is complex because the metabolic network is strongly interconnected, and a modification of central carbon metabolism often leads to an accumulation of metabolites that may have a detrimental effect on the wine's quality such as acetic acid (Quirós et al. 2014).

Various approaches have been developed: genetic engineering (Heux et al. 2006; Kutyna et al. 2010), the use of non-*Saccharomyces* yeasts (Contreras et al. 2014; Quirós et al. 2014) and more recently adaptive evolution-based strategies (Cadière et al. 2012; Tilloy et al. 2014).

Genetic engineering has been successfully used, and in fact there are already genetically modified yeasts that can produce wines with less alcohol and more glycerol and 2,3-butanediol (Ehsani et al. 2009). However, the use of GMO is usually viewed negatively by consumers, which is a major obstacle to the commercial use of these yeasts (Bauer et al. 2004; Fleet 2008).

The use of non-*Saccharomyces* yeasts is a current hot topic (Andorrà et al. 2012; Bely et al. 2013; Jolly et al. 2014). In recent years, the use of non-conventional yeasts such as *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Pichia kluyveri* for the production of quality wine has been increasingly frequent (Ciani and Maccarelli 1997; Jolly et al. 2006; Bely et al. 2008; Anfang et al. 2009). Indeed, the catalogues of most yeast manufacturers for wine contain an increasing presence of non-*Saccharomyces* yeasts as active dry yeast. The aim is to reproduce the advantages of spontaneous fermentation without any of its drawbacks and risks. Non-*Saccharomyces* yeasts have been reported as useful in improving some organoleptic characteristics of wine, such as flavour (Azzolini et al. 2012), texture (Giovani et al. 2012) and even the foaming properties of sparkling wines (González-Royo et al. 2014). The possible use of non-*Saccharomyces* yeasts to reduce the alcohol content has recently been proposed (Contreras et al. 2014; Quirós et al. 2014). The results are promising, and open up new possibilities for reducing the alcohol content of wine in the near future.

Finally, an alternative to genetic engineering is adaptive evolution-based strategies (Kutyna et al. 2012). In this case, yeasts are continuously and repeatedly cultured under a defined combination of conditions, from which strains that have specifically adapted to these conditions can be isolated (McBryde et al. 2006). Using a hyperosmotic medium, Tilloy et al. (2014) have generated a *S. cerevisiae*

strain yeast that produces appreciably less alcohol and more glycerol than natural *S. cerevisiae* yeasts.

Further studies are clearly required, but it is also quite probable that in the coming years, new strains of *S. cerevisiae* or non-*Saccharomyces* yeasts, used either alone or in combination, will be a new tool for reducing the alcohol content of wine in order to fine-tune the balance of some wines that usually reach very high alcohol levels.

8.5 Membrane-Based Technologies

Procedures for reducing alcohol based on membrane technology are currently probably the most used by the wine industry because they are easily applicable and affordable from the financial point of view (Saha et al. 2013). All these methods are based on passing the wine or the grape juice through a semipermeable membrane that fractionates the liquid into a permeate and a retentate. Most of these methods are useful for eliminating the ethanol from wine and in some cases for eliminating sugars from the grape juice. These methods can be classified into different categories, depending on the type of driving force and the porous size of the membrane:

8.5.1 Reverse Osmosis

This technique, which was the first membrane-based technology used commercially to remove ethanol from beverages (Meier 1992), consists of applying a high pressure through a semipermeable membrane with a pore size of around 0.1–1 nm, which retains molecules larger than a certain size (Catarino et al. 2007). The permeate will thereby contain only small molecules, such as water, ethanol and some mineral salts, whereas the retentate will retain all the other components of the wine (aromas, phenolic compounds, polysaccharides, etc.). Since water has a lower molecular weight than ethanol, it permeates faster and the permeate therefore has a lower ethanol content than the original wine, at around 0.7–1.5 % vol (Massot et al. 2008; Saha et al. 2013). The retentate consequently increases the concentration in all the components, even in alcohol. To compensate for this effect and achieve the desired level of ethanol reduction, the retentate must be restored, with the original water removed during the process. To do this, it is necessary to separate the water from alcohol, and this process is usually performed by thermal distillation, by means of a membrane contactor or by pervaporative extraction. This process provides a high-ethanol by-product and the original water which is added to the retentate. Modern equipment works in a cross-flow mode, so the wine flows tangentially to the membrane's surface and a portion of it passes selectively through the membrane. Since the permeate has a low ethanol concentration, it is necessary to recirculate the

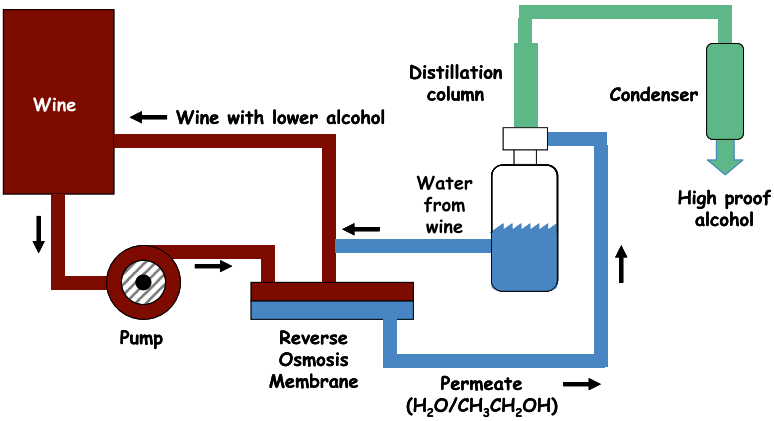

Fig. 8.1 Partial dealcoholisation of wine by reverse osmosis

wine several times to achieve the desired ethanol reduction. Figure 8.1 illustrates the reverse osmosis.

Today, reverse osmosis is probably the most widely used procedure to partially reduce ethanol in wines and other alcoholic beverages without appreciably affecting the composition of their other components and its organoleptic quality (Catarino et al. 2007; Labanda et al. 2009). This technique is now a reality, and some companies even rent this equipment to wineries (Zamora 2014). Table 8.1 shows an experimental assay of partial dealcoholisation by reverse osmosis with two red wines from the AOC Penedès and AOC Priorat (Gil et al. 2013a).

The results showed that the only significant differences were in alcohol content, while the other analytical parameters remained unchanged. These wines were also tasted by a trained sensory panel using the discrimination triangular test. In general, the tasters were able to distinguish between the control and the partially dealcoholised wines, but all the tasters confessed that it was quite much harder than they thought at the beginning of the test. It therefore appears that reverse osmosis may be a useful procedure for compensating for excess ethanol content in red wines, since it barely alters their composition and sensory characteristics. Moreover, the cost of the process can be considered affordable, since the equipment manufacturer provides the service at a price of 0.15 €/L for removing 1 % of ethanol. Reverse osmosis is therefore an interesting tool for improving the balance of wines from regions where grapes can easily reach a high alcohol content. This is particularly important nowadays, because climate change is increasingly creating a mismatch between the pulp and phenolic maturity of grapes.

However, this technique can hardly ever be applied to achieve non-alcoholic wines or wines with very low alcohol content, because the process loses efficiency as the alcohol content of the retentate falls (Pilipovik and Riverol 2005; Saha et al. 2013).

Reverse osmosis can also be used to remove sugar from grape juice, although nanofiltration is probably more widely used for that purpose.

Table 8.1 Partial dealcoholisation by reverse osmosis

	AOC Penedès			AOC Priorat		
Parameter	Control	−1 %	−2 %	Control	−1 %	−2 %
Ethanol content (%)	14.8 ± 0.2 A	13.8 ± 0.2 B	12.8 ± 0.2 C	16.2 ± 0.2 A	15.1 ± 0.2 B	14.1 ± 0.1 C
Titratable acidity (g/l)	4.8 ± 0.1 A	4.8 ± 0.1 A	4.9 ± 0.1 A	5.2 ± 0.1 A	5.2 ± 0.1 A	5.6 ± 0.1 B
Colour intensity	15.3 ± 1.5 A	15.6 ± 0.9 A	15.4 ± 0.7 A	15.4 ± 0.2 A	15.4 ± 0.4 A	14.5 ± 0.5 A
Hue	67.7 ± 1.1 A	67.9 ± 0.4 A	68.3 ± 1.5 A	59.3 ± 1.2 A	60.0 ± 0.4 A	59.2 ± 0.5 A
Anthocyanins (mg/l)	567 ± 41 A	546 ± 19 A	574 ± 14 A	200 ± 13 A	206 ± 23 A	226 ± 11 A
TPI	72.9 ± 2.5 A	73.9 ± 2.3 A	75.8 ± 20.6 A	62.4 ± 0.5 A	62.2 ± 0.2 A	62.1 ± 0.8 A
Proantho cyanidins (g/l)	1.8 ± 0.3 A	1.6 ± 0.2 A	1.7 ± 0.2 A	1.6 ± 0.2 A	1.7 ± 0.3 A	1.5 ± 0.2 A
mDP	6.8 ± 1.2 A	7.5 ± 1.8 A	7.2 ± 0.6 A	6.8 ± 1.8 A	5.8 ± 0.3 A	6.5 ± 0.7 A

All data are expressed as the average of three replicates ± standard deviation. Statistical analysis: one-factor ANOVA and Scheffe's test (both $p < 0.05$). Different letters indicate the existence of statistically significant differences

TPI total phenolic index, *mDP* mean degree of polymerisation of proanthocyanidins

8.5.2 Nanofiltration

This technique mainly differs from reverse osmosis in that the membrane pore size is higher (around 0.5–5 nm). While reverse osmosis can remove the smallest solute molecules, in the range of ≤0.0001 µm in diameter, nanofiltration removes molecules in the 0.001 µm range (Ferrarini et al. 2001). Nanofiltration can be used to remove alcohol from wine as an alternative to reverse osmosis, and enables a more extensive removal of alcohol in order to obtain alcohol-free wines (Gonçalves et al. 2013).

This technique can also be employed to enrich the must in sugars when the grapes have not ripened correctly (Pati et al. 2014) or to remove excess sugar when the opposite occurs (Bes et al. 2010). Figure 8.2 illustrates how nanofiltration can be used to reduce the potential alcohol content of grape juice.

This technique requires an initial ultrafiltration stage to obtain the required limpidity. The first retentate obtained by cross-flow ultrafiltration is redirected with the original must, whereas the permeate is directed to the nanofiltration equipment. Afterwards, nanofiltration provides a new retentate which contains nearly all the sugars, and a permeate containing the grape juice water with some of their acids. This technique therefore allows the sugar content of must (and alcohol in wine) to be increased or reduced as needed: by adding the grape water if it is necessary to reduce the sugar content, or adding the sugar concentrate in the opposite case.

However, using nanofiltration or reverse osmosis for reducing sugars in grape juice has a major financial drawback. 7 % of the total volume of grape juice is lost as sugar concentrate for every 1 % vol of dealcoholisation.

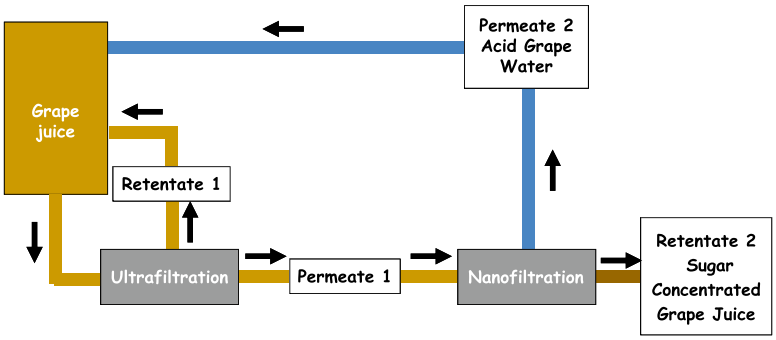

Fig. 8.2 Removal of sugar from grape juice by nanofiltration

8.5.3 Pervaporation

Pervaporation is a method for separating mixtures of liquids by partial vaporisation through a membrane. Pervaporation is commonly used to remove organics from aqueous streams, for the dehydration of organic solvents and to separate heat-sensitive products (Brüschke 1990). For this reason, it can be used for the partial or even total dealcoholisation of wines (Karlsson and Trägårdh 1996; Takács et al. 2007).

Pervaporation involves the separation of two or more components through a membrane by differing rates of diffusion through a thin polymer, and an evaporative phase change comparable to a simple flash step. A concentrate and vapour pressure gradient is used to enable one component to preferentially permeate through the membrane. A vacuum applied to the permeate side is coupled with the immediate condensation of the permeated vapours. Pervaporation is typically suited to separating a minor component of a liquid mixture, meaning that high selectivity through the membrane is essential.

Pervaporation involves three major steps (Karlsson and Trägårdh 1996):

1. Sorption of the permeate at the interface of the solution feed and the membrane
2. Diffusion across the membrane due to concentration gradients
3. Desorption into a vapour phase on the permeate side of the membrane

Figure 8.3 shows a schematic diagram of the pervaporation process.

Pervaporation can be performed at low or ambient temperatures, although most alcohol removal procedures have been conducted at temperatures of or exceeding 30 °C (Tan et al., 2005; Takács et al. 2007). Unfortunately, aroma compounds are also volatile substances and consequently significant aroma losses may occur when pervaporation is applied to wine (Karlsson et al. 1995; Saha et al. 2013), especially if the operating temperature is high (Catarino et al. 2009).

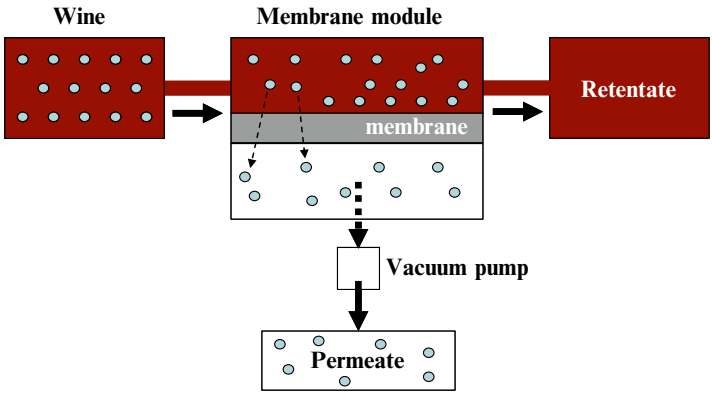

Fig. 8.3 Pervaporation process

8.5.4 Osmotic Distillation

Osmotic distillation is a separation process in which a liquid mixture containing volatile substances is contacted with a microporous hydrophobic membrane. This hydrophobic membrane, also named contactor, is non-wettable and water consequently cannot pass through it. The pores of the membrane are filled with air and it is through these air-filled pores where volatile substances can migrate to the opposite side of the membrane, where they condense (Varavuth et al. 2009; Stassi et al. 2013).

The hydrophobic membranes, used in the removal of ethanol from wine, are made of polypropylene or polyvinylidenefluoride, and the stripping fluid is degassed water (Diban et al. 2008). Figure 8.4 shows a schematic diagram of direct osmotic distillation.

Osmotic distillation can be performed at ambient temperatures with no need for high pressure, which is a major advantage because its financial cost is relatively low and the thermal degradation of the aroma is limited. However, some wine aromas can migrate through the membrane contactor, leading to a non-negligible loss of aromas (Diban et al. 2008, 2013), especially of ethyl esters that are substantially reduced (Fedrizzi et al. 2014).

8.6 Vacuum Distillation Procedures

Distillation has probably been the most common method for removing alcohol from wine. All dealcoholisation procedures were initially carried out by atmospheric distillation. However, these techniques require the wine to be heated to high temperatures, which seriously affects its quality. For this reason, atmospheric distillation was replaced by vacuum distillation which enables the alcohol to be removed at much lower temperatures (Pickering 2000) and consequently affects the wine

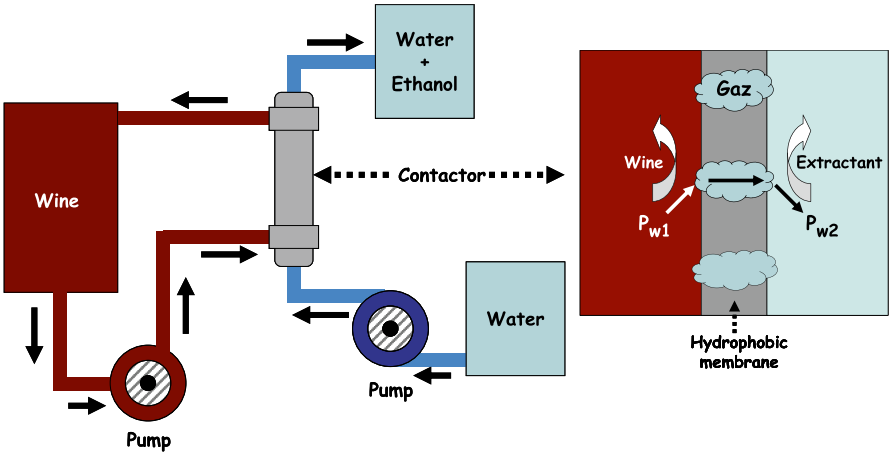

Fig. 8.4 Osmotic distillation

quality to a lesser extent. There have been numerous variations on and modifications of the distillation and evaporation principle, most of which have been patented. Nevertheless, since its inception the spinning cone column has displaced the other procedures, so that it nowadays practically monopolises the market for wine dealcoholisation by evaporation (Wright and Pyle 1996; Prince et al. 1997).

The spinning cone column is a multi-stage strip column which was initially developed in the USA in the 1930s and subsequently modified in Australia (Gray 1993; Pickering 2000). The spinning cone column consists of a vertical column with a countercurrent flow system that contains a succession of alternate rotating and stationary metal cones. The liquid flows down the upper surfaces of the stationary cones under the influence of gravity, and moves up the upper surfaces of the rotating cones in a thin film by the action of the applied centrifugal force. This process increases the surface of the liquid a great deal, which favours the evaporation of its volatile components. The spinning cone column operates in a vacuum, so the volatile aroma components are transferred to the gas phase in a relatively high vacuum and at low temperatures (Saha et al. 2013). Figure 8.5 shows the operating diagram of the spinning cone column and Fig. 8.6 shows a detailed view of the gas-liquid interchanges that take place in the spinning cones of the column.

The spinning cone column can be used for partial dealcoholisation and to obtain alcohol-free wines. It is possible to reduce the ethanol concentration from 15 % v/v to less than 1 % vol. The spinning cone column also includes a system for recovering the volatile aroma that evaporates during the dealcoholisation process. Dealcoholisation is usually carried out in two steps. The first one is performed in a high vacuum (around 0.04 atm) and at a low temperature (around 26–28 °C) in order to recover the more volatile aroma. The second step takes place at a higher temperature (around 38 °C) in order to remove ethanol from the wine (Belisario-Sánchez et al. 2009).

The entire volume of the wine must be treated in order to obtain alcohol-free wines. However it is only necessary to treat a portion of the wine to partially deal-

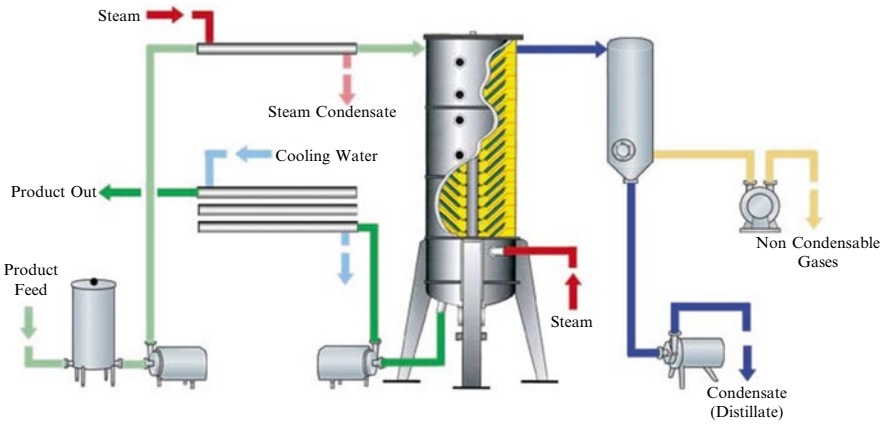

Fig. 8.5 Operating diagram of the spinning cone column

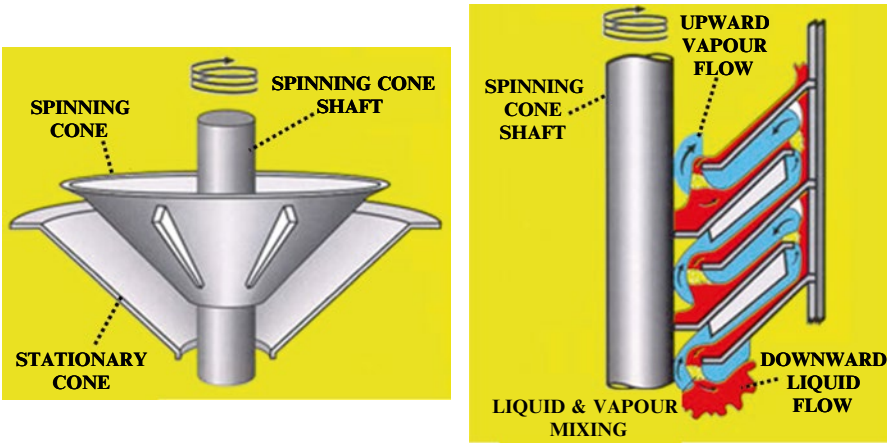

Fig. 8.6 Detail of the spinning cone column

coholise it. The final adjustment of the ethanol content is made by blending the treated and non-treated wine with the recovered aroma (Pyle 1994).

8.7 Other Procedures

The most obvious solution is to harvest grapes at an early stage of ripening. However, this is not a good solution because the grapes may not have reached an adequate phenolic and aromatic maturity, which would produce bitter and herbaceous wines (Canals et al. 2008; Zamora 2014).

Another possibility is to add water to the grape juice before fermentation begins. This reduces the sugar concentration, but has a generally negative effect on the wine's quality because it dilutes all the other compounds, and although this practice is authorised in some countries, it is strictly forbidden in others. It is also possible to reduce this problem by blending high-alcohol wines with low-alcohol wines. However, a sufficient volume of low-alcohol wine which does not have the disadvantages described above is required.

Glucose oxidase (EC 1.1.3.4) has also been proposed as a way of obtaining less alcoholic wines (Pickering et al. 1998). However, it has two drawbacks. Glucose is oxidised by generating a very high concentration of gluconic acid (Schmidtke et al. 2012). Moreover, it uses oxygen as a substrate, meaning that the grape juice needs to be aerated, which may oxidise other wine components (Pickering et al. 1999). Both aspects affect seriously the organoleptic quality of the wine, and consequently this procedure is a dead end.

Supercritical liquid extraction has also been proposed as a way of reducing alcohol content in wines (Ruiz-Rodriguez et al. 2010). However, this procedure requires considerable energy inputs and involves highly specialised plant and equipment, making it unprofitable from an economic point of view (Pickering 2000).

Another technique proposed for partial wine dealcoholisation is stripping (Pickering 2000). The principle behind stripping is to submit the wine to a strong bubbling with carbon dioxide or other gases to encourage the evaporation of ethanol. However this procedure also removes other volatile compounds, affecting the aromatic quality of the wine (Aguera et al. 2010). Aroma losses can be minimised by applying this technique in the middle of alcoholic fermentation (around 6 % vol), when not all aroma compounds have been synthesised.

An additional thermal method is freeze concentration. Water in wine can be removed by freezing and the alcohol in the residual liquid can be removed by vacuum distillation. This process is relatively delicate and expensive (Pickering 2000).

Another possible strategy is to use unripe grapes harvested during cluster thinning as a method for reducing alcohol content (Kontoudakis et al. 2011b). Grapes from cluster thinning can be used to produce a very acidic and low-alcohol wine that must be treated with high doses of charcoal and bentonite to avoid problems caused by the grapes' lack of maturity, such as bitterness and herbaceous odours. This low-alcohol wine can be blended with grape juice with a high sugar concentration during alcoholic fermentation in order to decrease its final alcohol content. This procedure has also the advantage of decreasing wine pH. The results obtained in red wines are promising, and confirm that this procedure may be useful for partial reduction of their alcohol content. The colour of the reduced-alcohol wines was better than their controls, and their phenolic composition was similar. Moreover, this procedure does not require additional equipment and is easy to apply in standard wineries. Further experimentation is needed to better adapt the process in order to obtain more balanced wines without any problems of excess alcohol and high pH.

8.8 Conclusions

There is clearly a market demand for a reduction in the excessive alcohol content of some wines, especially red wines. Many of the procedures described here may be useful for accomplishing this without appreciably affecting their sensory quality. These techniques are gradually being introduced in wineries, and the presence in the market of wines with an adjusted alcohol content is increasing.

Another question is the future of low-alcohol wines and alcohol-free wines. Classic wine consumers believe that dealcoholised wines are organoleptically poorer, which is a significant commercial barrier (d'Hauteville 1994; Meillon et al. 2010a; Stasi et al. 2014). Although dealcoholisation procedures have greatly improved, and they increasingly preserve their aromatic composition, low-alcohol wines undergo changes in mouthfeel and in aroma perception. The reason for this is very simple. Ethanol is a major contributor to the sensations of texture and aroma, and its absence in the case of alcohol-free wine or its low concentration in low-alcohol wines completely alters the sensory balance. For this reason, ethanol-reduced wines are usually blended with grape juice to enhance the aromatic intensity and improve the mouthfeel.

Another consideration is that ethanol is an antimicrobial agent and its removal from wine increases the risk of microbial growth a lot. And this risk is even higher if some sugar has been added to balance the lack of alcohol. For that reason low-alcohol wines and especially alcohol-free wines need to be produced, conserved and packaged in highly controlled conditions. Otherwise, the development not only of spoilage microorganisms but also of pathogenic germs, which cannot grow in conventional wines, could occur.

Despite all these disadvantages, several commercial low-alcohol and alcohol-free wine have been marketed with success. Undoubtedly there are many potential consumers for this kind of products that are probably very different than traditional wine lovers. The challenge for wineries is therefore not only knowing how to make this kind of wines but knowing how to find this new niche market as well. Only in this way wineries will achieve success in diversifying its production.

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Chapter 9
Sustainability and Organic Wine Production

Monica Laureati and Ella Pagliarini

9.1 Introduction

In recent years, consumers have become increasingly concerned about the effects of conventional agricultural production practices on both human and environmental health. Many agribusinesses have responded to these concerns either to retain or to attract customers and to differentiate their products in crowded marketplaces (Forbes et al. 2009). Consequently, produce obtained from organic farming methods has been rapidly increasing in developed countries. Indeed, although the economic crisis has recently had a major impact on food trade, a reverse trend has been observed in the market for organically grown products (Eurostat 2013). This may be explained by the fact that organic food adequately meets all requirements for quality, authenticity, and healthiness (Forbes et al. 2009). Organic farming is a method of production that puts the highest emphasis on environmental protection, and, with regard to livestock production, animal welfare considerations. It avoids or largely reduces the use of synthetic chemical inputs such as fertilizers, pesticides, additives, and medical products. Farming is considered to be organic at the European Union (EU) level only if it complies with Council Regulation (EC) No. 834/2007, which set up a comprehensive framework for the organic production of crops and livestock and for the labeling, processing, and marketing of organic products while also governing the import of organic products into the EU. The detailed rules for the implementation of this Regulation are laid out in the Commission Regulation (EC) No. 889/2008.

Currently, viticulture is also experiencing a gradual shift to more sustainable production patterns. Many producers have initiated or have already accomplished the conversion to field operations that improve the environmental profile of wine production. Hence, many vineyards have initiated the application of both organic

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and biodynamic viticulture as novel and attractive agricultural techniques (Villanueva-Rey et al. 2014). Organic viticulture is characterized by the avoidance of mineral fertilizers and synthetic plant protection substances. Organic products, including those related to viticulture and the wine production sector, are regulated by the Member States in the EU based on a common legislation, i.e., the two previously mentioned European Commission Regulations and the recent Commission Regulation (EC) No. 203/2012 that is specific for organic wine (see Sect. 9.3). Biodynamic agriculture was developed in the 1920s based on a set of conferences by the philosopher Rudolf Steiner (Stockdale et al. 2001). This type of agriculture takes a holistic approach concerning the exploitation of natural resources, taking into consideration the sustainability of different elements, such as the crops themselves, the preservation of animal life, or the maintenance of a high-quality soil, to recover, preserve, or improve ecological harmony. This is achieved through the reduction of external inputs into the production system, the use of a set of preparations to apply to crops to aid fertilization, and the application of other homeopathic treatments based on infusions or plant extracts (Stockdale et al. 2001; Villanueva-Rey et al. 2014). Cultivation sites that are certified as being biodynamic need to be previously certified as organic agriculture production sites (Commission Regulation 2007, 2008, 2012) and have to go through a 3-year conversion period. For the sake of clarity, in this chapter, the terms *organic* and *biodynamic* related to wine production are used in contrast to the term *conventional* (nonorganic wines) to indicate different agricultural practices.

9.2 The Market for Organic Wine

Although organic wine still represents a niche market, its production has increased considerably in the last few years. According to ISMEA-IAMB data, in 2005, approximately 110,000 ha were dedicated to vines cultivated according to organic practices worldwide, with a predominance of countries from the Mediterranean area (Table 9.1). Until recently, very little data were available about the actual organic wine market and market needs as well as the future market trends. An indication of the organic wine market structure was given by the transnational study named Orwine, which was carried out within the six-framework program from mid-2006 until the end of 2007 (http://www.orwine.org). This study chiefly involved four European countries, Italy, France, Germany, and Switzerland. The results from the survey indicated that the consumption of organic wine is growing in all targeted countries. The purchase of organic wine bottles by Italian consumers in 2006 accounted for 245,000 L, for a total value of 1.2 M€. Between 2003 and 2005, in France, the organic wine market grew from 12 to 16 %, whereas a growth rate from 25 to 40 % was reported in Germany. In Switzerland, 55 % of the organic wines are imported, especially from France, Italy, and Spain. The majority of organic wines traded are red wines and bottled wines. The most relevant price range for organic wine is within the price point of 5–10€ per bottle. Regarding sale channels, most

Table 9.1 Organic vine surface distribution (source ISMEA-IAMB 2008)

Country	Hectares	% Worldwide	% Country
Italy	33,885	30.9	3.4
France	18,133	16.5	1.9
Spain	15,991	14.6	1.2
USA	9209	8.4	0.6
Moldova	8155	7.4	73.6
Turkey	4624	4.2	0.3
Syria	4000	3.6	19.5
Greece	3758	3.4	2.7
Germany	2600	2.4	2.4
China	2000	1.8	1.0
Chile	1892	1.7	1.7
Austria	1657	1.5	3.4
Portugal	1308	1.2	0.4
Hungary	594	0.5	0.5
Switzerland	388	0.4	2.4
New Zealand	299	0.3	2.0
Romania	257	0.2	0.3
Argentina	273	0.2	0.1
Slovenia	67	0.1	0.3
Slovakia	91	0.1	0.1
Israel	100	0.1	1.5
Ireland	100	0.1	0.3
Cyprus	93	0.1	.
Canada	69	0.1	0.9
Taiwan	1	0.0	0.1
Serbia-Montenegro	6	0.0	1.0
Malta	1	0.0	7.1
Macedonia	1	0.0	0.4
Luxembourg	6	0.0	0.2
Lebanon	12	0.0	0.5
Georgia	31	0.0	23.8
Croatia	30	0.0	0.1
Czech Republic	48	0.0	0.3
Azerbaijan	50	0.0	0.3
Albania	5	0.0	0.4
Total	109,734	100.0 %	

organic wines are sold in specialized organic shops or through direct sales and less frequently in supermarkets or discounters. The export of organic wines was found to be important only for Italy and France. In Italy, almost 50 % of producers declare that export represents more than 30 % of their sales, whereas in France, approximately 70 % of organic wines are exported.

The importance of the organic wine import–export market was also highlighted in a recent survey by Nomisma (2013), reporting that the US organic wine import

market accounted for a total of 193 M€ in 2013, which represents 5.2 % of the total US bottled wine import market. Organic wines exported to the USA are predominantly from France (33.7 %) and Italy (29.3 %), followed by New Zealand (7.6 %) and Spain (7.5 %).

9.3 Organic Wine Legislation

The sizeable increase in the organic wine market that occurred in the last few years is not only due to higher environmental consciousness by consumers but also due to the publication of Commission Regulation (EC) No. 203/2012. With this new regulation, which applies at the 2012 harvest and thereafter, organic wine growers are allowed to use the term *organic wine* on their labels, whereas before this regulation, only *wine from organic grapes* could be sold. The wine producers certified as organic by the certification bodies must also show the EU organic logo on their labels as well as the code number of their certifier, and they must respect other wine labeling rules (Gaeta and Corsinovi 2014). Although there were already rules for *wine made from organic grapes* (Commission Regulation 2007, 2008), they did not cover wine-making practices. Advantageously, the new rules have improved transparency and better consumer recognition. Not only do these changes facilitate the internal market, but they also strengthen the position of EU organic wines at the international level because many other wine-producing countries (USA, Chile, Australia, and South Africa) have already established standards for organic wines. The new regulation establishes a subset of enological practices and applicable restrictions for organic wines defined by implementing Commission Regulations (EC) No. 606/2009 and 144/2013. For example, sorbic acid, desulfurization, and heat treatments are not allowed, and the level of sulfites in organic wine must be at least 30–50 mg/L lower than their conventional equivalent (depending on the residual sugar content). Other than this subset of specifications, the general wine-making rules defined in the regulations also apply.

9.4 Sustainability in the Wine Industry

A great deal of interest in sustainability issues has been expressed globally, especially in the last decade. Considering the keyword "food sustainability" from 1995 to 2004, without applying any filter, Scopus database (http://www.scopus.com/) returned approximately 960 journal articles, whereas from 2005 to 2014 they were more than 5800. Political attention and institutions' interest are also growing, and it will continue to grow as long as sustainability is a crucial issue for economic growth and development; in fact, sustainability is a strategic goal in economic and social policies on an international level (Laureati et al. 2013). The roots of sustainable practices go back to the book by Meadows et al. (1972), The Limits to Growth, and

the article by Goldsmith et al. (1972). According to the official definition provided in 1987 by the United Nations World Commission on Environment and Development (WCED), sustainable development is "a development that meets the needs of the present without compromising the ability of future generations to meet their own needs" (United Nations 1987).

Sustainability plays an important role in the wine business because the wine industry has had to cope with several problems in the twenty-first century that include the environmental dimension of wine production, soil and water management, agrochemical use, and solid waste (Szolnoki 2013). In addition, reducing the use of pesticides in agriculture has been highlighted as a major factor for preventing pollution. Consequently, there is strong demand for vine growers to engage in more environmentally sustainable wine production practices.

Sustainability is a broad concept that is not easy to contextualize. The often-cited three-dimensional concept of sustainability (United Nations 2005) defines the three main fields of sustainability as environmental, economic, and social. This concept also applies to wine production, and thus sustainable wine-growing and wine-making practices are those sensitive to the environment, responsive to the needs and interests of society, and economically feasible to implement and maintain (Zucca et al. 2009). According to Szolnoki (2013), the first sustainable wine-growing program was implemented in 1992 in California, which resulted in the *California Code of Sustainable Winegrowing Practices*. This was followed by the *Wine Sustainable Policy* in New Zealand, the *Integrated Production of Wine Scheme* in South Africa, the *Vignerons en Développement Durable* in France, and the *Winemaker Federation of Australia*. Furthermore, OIV resolution CST 1/2004 established guidelines for the production of grapes, wines, spirits, and other vine products in accordance with the principles of sustainable development as applied to viniculture, which was extended in 2008. These numerous institutions and organizations with completely different strategies and different practices have made international and sometimes even national comparison extremely complicated (Szolnoki 2013).

The concept of sustainability seems to create significant confusion not only among consumers but also among producers and stakeholders. For instance, Forbes et al. (2009) reported that customers like the concept of sustainable wine making, but they lack a clear idea of what sustainability indicates in practice or in the processes wineries apply to achieve it. Loveless et al. (2011) conducted a study in five countries to analyze the relative importance of sustainability compared with other important attributes (e.g., taste, region, brand, price, quality control, and traceability). They found that even though sustainability is less important to consumers than other characteristics, a segment representing almost 30 % of wine consumers considered sustainable claims in the wine industry to be valuable. Zucca et al. (2009) reported a strong consumer intention regarding wine that is produced using *green* production practices in the USA; however, the result may differ in other countries. Szolnoki (2013) reported that wine producers, especially those from small wineries certified as organic, mainly associated the term sustainability with the environmental dimension, whereas some wineries, especially cooperatives or bigger companies, also took economic and social dimensions into consideration. This author also highlighted

ambiguity regarding the production management systems because many wine producers confused the terms organic, biodynamic, and sustainable. Finally, a common finding in the scientific literature on sustainability is the perceived lack of information communication between relevant organizations, producers, and consumers. This deficiency is highly important because sustainability is a positive concept in consumers' minds but consumers show poor awareness of the problems related to it (Forbes et al. 2009; Szolnoki 2013; Vermeir and Verbeke 2006; Zucca et al. 2009). Information barriers are a huge challenge that the sustainable wine industry will face in the future.

9.5 Quality Aspects of Organic Wine

Food quality and what is meant by quality in the context of organic food production systems is one area that has received ample attention in the debate on differences between organically and conventionally produced foods. It is a common belief that wines from organic viticulture are of lower quality than conventional wines, both concerning sensory characteristics and a supposedly higher content of compounds harmful for human health (e.g., ochratoxin A, biogenic amines). However, these opinions are mainly based on hypothetical considerations because of the lack of scientific data on the analytical and sensory characterization of organic wines as well as the comparison between organic and conventional products (Bourn and Prescott 2002).

The studies comparing wine derived from organic and conventional growing systems focused mainly on three quality aspects (Table 9.2): food safety, nutritional value, and sensory quality. The aspects dealing with the quality of organic and conventional wines are reported in the following sections as follows: (1) factors which are not directly perceivable by the consumer at the moment of purchase or consumption, such as food safety and health aspects (see Sect. 9.5.1), and (2) factors which are directly perceivable by the consumer, such as sensory quality and aspects related to labeling and price (see Sect. 9.5.2).

9.5.1 Health and Food Safety Aspects of Organic Wine Production

Few comparative studies have examined the quality of wine and grape must from organic and conventional production practices. These studies have mainly focused on the contamination of plants by pesticides and the presence of substances that can be harmful (e.g., sulfites, biogenic amines) or beneficial (e.g., phenol contents) to the consumer health.

Regarding food safety, one of the most discussed difference between conventional and organic wine is the sulfur dioxide level. Since the beginning of modern enology,

Table 9.2 List of the most relevant studies comparing organic (org) and conventional (conv) wines for food safety, nutritional, and sensory aspects

Quality aspect	Article (year)	Parameters	Main outcome
Food safety	Chiodini et al. (2006)	OTA	org = conv
	García-Marino et al. (2010)	BA	org > conv
	Kalkan Yildirim et al. (2007)	BA	org = conv
	Miceli et al. (2003)	OTA	org < conv
	Ponsone et al. (2007)	OTA	org = conv
	Tinttunen and Lehtonen (2001)	SO_2	org < conv
	Tassoni et al. (2013)	BA	org = conv
	Vrček et al. (2011)	Me	org = conv
	Yañez et al. (2012)	BA	org = conv
Nutritional value	Laureati et al. (2014)	PHE	org > conv
	Miceli et al. (2003)	RES, PHE, OX	org > conv
	Mulero et al. (2009)	RES, PHE, OX	org = conv
	Mulero et al. (2010)	PHE, OX	org = conv
	Tassoni et al. (2013)	RES, PHE, OX	org = conv
	Tinttunen and Lehtonen (2001)	RES, PHE	org > conv
	Vrček et al. (2011)	RES, PHE, OX	org > conv
	Zafrilla et al. (2003)	PHE, OX	org = conv
Sensory properties	Dupin et al. (2000)	Odor	org < conv
	Laureati et al. (2014)	Sensory profiling	org = conv
	Moyano et al. (2009)	Odor	org < conv
	Pagliarini et al. (2013)	Sensory profiling, preference	org = conv

OTA = ochratoxin A; BA = biogenic amines; OX = antioxidant activity; PHE = phenolic compounds; RES = resveratrol; Me = metal content; SO_2 = sulfite content

sulfur dioxide has always been considered a fundamental additive for its antioxidant, anti-oxidasic, and antimicrobial properties. Nevertheless, it is also a poisonous and allergenic substance. Therefore, the growing interest of consumers in the relationship between this additive and the healthiness of wine and the consequent awareness of consumers regarding the toxicity of sulfites is forcing wine makers to lower the overall amounts of sulfur dioxide in their products (Comuzzo et al. 2013). Because organic wine is generally considered more "natural" and "healthy," the level of sulfites is particularly critical for organic wine producers. Despite the strong impact that SO_2 can have on consumer perception and health, there are very few articles reporting the sulfite concentrations in organic wines and their relationship with the other wine quality control parameters. Recently, Comuzzo et al. (2013) conducted a compositional survey on 1000 wines from organic viticulture in different European countries to assemble an extensive dataset on organic wine basic quality control parameters and several compounds involved in health-related aspects (ochratoxin A and biogenic amines). The authors found that the quality control parameters (e.g., alcoholic content, reducing sugars, total acidity and pH, volatile acidity, and malic and lactic acids) generally agreed with the values normally detected for conventional wines and

that the total sulfur dioxide was lower than 110–120 mg/L in most samples. Tinttunen and Lehtonen (2001) compared the SO_2 levels in organic and conventional wines and found a lower sulfite content in organic wines.

The presence of heavy metals is another important issue when comparing wines from traditionally and organically grown grapes. Many consumers implicitly assume that organic foods have lower levels of pollutants compared with conventionally grown products. However, to our knowledge, the metal content in organic and traditional wines was compared only in the study by Vrćek et al. (2011), who found that both wine productions were comparable and well within the toxicological safety limits.

Regarding ochratoxin A, literature data report that the presence of this toxin is not a generalized problem for organic wine production (Comuzzo et al. 2013; Chiodini et al. 2006; Miceli et al. 2003; Ponsone et al. 2007). However, the risk of ochratoxin A pollution may be higher in certain southern European regions (Comuzzo et al. 2013).

On the contrary, the presence of biogenic amines is a serious problem for organic wine making because high levels of these microbial metabolites were found in several organic wines (Comuzzo et al. 2013). Biogenic amines are undesirable in all foods and beverages because if absorbed at concentrations that are too high, they may cause headaches, respiratory distress, heart palpitations, hypertension or hypotension, and several allergenic disorders (EFSA 2011). García-Marino et al. (2010) detected significantly higher levels of biogenic amines in organic wines than in conventional ones, but only one organic sample was analyzed in this study. Conversely, Tassoni et al. (2013) evaluated biogenic amines levels in conventionally grown grapes and their related wines compared to organic and biodynamic ones and found no significant differences. Kalkan Yildirim et al. (2007) found that the biogenic amines profiles were similar in organic and conventional wines, although organic wines showed a significantly higher level of putrescine but no presence of agmatine. Yañez et al. (2012) reported that the wines produced with conventionally cultured grapes had a general tendency, though not significant, to have a greater concentration of some biogenic amines than those produced with organically cultured grapes. Therefore, it appears that the results regarding microbial metabolites in conventional and organic wines are somewhat controversial, and further research is needed to draw more general conclusions.

Regarding nutritional value, the health effects of wine consumption have been studied in depth over the last decade, and special attention has been given to protection against cancer and cardiovascular disease. The protective effects of wine have been attributed to phenolic compounds that are efficient scavengers of free radicals and breakers of lipid peroxidative chain reactions. In addition to antioxidant activity, phenols also have anti-inflammatory effects and may protect low-density lipoproteins (LDL) against oxidative modification (Akçay et al. 2004).

Several studies performed to compare phenolic compound content and antioxidant activity of organic and conventional wines reported a higher nutritional value of organic wine (Laureati et al. 2014; Miceli et al. 2003; Tinttunen and Lehtonen 2001; Vrček et al. 2011). However, despite the differences found between the nutritional

value of organic and conventional wine, the effects produced on human antioxidant capacity by the consumption of the two types of wine are comparable (Akçay et al. 2004). The higher content of phenolic compounds in organic wine than in conventional wine can be explained by the fundamental differences between the two production practices. Organic systems emphasize the accumulation of organic matter over time through the use of cover crops, manure, and composts. Conventional farms utilize fertilizers containing soluble inorganic nitrogen and other nutrients that are more directly available to the plants, thus influencing the synthesis of secondary plant metabolites, proteins, and soluble solids (Rapisarda et al. 2005). Additionally, organically produced plants have a longer ripening period than conventional plants because of a slower release of the supplied nutrients (Brandt and Mølgaard 2001). Because secondary plant metabolites such as polyphenols are formed in the ripening period, it is likely that the content of these compounds is higher in organically grown plants. However, this is not always the case, as other studies failed to find significant differences in antioxidant activity between organic and conventional wines (Mulero et al. 2009 2010; Tassoni et al. 2013; Zafrilla et al. 2003). This data disagreement could be attributed to several variables, such as vine, area of origin, and vintage. Thus, the question whether organic and conventional wines differ in their nutritional quality is still an issue to pursue.

9.5.2 Consumer Perception of Organic Wine

9.5.2.1 Does the Consumer Perceive Organic Wine to Be of Better Sensory Quality than Conventional Wine?

Both the scientific community and consumers have always been interested in the sensory analysis of wine. There have been many studies carried out on different aspects connected with wine tasting, but relatively little attention has been given to the sensory characteristics of wines derived from organically and conventionally grown grapes.

Organic and conventional fruits and vegetables may differ on a variety of sensory aspects (Bourn and Prescott 2002). Generally speaking, there are two opposing opinions regarding the sensory properties of organic and conventional food. On one hand, organically grown fruits and vegetables, and thus grapes, not subjected to pesticides to protect against pests may be more susceptible to microbiological contamination than conventional grapes, thus resulting in products with lower sensory quality. On the other hand, organic methods can potentially produce fruits and vegetables with better taste and flavor due, for instance, to low nitrogen, phosphorus, and potassium (NPK) fertilization, which results in low yields and a high concentration of sugars, total dissolved solids, and dry matter (Basker 1992; Haglund et al. 1999). Nevertheless, literature data failed to find consistent differences between organic and conventional products from both hedonic and sensory points of view (Laureati et al. 2014). Therefore, the assumption of organic food

having a better taste may be explained by the consumer's expectation of a healthier and safer product evoked by the "organic food" label (Deliza and MacFie 1996; Pagliarini et al. 2013).

Studies dealing with the sensory properties of organic wine are very limited. Callejon et al. (2010) found that the use of autochthonous yeasts during the fermentation of organic grapes improved the sensory quality of wines compared to commercial yeasts. However, no comparison was made with the relevant conventional grapes. Parpinello et al. (2015) compared color, aroma, and taste properties as well as preference for biodynamic and organic wines. Differences were found only in the color intensity, but consumers showed no preference for any wine.

The few studies that have compared the sensory properties of wines derived from organically and conventionally grown grapes are reported in Table 9.2. Moyano et al. (2009) examined the aroma profile of sherry wines that had been cultivated conventionally and organically, and they found that organic wines had a sensory profile similar to that of the conventional ones but lower odor intensity. The same finding was reported by Dupin et al. (2000), who examined German wines and found that organic products tended to be less aromatic than conventional ones. More recently, Pagliarini et al. (2013) identified and described the sensory properties that characterize organically and traditionally grown Romagna Sangiovese red wines. The differences detected by the authors between the two types of wine were only marginal and did not influence consumer preference (Fig. 9.1). This outcome suggests that consumers are not able to discriminate among organic and conventional wines from a hedonic point of view. Importantly, the wines used in Pagliarini and colleagues' study were evaluated under blind conditions without any information concerning production method. Thus, consumer preference derived mainly from the mere sensory perception of the wines without any preconceived ideas from their knowledge about the product. Certainly, this does not reflect the consumer's actual purchase because, at the moment of choice, the consumer seldom bases choice on taste but rather on label information as well as price, vintage, etc. It is likely that, if the consumers would have been informed about the production practices during acceptability evaluation, differences between organic and conventional wines may have arisen. This assumption is supported by previous literature that compared the hedonic qualities of organically and conventionally produced food, e.g., yogurt (Laureati et al. 2013), cheese (Napolitano et al. 2010a), and meat (Napolitano et al. 2010b). In these studies, the preference of organic and conventional products was evaluated under different information conditions: the blind condition (i.e., consumers taste and judge the product without any type of information); the expected condition (i.e., consumers do not taste the product and judge it only on the basis of written or visual information); and the informed condition (i.e., consumers taste and judge the product after reading written information and/or observing an image). The main outcome of these studies is that organic products are liked more than their conventional counterparts but only in informed conditions when the consumers know that they are tasting an organic food. This finding confirms the hypothesis that organic products are liked more because of the "healthier" connotation that they have in the consumer's mind rather than for an actual preference based on perceptual

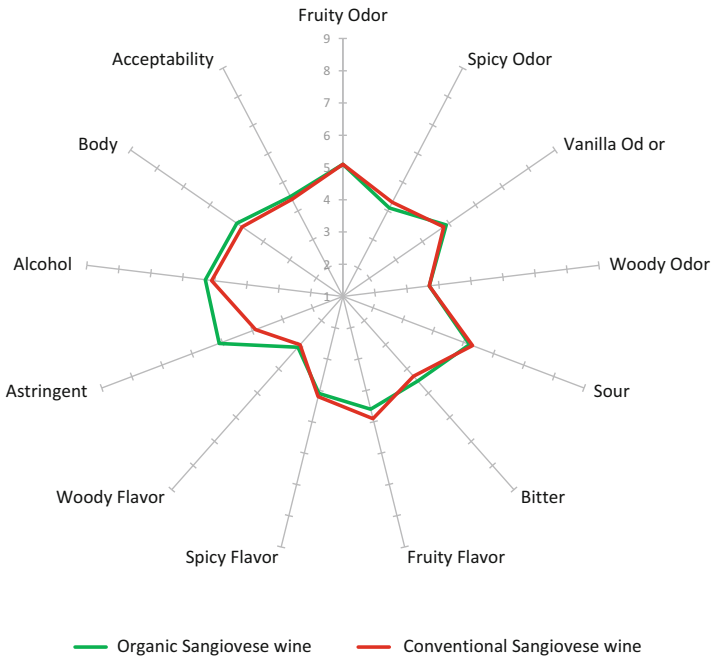

Fig. 9.1 Mean values of sensory descriptors and acceptability ratings for organic and conventional Sangiovese red wines (modified from Pagliarini et al. 2013)

attributes (Pagliarini et al. 2013). Unfortunately, there are very few studies on the hedonic appreciation of organic and conventional wines; thus further research is needed to draw general conclusions.

9.5.2.2 Do Consumers Care about Eco-Labels and Logos Indicating Organic Wine Production?

In the market of organic wine and, more generally, organic food, consumer's trust is a delicate issue. Indeed, sustainability and thus organic production are credence attributes because consumers cannot determine by themselves whether a wine has been produced using environmentally sustainable practices (Janssen and Hamm 2012). Thus, consumers can only choose to buy environmentally sustainable wine once they are provided with accurate, understandable, and trustworthy information. In this context, the information provided by logos included on labels in combination with awareness and concern about environmental issues could help consumers to make better choices when purchasing organic products (Ginon et al. 2014). However,the usefulness of eco-labels has been questioned because consumers usually have limited knowledge of agricultural production practices and, in addition, the benefits associated with sustainable products are often poorly communicated to them (Vermeir and Verbeke 2006).

a) European Union (new) b) European Union (old) c) United States d) Canada e) France f) Japan

Fig. 9.2 Examples of organic certification logos: (**a**) New mandatory EU organic logo of the EU (since July 2010); (**b**) old voluntary organic EU logo; (**c**) US Department of Agriculture logo; (**d**) Canadian federal organic logo; (**e**) French public organic farming logo; (**f**) Japanese Agricultural Standard logo

Organic certification is a long-standing tradition in many European countries. As previously mentioned, only the products that comply with the principles of organic production, certification, and labeling by Regulation (EC) No. 834/2007 (and respective implementing regulations) can be labeled and sold as organic food in the EU. Since July 2010, all organic products, and thus organic wine, produced and sold in the EU must be labeled with the new mandatory EU logo (Regulation (EC) No. 834/2007), which replaced the old voluntary EU logo. There are also several other voluntary organic certification logos in many European countries, which are owned by governmental and private organizations (Fig. 9.2). The variety of organic logos in the market raises the question of whether consumers prefer products with certain organic certification logos over others. This information is important for wine makers deciding on whether they should seek certification according to another organic scheme in addition to the mandatory certification. Ginon et al. (2014) performed a study aimed at evaluating how French consumers perceive logos indicating environmentally sustainable wine production. Twelve logos were considered, among which were logos specifically referring to wine production as well as logos more generally related to sustainable practices. Overall, a lack of knowledge among consumers regarding logos indicating environmental sustainability was observed. Only 2 of the 12 logos were known and conveyed a message of environmental sustainability, specifically the old European logo and the French organic farming logo. The new European logo was quite unknown among consumers, most likely because it is somewhat "anonymous" and does not explicitly refer to concepts of sustainability or organic production. None of the sustainable wine practices logos were known by the consumers. This outcome may be a signal that the large number of logos available in the market creates confusion among consumers and most likely contributes to reduce their credibility, as also observed by Delmas and Grant (2014). The trust in organic logos and certification schemes is a fundamental issue for increasing consumer willingness to purchase organic products. Consumers are typically willing to pay premium prices for well known and trustworthy logos with perceived strict organic standards and control system (Janssen and Hamm 2012). Thus, there is an urgent need for the organizations owning a labeling scheme related to environmental sustainability in wine production to improve their design information and communication strategies to help consumers make better choices while shopping for wines.

9.5.2.3 Is the Consumer Willing to Pay Extra for Organic Wine?

Organic wines are usually sold at a higher price than conventional wines. Effectively, organic grapes, being not subjected to pesticides, are more susceptible to pests and diseases, which can translate into low yields.

Selling sustainable wine at a higher price is considered one of the most effective incentives to encourage vine growers to adopt environmentally friendly production practices. However, the success of this approach depends on consumer's willingness to pay a premium price for organic wine (Ginon et al. 2014). Certain authors are skeptical that consumers will accept paying more for sustainable products. For instance, in a study of attitudes toward Colorado wines, the premium that consumers were willing to pay for an environmentally friendly wine was calculated at just 13 cents over the per bottle base price of $10 (Loureiro 2003). In contrast, Pagliarini et al. (2013) interviewed 100 Italian habitual consumers of wine and found that 23 % of them were willing to pay an extra premium of less than 10 % for organic wines, another 39 % were prepared to pay between 10 and 20 % more for such wines, and a small percentage of consumers (4 %) declared willingness to pay an additional premium of up to 20–30 %. A study conducted on Spanish consumers reported that the average premium they were willing to pay for an organic wine was approximately 16 % above the price of a conventional wine (Brugarolas Molla-Bauza et al. 2005). Similar findings were reported by Forbes et al. (2009), who showed that 73 % of the New Zealand consumers involved in their study indicated that they would be prepared to pay more for an environmentally sustainable wine. In particular, approximately one-third of respondents were prepared to pay a premium of up to 5 % for sustainably produced wines, and another third were prepared to pay between 6 and 10 % more for such products. In a recent review by Lockshin and Corsi (2012), Australian wine consumers were reported to be willing to pay a price premium of +22 % for an organic wine compared to a conventional wine. Finally, Olsen et al. (2012) found that US consumers are willing to make a sacrifice to pay a premium price for organic wines because they believe that organic products are better for the environment; therefore, cognitive factors, such as personal expectancies toward organic wine, play a fundamental role in consumer's purchase intention. Accordingly, Vermeir and Verbeke (2006) reported that consumers with higher environmental involvement were more likely to purchase sustainable products and suggested that the level of involvement could be increased through the provision of information from green producers. Similar findings were also observed by Laureati et al. (2013), who evidenced that "sustainable" consumers expressed higher hedonic expectations for organic products than "non-sustainable" subjects.

Despite the general positive outcome on consumer's willingness to pay for organic wine, previous studies demonstrate widespread inconsistency between consumer's beliefs, opinions, values, and actual behaviors; in other words, the consumer's attitudes are often a poor indicator of actual marketplace purchase behavior (De Barcellos et al. 2011; Vermeir and Verbeke 2006). Thus, systematic research dealing with actual consumers' wine purchase intention in realistic environments is necessary to represent real-life buying situations.

9.6 Conclusions

Reviewing the scientific literature regarding sustainability and organic wine production led to the following main considerations:

1. There has been considerable interest in organic wine in response to increased consumer's awareness about health and the environment.
2. Although the increased demand for organic wine produced a greater quantity of scientific studies on the quality aspects of organic and conventional wines, the findings disagree too much to draw general conclusions. It seems that organic wine is of equal or even better nutritional quality than conventional wine as most of the literature noted a similar or higher content of phenolic compounds and antioxidant activity. The results regarding food safety and sensory quality show too much disagreement and are too few in number, respectively, to conclude on whether organic wine is of better quality than conventional wine. Further research comparing organic and conventional wine quality is strongly recommended.
3. Sustainability is a positive concept in consumers' minds; however, consumers show poor awareness of the problems related to it, and it is difficult to find a unique and generally accepted definition of the concept. Furthermore, there is a general lack of information regarding sustainability issues among consumers and the different logos used to transmit information about wine sustainability have little power on the consumer.
4. A general positive attitude to pay more for organic wine is observed in European and extra-European countries, mainly for health and environmental reasons but also because consumers are interested in helping producers who adopt these innovations. Moreover, a greater predisposition to pay an additional charge for organic wine may be achieved by increasing consumer involvement in sustainability issues through a careful program of information dispersal.

In conclusion, organic wine has good market prospects, but information barriers are a huge challenge that the sustainable wine industry will face in the future.

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Chapter 10
Dietary Supplements/Nutraceuticals Made from Grapes and Wines

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10.1 Introduction

In the recent years, there has been a significant increase in demand for healthy foods from natural plant sources. It is important to note that the use of natural products with nutritional values has gained increased interest among well-informed consumers as a way of improving healthy lifestyle (Koch et al. 2014). The word nutraceutical refers to the composition of the terms nutrient and pharmaceutical, which is defined as "a food or part of a food that provides medical or health benefits, including the prevention and/or treatment of a disease" (Kalra 2003). However, a common definition of nutraceuticals and a valid international regulation and classification of this category of products do not exist (Koch et al. 2014). It is clear that nutraceuticals are by definition very close to functional foods, which are foods manufactured or prepared using "scientific intelligence." Nutraceuticals are also close to botanicals, which are plants or plant parts valued for their medicinal or therapeutic properties, flavor, and/or scent. In addition, nutraceuticals are close to dietary supplements, which also refer to products that are intended to complement the diet and they contain one or more of the following dietary ingredients: vitamins, minerals, herbs, other botanicals, amino acids, and dietary substances. Dietary supplements are intended to be ingested in the form of pills, capsules, tablets, or liquid and are not intended for use as conventional foods. Therefore, the confusion in defining nutraceuticals raises the mandatory question of coming up with a strict definition for "nutraceuticals," and properly regulating the use of this terminology (Koch et al. 2014). However, to date the term "nutraceuticals" has been widely accepted for marketing purposes to describe health beneficial foods or food additives with health-promoting properties.

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Grape is one of the most widely cultivated fruit species in the world. It contains a unique mixture of biologically active phytochemicals with prominent health beneficial effects on human body. Thus, grapes and its by-products are perfect candidates for nutraceutical formulations. Currently wines and grape juices are the major processed products from grapes. It is worth pointing out that wine and juice production generates significant amounts of waste, which unutilized properly can contribute to serious ecological and economic problems (Devesa-Rey et al. 2011). Meanwhile, more than 70 % of grape phenolics are retained in skins and seeds, making the winery waste an inexpensive source for the recovery of valuable biologically active compounds (Georgiev et al. 2014a; Dwyer et al. 2014). Thus, lately scientists and the industry have joined their efforts to utilize the wastes from wineries by developing different value-added nutraceutical products. For example, the market potential of winery waste produced in Canada during the 2011 season was estimated to be $499,273,431.1 in Ontario and $185,235,692.8 in British Columbia (Dwyer et al. 2014). This chapter describes the main types of nutraceuticals and other products generated from grapes, wines and winery by-products, as well as their biological activities and modern production processes.

10.2 Nutraceuticals from Grapes and Wines

Recent scientific data indicates that the phenolic compounds are responsible for the benefits ascribed to wine in the Mediterranean diet (Chiva-Blanch et al. 2013a, b; Kondrashov et al. 2012; Noguer et al. 2012; De Curtis et al. 2005). The discovery has prompted scientists to investigate the options for producing nonalcoholic wines, grape juice, powders, extracts, and other products with nutraceutical properties (Fig. 10.1) (Kondrashov et al. 2012; Rho and Kim 2006; Varghese et al. 2014; Chanukya and Rastogi 2013; Sanchez et al. 2013; Shiby et al. 2013).

The waste from the grape juice and wine productions includes various types of residue generated at different technological stages and could be summarized as pomace (a by-product obtained after pressing grapes) and tartrate sludge (filter trim) in grape juice production. These waste materials also include nonfermented or fermented pomace (grape marc) and wine lees, which are all sediments formed after fermentation, during storage or after malolactic fermentation in winemaking (Fig. 10.1). Juice and winery waste have been widely underestimated and underutilized when used in alcohol distillation, as compost and soil fertilizers or simply disposed as organic waste. This has generated serious waste-management problems for the production companies (Devesa-Rey et al. 2011). Nowadays, because of their high polyphenolic contents, those by-products, and especially the grape pomace, have been considered as an inexpensive source of valuable bioactive components or used in nutraceuticals formulations (Fig. 10.1). Various nutraceutical products such as extracts, powders, and dietary fibers could be produced directly from pomace. Grape seeds, skins, and stalks may be recovered from grape pomace and used as raw materials for production of other grape-derived nutraceutical products (Fig. 10.1).

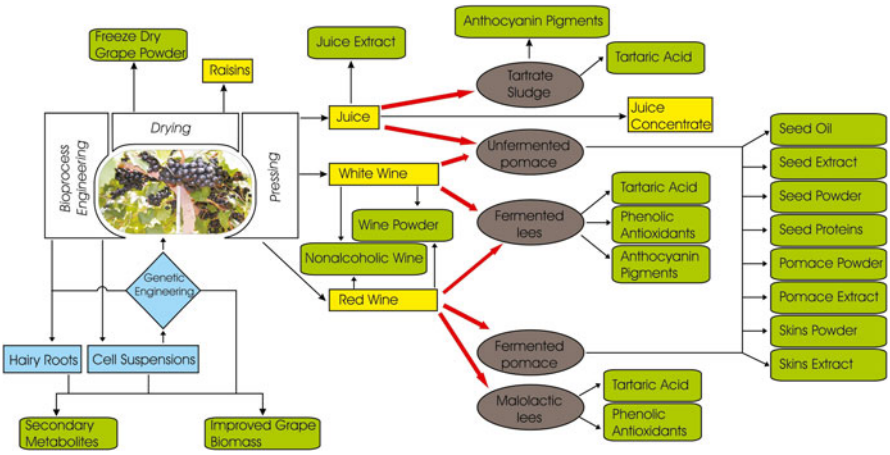

Fig. 10.1 Origin and connections between the major grape products and grape-derived nutraceuticals. Color codes: *Yellow boxes*—traditional grape products; *Green boxes*—grape-derived nutraceuticals; *Brown boxes*—grape by-products; *Blue boxes*—in vitro biotechnological approaches for production of "new generation grape nutraceuticals"; *Red arrows*—technological wastes

Despite the fact that wine lees mainly consist of dead yeast and/or bacterial cells which are rich in proteins, they have poor nutritional value because of the formation of non-digestible tannin-protein precipitates. However, recently the ultrasound- and microwave-assisted extraction in combination with spray-drying have been successfully applied for recovery of anthocyanin pigments and polyphenolic antioxidants from winery lees (Tao et al. 2014; Pérez-Serradilla and Luque de Castro 2011).

More importantly, the advancement in understanding the complex mechanisms involved in secondary metabolism in grapes and the fast progress in modern plant cell biotechnology hold a promising perspective for the development of "new generation" of high-quality grape-derived nutraceutical products (Ananga et al. 2013a, b). The use of classical in vitro techniques, high-end bioprocess engineering technology, and molecular methods of genetic and metabolic engineering can be combined to enable continuous production of nutritionally improved grape biomass or for large-scale biosynthesis of rare grape-derived metabolites (Fig. 10.1).

10.2.1 Nonalcoholic and Powdered Wines

The production of low-alcohol content or alcohol-free wine could extend the range of consumers who rely on these remarkable grape products. Removing the alcohol could make wine and its health benefits accessible for a range of social groups who normally do not consume alcohol such as young adolescences, nondrinkers, intensively working people, drivers, and some religious groups. However, the existing techniques for wine dealcoholization usually result in products with declined

quality and sensory characteristics, since the ethanol removal can be accompanied with the removal of most of the volatile aroma compounds (Takács et al. 2007; Catarino and Mendes 2011). Several advanced methods, including membrane separation by reverse osmosis and nano-filtration, spinning cone column distillation technique, liquid emulsion membrane extraction and pervaporation (separation on nonporous polymer membranes by partial evaporation), have been employed and adapted for wine dealcoholization (Catarino and Mendes 2011; Belisario-Sánchez et al. 2012). To protect the organoleptic properties of dealcoholized wines, a production of low-alcoholic wine obtained by blending nonalcoholic wine with original wine and adding of the aroma compounds previously recovered from dealcoholization process could be a reasonable solution. However, these techniques require the investments in expensive equipment, which significantly increases the price of the final product. Nevertheless, because of the growing problem with the wine surplus in European countries, and because of the strict regulations which define higher taxes for the wines with high ethanol content (over 14.5 vol.%), many winemakers have been interested in reorganization of their technology to produce nonalcoholic wines as high value-added nutraceutical products.

Another nutraceutical product, obtained by wine, is the freeze-dried wine powder. However, the freeze-dried wine can form highly hygroscopic amorphous clay. Recently, encapsulation in maltodextrin matrix was proposed as an effective way to produce a freeze-dried wine in the form of stable free-flowing powder (Sanchez et al. 2013). When compared to the same amount of red wine, this product was found to have 3.7-folds more polyphenols and can be added to other foods as an alcohol-free source of wine polyphenols.

10.2.2 Pomace

More than 80 % of the grapes produced worldwide are being utilized for winemaking (Mildner-Szkudlarz et al. 2013). Following the vinification technology, a huge part of grape biomass (20–25 %) is usually discarded in the form of pomace (Yu and Ahmedna 2013), which keeps more than 70 % of all grape phenolics (Deng et al. 2011). Pomace consists of the pressed seeds, skins, and stalks remaining after grape processing into wine and juice. The pomaces from grape juice and white wines are separated immediately after grape pressing, whereas the pomaces from red wines undergo maceration and partial fermentation for a given period before they are discarded. Nevertheless, comparative study between pomaces obtained from red and white wine production showed that pomaces from red wines have higher phenolics, flavonoids, anthocyanins, and proanthocyanidins; therefore, they have better antioxidant properties (Deng et al. 2011). There is a variation in the composition of polyphenols from grape pomace, and it depends on the grape varieties, growing location, climate, developmental stage of berries, and the type of fermentation process. In most cases, pomaces from red varieties are usually rich in anthocyanins,

flavonoids, and catechin, whereas flavan-3-ols (gallocatechin, epigallocatechin, procyanidins B1, B2, B4 and C1, and catechin) are the most abundant polyphenols in white grape pomaces (Yu and Ahmedna 2013). The phenolic compositions of pomaces from North American native muscadine grapes (*V. rotundifolia*) are significantly different than those from European grapes (*V. vinifera*) and consist mainly of anthocyanin diglucosides, ellagic acid, gallic acid, (+)-epicatechin, (+)-epigallocatechin, catechin, myricetin, quercetin, kaempferol, and some anthocyanidins (Yu and Ahmedna 2013). Pomace can be used for direct formulation of nutraceuticals in the form of dry pomace powder extracts, or it can also be used as a raw source of grape seeds, skins, and stalks after mechanical separation. Dry pomace powder is also a bulk nutraceutical product, obtained after drying (air drying under shade, furnace drying at 60 °C, or freeze-drying could be used) and grinding of raw grape pomace (Nishiumi et al. 2012; Anastasiadi et al. 2008). To produce dry pomace extracts, polyphenols from pomace are usually extracted by organic solvents (methanol or ethanol) that are slightly acidified (acetic acid or hydrochloric acid may be used) and then the solvent has to be removed by evaporation and the residues are dried by heating or freeze-drying. Recently, an aqueous extraction with cyclodextrins was reported as an effective recovery system for grape pomace polyphenols with low polarity, and in addition to that, the possible application of nutraceuticals produced from biologically active powder has been suggested (Ratnasooriya and Rupasinghe 2012). Nevertheless, grape pomace powders and extracts can be directly incorporated in food products such as breads, muffins, brownies, yogurt, salad dressing, fruit candies, and biscuits. This can contribute to a significant increase in their shelf-life and nutritional, sensory, and physicochemical properties (Tseng and Zhao 2013; Sant'Anna et al. 2014; Walker et al. 2014).

10.2.3 Grape Seed Products

Grape seeds can be recovered from the pomace after separation by sieves. Most of the grape polyphenols (60–70 %) are accumulated in the seeds which makes them a valuable source for recovery of biologically active compounds (Ali et al. 2010). Grape seeds are rich in procyanidins, gallic acid, tocopherols, linoleic acid, catechin, epicatechin, epicatechin gallate, quercetin, and may have traces of stilbenoids. The seeds can be dried and then ground to produce grape seed powder as bulk nutraceuticals, or can be used for production of grape seed oils or dry grape seed extracts (Ross et al. 2011). Grape seed oils are characterized with high content of polyunsaturated fatty acids (64–74 %) and can be considered as a nutraceutical products with strong antioxidant capacity because they are rich on phenolics and tocopherols (Fernandes et al. 2013). Oil can be isolated from the grape seeds by chemical extraction (usually with hexane) or by mechanical pressing. The chemical extraction produces more purified oils, which after refining have better shelf-life. Different techniques, including Soxhlet extraction, pressurized liquid extraction, and

supercritical fluid extraction, have been adapted and employed to produce grape seed oils (Freitas et al. 2013). However, the conventional extraction with Soxhlet apparatus has been found to extract maximal amounts of tocopherols (Passos et al. 2010). Cold-pressing is the most popular mechanical method for extracting oil from the grape seeds. The cold-pressing involves no heat or chemical treatment and is based on mechanical separation of the oil from the seeds to produce "extra-virgin" oil. The extra-virgin grape seed oils are considered to be a better source of antioxidants than the conventional oils obtained by chemical extraction (Lutterodt et al. 2011). Moreover, the defatted seed flours retain their biological active polyphenols and could be directly used as bulk nutraceuticals with equal pharmacological and nutritional properties as the grape seed powders. Because of the presence of phenolic compounds, cold-pressed grape seed oils have strong antioxidant activity and pleasant vinous and fruity aroma (Matthäus 2008). However, the same phenolics are also the reason for their low shelf-life, since the flavor can easily change into astringent, bitter, or ethyl acetate sensory during storage.

Proanthocyanidin-rich extracts can be obtained by extraction of grape seeds with appropriate solvents (usually water-alcohol mixtures) followed by concentration or drying into powder and can be directly used as nutraceuticals (Chen et al. 2012; Shrotriya et al. 2012). Grape seeds also contain high levels of dietary proteins, which after purification can be used to improve the nutritional and sensory quality of certain foods (Zhou et al. 2011). Grape seed powders, grape seed oils, and grape seed extracts have been applied as direct additives in frankfurters, yogurt, bakery, and cookies to enhance their quality and antioxidant and nutritional properties (Özvural and Vural 2014; Chouchouli et al. 2013; Davidov-Pardo et al. 2012).

10.2.4 Grape Skin Products

Grape skins can be recovered from dry pomace by mechanical separation with vibrating sieves. Isolated skins can be dried and powdered as bulk nutraceutical products or can be used as raw materials for obtaining partially purified antioxidants. In fact, dried grape skins have been found to be better sources of resveratrol than the red grapes themselves (Khurana et al. 2013). It is important to note that most of the anthocyanins in grapes accumulated in the grape skins and only a small amount of them are extracted in wines during pressing and maceration (Georgiev et al. 2014a). The skins of red varieties are rich in anthocyanins, rutin, quercetin derivatives, kaempferol derivatives, catechin, and chlorogenic acid, whereas the white grapes do not have anthocyanins but are rich in phenolic acids, flavan-3-ols, and flavonols (Ali et al. 2010). Thus, the red grape skins have been used for anthocyanin extraction and preparation of natural food colorants (E 163) with antioxidant properties (Devesa-Rey et al. 2011). Recently it was found that the grape skins can be used as valuable raw material for isolation of oleanolic acid (Mendes et al. 2013). Supercritical fluid extraction has been successfully applied for oleanolic acid extraction from grape

skins and the yield extracted was comparable with that of the classical solid–liquid extraction techniques (Chronopoulou et al. 2013).

10.2.5 Antioxidant Dietary Fibers

Dietary fibers are food supplements of growing interest because of their low energy value and significant contribution to health (Zhu et al. 2015). Dietary fibers from grape by-products have been characterized with high antioxidant capacity, which distinguishes them from the most known dietary fibers. To better classify dietary fibers with additional health-promoting properties, a concept of "Antioxidant dietary fiber" was developed (Saura-Calixto 1998). Antioxidant dietary fibers are products containing significant amounts of natural antioxidants associated with the fiber matrix. According to the proposed definition, the antioxidant dietary fiber should have: (1) dietary fiber content higher than 50 % on a dry matter basis; (2) 1 g of antioxidant dietary fiber should have an antioxidant capacity that can inhibit lipid oxidation equivalent to minimum 200 mg of vitamin E and a free radical scavenging capacity equivalent to minimum 50 mg of vitamin E; and (3) the antioxidant capacity must also be derived from the natural constituents of the material neither by added antioxidants nor by added constituents (Saura-Calixto 1998). Several supplements of soluble and/or insoluble antioxidant dietary fibers have been developed from grape pomace and grape stalks, and all of them have shown promising potential as nutraceuticals (Table 10.1). Grape stalks are mainly composed of cellulose, hemicelluloses, lignin, and tannins. Research has found that stalks from red grapes (*V. vinifera*, var. Manto Negro) contain higher amounts of insoluble dietary fibers than pomace (73.5 % of DW in stalks vs. 63.7 % of DW in pomace), and the latter has been defined as a better source of soluble dietary fiber (10.8 % of DW in pomace vs. 3.77 % of DW in stalks) (Llobera and Cañellas 2007). The combination of soluble and insoluble fibers in grapes makes them an attractive source for development of nutraceuticals acting as a long-term delivery system of bioavailable antioxidants in functional foods or pharmaceuticals.

10.3 Biological Activities of Grape-Derived Nutraceuticals

Biological activities of grape-derived nutraceuticals are predetermined by the nature and activities of the phytochemicals they are composed of. Several biological activities, such as antioxidant, anti-inflammatory, cardioprotective, hepatoprotective, antiproliferative, antiallergenic, antiviral, anticancer, anti-obesity, and antimicrobial have been assigned to flavonoids and other phenolic compounds found in grapes (Georgiev et al. 2014a; Scola et al. 2013). Recent examples supporting the in vivo biological effects of some grape-derived nutraceuticals as well as their origin and basic phytochemical profiles are summarized in Table 10.1.

Table 10.1 Recent examples of some grape-derived nutraceuticals and evidences for their biological activities

Nutraceutical/supplement	Source	Main compounds	Biological activity	Type of assay	Reference
ActiVin® IH 636 grape seed proanthocyanidin extract	California grapes	75–80 % oligomeric proanthocyanidins, 3–5 % monomeric proanthocyanidins	Anti-allergic, immunomodulatory, reduce Ca^{2+} influx, increase cAMP level	RBL-2H3 cells	Chen et al. (2012)
ActiVin® IH 636 grape seed proanthocyanidin extract	California grapes	75–80 % oligomeric proanthocyanidins, 3–5 % monomeric proanthocyanidins	Anticancer, selectively increase DNA damage and apoptosis in head and neck squamous cell carcinoma	HNSCC Detroit 562 and FaDu cells, athymic nu/nu mice	Shrotriya et al. (2012)
Black grapes concentrated extract	Dried skin, seeds, and pulps from fresh black grape berries (*V. vinifera*)	Gallic acid, catechin, epicatechin, ellagic acid, myricetin	Counteract lead-induced oxidative stress, Improvement of hepatocytes and nephrons	Sprague–Dawley rats	Lakshmi et al. (2013)
Cold-pressed muscadine seed oil	Muscadine grape seeds from pomace	Linoleic acid, oleic acid, stearic acid, palmitic acid	Antioxidant, antiproliferative	DPPH, HT-29 human colorectal adenocarcinoma cells	Ali et al. (2010)
Concentrate of soluble fibers	White grape pomace	Rhamnose, arabinose, xylose, mannose, galactose, glucose	Antioxidant	TEAC	da Silva Ferreira et al. (2013)
Concord grape juice	Hot pressed concord grapes	Polyphenols, anthocyanins, proanthocyanidins	Enhance neurocognitive function in older adults with mild memory decline, Increase neural activation in cortical regions, improve memory function	Clinical study with 11 men and 10 women (68–90 years)	Krikorian et al. (2012)
Dried stalks powder	Red grape stalks (*V. vinifera* Manto Negro var.)	Polyphenols, soluble dietary fiber, insoluble dietary fiber, uronic acids, Klason lignin	Antioxidant	DPPH	Llobera and Cañellas (2007)
Extracts from air dried pomace powder	White and red grape pomace	Flavonoids, stilbenes, and phenolic acids	Antilisterial	*Listeria monocytogenes* Scott A	Anastasiadi et al. (2008)

Extracts from air dried fresh seeds powder	White and red fresh grape berries	Monomeric flavan-3-ols, (+)-catechin, (−)-epicatechin, epicatechin gallate, procyanidins B2 and B3	Antilisterial	*Listeria monocytogenes* Scott A	Anastasiadi et al. (2008)
Extracts from air dried stalks powder	White and red grapes	*Trans*-resveratrol, ε-viniferin, (+)-catechin, and procyanidin B3	Antilisterial	*Listeria monocytogenes* Scott A	Anastasiadi et al. (2008)
Extracts from freeze-dried berry powder;	White and red fresh grape berries	Flavonoids and 3-O-flavonol glycosides	Antilisterial	*Listeria monocytogenes* Scott A	Anastasiadi et al. (2008)
Freeze-dried grape berries powder; freeze-dried nonfermented pomace; freeze-dried grape juice	Fresh grapes Campbell Early (*V. labruscana* Bailey)	Flavonoids, B-carotene, tocopherols, soluble and insoluble dietary fibers	Antioxidant, antiaging, promote liver and red blood cell antioxidant enzyme activities, decrease lipid peroxide content in blood plasma, decrease DNA damage to kidney tissue	Sprague–Dawley rats	Rho and Kim (2006)
Freeze-dried grape powder	Grape berries	Phenols, flavans, anthocyanin, quercetin, myricetin, kaempferol, resveratrol	Antioxidative; increase plasma IL-10 and adiponectin levels	Clinical study with 24 men (30–70 years)	Barona et al. (2012)
Freeze-dried grape powder	Red, green, and blue-purple seeded and seedless California grapes	Catechin, catechin gallate, epicatechin, epicatechin gallate, kaempferol 3-O-glucoside, rutin, quercetin 3-O-glucoside, quercetin, procyanidin B2, delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, petunidin 3-O-glucoside, peonidin 3-O-glucoside, malvidin 3-O-glucoside, gallic acid	Anti-inflammatory, anti-obesity, improve glucose tolerance acutely, reduce markers of inflammation chronically	C57BL/6J mice; abdominal WAT from nondiabetic and nonobese Caucasian and African-American women	Chuang and McIntosh (2011)

(continued)

Table 10.1 (continued)

Nutraceutical/supplement	Source	Main compounds	Biological activity	Type of assay	Reference
Freeze-dried grape powder	Red, green, and blue-purple seeded and seedless California grapes	Phenolics, flavanols, anthocyanins, quercetin, myricetin, kaempferol, resveratrol	Attenuate atherosclerosis development, reduce macrophage atherogenicity, reduce serum oxidative stress, reduce macrophage uptake of oxidized LDL, increase serum antioxidant capacity	Apolipoprotein E deficient (E^0) mice	Fuhrman et al. (2005)
Freeze-dried grape powder	Red, green, and blue-purple seeded and seedless California grapes	Phenolics, flavanols, anthocyanins, quercetin, myricetin, kaempferol, resveratrol	Anti-obesity, may induce beneficial alterations in potentially atherogenic lipid subfractions	Clinical study with healthy obese male and female volunteers (20–60 years)	Zunino et al. (2014)
Freeze-dried grape seed powder	Fresh berries *Vitis labruscana* Bailey, Campbell Early	Proanthocyanidins, procyanidins, tocopherols	Protective effects on cardiovascular disease, protective effect against oxidative stress, decrease plasma and hepatic lipid peroxide concentrations, suppress lipid peroxidation	Sprague–Dawley rats	Choi et al. (2012)
Freeze-dried grape skin extract	*V. vinifera* grape skins	Polyphenols	Prevent obesity and the risk of obesity-associated diseases, suppress adipogenesis and lipogenesis in adipocytes	3T3-L1 preadipocytes cells	Jeong et al. (2012)
Freeze-dried resveratrol-amplified grape skin extract powder	Campbell Early grape (*V. labruscana* Bailey) illuminated with UV light after postharvest	Resveratrol, phenolics	Anti-obesity, attenuate adipogenic differentiation, regulate lipid metabolism	3T3-L1 preadipocytes cells	Zhang et al. (2012)

Fresh grape extract	Fresh berries *V. vinifera* Merlot, Syrah, Cabernet franc, Cabernet Sauvignon	Phenolics, flavonoids, anthocyanins	Antioxidant, antimicrobial	DPPH; *Escherichia coli* ATCC 10536, *Salmonella arizonae* ATCC 13314, *Listeria monocytogenes* ATCC 19111, *Candida albicans* ATCC 10231	Nada et al. (2012)
Gervital® Dried grape seed extract powder	Grape seeds from pomace	Polyphenols	Antivenom, inhibit the toxic effects of Egyptian sand viper (*Cerastes cerastes cerastes*) neurotoxin	Swiss albino mice	Mahmoud (2013)
Grape antioxidant dietary fiber	Red grapes (Cencibel variety) grown in Spain	Soluble and insoluble dietary fibers, polyphenols, phenolic acids, anthocyanidins, proanthocyanidins, catechins, flavonoids	Protective effects on cardiovascular disease, reduction in lipid profile and blood pressure	Randomized, controlled, parallel-group trial with 43 nonsmokers (27 women and 16 men with an average age of 33.7 years)	Jiménez et al. (2008)
Grape antioxidant dietary fiber	Pomace from red grapes (*V. vinifera*, Cencibel var.)	Polyphenols, soluble dietary fiber, insoluble dietary fiber, proanthocyanidins, protein, fats	Prevent mitochondrial apoptotic pathways, Modulate antioxidant enzyme system, reduce oxidative damage, reduce oxidative environment of the colonic mucosa	Wistar rats	López-Oliva et al. (2013)
Grape pomace extract	Dried pomace of red and white grapes (*V. vinifera*)	Polyphenols, anthocyanins	Anti-inflammatory, suppress inflammatory responses	Sprague–Dawley rats	Nishiumi et al. (2012)

(continued)

Table 10.1 (continued)

Nutraceutical/supplement	Source	Main compounds	Biological activity	Type of assay	Reference
Grape seed extract powder	Grape seeds from pomace	Polyphenols, flavonoids, catechin, proanthocyanidins, flavanones, flavones, flavonols, isoflavones, stilbenes, lignans	Antimicrobial	*Alicyclobacillus acidoterrestris* DSM 3922	Molva and Baysal (2015)
Grape seed oil	Grape seeds	C16:0, 8 %; C18:0, 4 %; C18:1, 15 %; C18:2, 73 %	Decrease the levels of liver cholesterol	Rats	Asadi et al. (2010)
Grape seed oil	Fresh red grape seeds from: Aragonês, Cornifesto, Marufo, Periquita, Tinta Barroca, Tinta Carvalha, Tinto Cão, Touriga Francesa, Touriga Nacional, Trincadeira Preta	γ-tocotrienol, α-tocopherol, α-tocotrienol, linoleic acid, oleic acid, palmitic acid, stearic acid	Antioxidant	DPPH, ABTS	Fernandes et al. (2013)
Grape seed powder extract	Fresh berries *V. vinifera* Roumy Ahmer var.	Polyphenols, proanthocyanidins	Hepatoprotective, antioxidant, enhance antioxidant defenses	Swiss albino mice	Hassan (2012)
Grape skin extract, JF-NATURAL Corporation (Tianjin, CN)	Grape skins	Flavonoids, anthocyanins, catechin, epicatechin, epicatechin gallate, procyanidin B1, procyanidin B2, quercetin 4-glucoside resveratrol	Chemotherapeutic effect against breast cancer with metastases	4T1 cells, female BALB/c mice	Sun et al. (2012)
Grape skin tea infusion	Fermented pinot Noir pomace	Catechin, epicatechin, gallic acid, caffeic acid	Antiviral	MDCK cells, influenza virus	Bekhit et al. (2011)

Grape skin tea infusion	Nonfermented pinot Gris pomace	Catechin, epicatechin, epigallocatechin, caffeic acid, rutin	Antiviral	MDCK cells, influenza virus	Bekhit et al. (2011)
Meganatural-Az® grape seed polyphenolic extract spray dried powder	Grape seeds from California grapes	Catechin, epicatechin, epigallocatechin, epicatechin gallate	Disrupting the ultrastructure of native paired helical filaments found in Alzheimer's disease brain, neutralize phospho-epitopes	Human tissue from autopsy brains diagnosed with AD	Ksiezak-Reding et al. (2012)
Meganatural-Az® grape seed polyphenolic extract spray dried powder	Grape seeds from California grapes	Catechin, epicatechin, epigallocatechin, epicatechin gallate	Inhibit demineralization and promote remineralization of artificial root carious lesions, increase microhardness of the lesions	Extracted human third molars	Xie et al. (2008)
Muscadine grape skin powder	Nonfermented pomace from *Vitis rotundifolia* var. Ison	Phenolics, ellagic acid	Anti-inflammatory, inhibit the release of superoxide and cytokines	Human peripheral blood mononuclear cells, Sprague–Dawley rats	Greenspan et al. (2005)
Nonalcoholic red wine	Red wine	Polyphenols	Protective effects on cardiovascular disease, beneficial effect on insulin resistance	Clinical study with 67 men (55–75 years)	Chiva-Blanch et al. (2013a, b)
Nonalcoholic red wine	Red wine (Montepulciano d'Abruzzo)	Phenolics, flavonoids, anthocyanins	Prevent arterial thrombosis, reduce platelet adhesion to fibrillar collagen	Normolipidemic (FNL) Sprague–Dawley rats	De Curtis et al. (2005)

(continued)

Table 10.1 (continued)

Nutraceutical/supplement	Source	Main compounds	Biological activity	Type of assay	Reference
Nonalcoholic red wine	Spain red wine	Phenolics, anthocyanins	Increase the activity of antioxidant enzymes, protect from oxidative stress	Clinical study with eight volunteers (25–40 years)	Noguer et al. (2012)
Nonalcoholic red wine extract	Alibernet red wine	Polyphenols, potassium, zinc, magnesium, calcium	Increase NOS and SOD activities, increase NO production	Normotensive Wistar Kyoto (WKY) rats, spontaneously hypertensive rats (SHR)	Kondrashov et al. (2012)
Stilbenoids cell cultures extract	*V. vinifera* cell cultures of MAL, ITA, and A. Lavallee	Piceid, *trans*-resveratrol, ε-Viniferin, δ-viniferin	Antitumoral, antiproliferative, induce cytotoxicity in cancer cells	MCF7 (human breast cancer cell line), HEPG2 (human liver hepatocellular carcinoma cell line), MRC5 (normal human fibroblast cell line derived from fetal lung tissue)	Giovannelli et al. (2014)
StilVid® resveratrol-enriched grape extract (Actafarma S.L.)	Grapes illuminated with UV light after postharvest	Resveratrol, procyanidins, anthocyanins, flavonols, hydroxycinnamic acids	Exert cardiovascular benefits in stable patients with coronary artery disease, increase serum adiponectin, inhibit atherothrombotic Signals in peripheral blood mononuclear cells	Clinical trial with 75 patient (18–80 years)	Tomé-Carneiro et al. (2013)

10.3.1 Antioxidant

The only unique and common characteristic of all grape-derived nutraceuticals is their exceptionally high phenolic content. In grapes, phenolics are found in a unique mixture of poly- and simple phenols, and their composition can significantly vary from one cultivar to another (Georgiev et al. 2014a; Ananga et al. 2013a, b). But, the antioxidant quality of different winery by-products is strongly dependent on the phenolic composition of the grape varieties utilized in the winemaking process. For example, nonfermented grape pomace from *V. vinifera* var. Cabernet Sauvignon showed significantly higher total phenolic content and better antioxidant score than the nonfermented pomaces from Merlot and Tanat, and all varieties were cultivated in Brazil (Iora et al. 2014). Rockenbach et al. (2011) investigated phenolic profiles and antioxidant activities of freeze-dried skin and seed powders from nonfermented pomaces from five *V. vinifera* (Primitivo, Sangiovese, Pinot Noir, Negro Amaro, and Cabernet Sauvignon) and one *V. labrusca* (Isabel) varieties. These are varieties that are widely used for making wines in Brazil. It has also been found that the grape seed powders have higher total phenolic contents (composed mainly of flavanols) than the skin powders (composed mainly of anthocyanins), which is in strong correlations with their antioxidant capacity. Maceration process during vinification can decrease the phenolic content of pomace because of enzyme degradation and improved extraction of polar polyphenols from the grape skin and seeds into wine. Comparative study on phenolics in fermented pomaces and mature grapes of six *V. vinifera* varieties (Grenache, Syrah, Carignan, Mourvèdre, Counoise, and Alicante) showed that, despite the intensive extraction during fermentation, most of the phenolics (mainly flavanols and anthocyanins) were retained by fermented skins and seeds (Ky et al. 2014). The authors found that after 11 days of fermentation, the total phenolic content in Alicante grape skins and seeds decreased with almost the same rate (39.6 % in skins—from 52.3 mg GAE/g DW to 31.6 mg GAE/g DW, and 41.8 % in seeds—from 76.4 mg GAE/g DW to 44.5 mg GAE/g DW); nevertheless, the fermented skins and seeds still showed strong antioxidant capacity.

Since the pomace is a commercial by-product, it should be sterilized before it is utilized for production of food grade nutraceuticals. Recent research showed that dry air heating in furnace and autoclave treatment does not have significant effects on antioxidant capacity of heat-treated pomace and grape seed extract (Chamorro et al. 2012). This phenomenon was probably due to the formation of novel compounds such as Maillard products, associated with the observed increase of antioxidant activity in thermal processed products (Chamorro et al. 2012).

Dietary fibers, extracted from grape pomace, are considered as other popular grape-derived products with strong antioxidant action (Table 10.1). A study on antioxidant capacity of dietary fibers obtained from skins of nonfermented white grape pomaces and fermented red grape pomaces showed that the fibers from red varieties (Cabernet Sauvignon, Pinot Noir and Merlot) had more phenolics and better antioxidant scores than the fibers from white varieties (Morio Muscat and Muller Thurgau) (Deng et al. 2011). Considering the strong antioxidant potential of red

grape pomace skin dietary fibers, the authors proposed that they should be used as nutraceuticals, medicines, and/or food supplements.

Antioxidant properties of dietary fibers could vary significantly depending on the employed extraction procedure. For example, a hot aqueous extraction (90 °C, ratio substrate/solvent of 1:4) of white grape pomace (*V. vinifera* var. Chardonnay), followed by membranes concentration, improved the total antioxidant activity of produced fibers (da Silva Ferreira et al. 2013). Moreover, the antioxidant score could be additionally boosted by post-processing treatment for reduction of particle size and increasing the contact area of the fibers (Zhu et al. 2014). Recently, it was demonstrated that superfine grinding treatment by mini-type airflow pulverization system can reduce the particle size of insoluble dietary fiber from fermented red grape pomace (*V. vinifera* var. Cabernet Sauvignon) to submicron size, and thus to increase significantly the antioxidant capacity of obtained product (Zhu et al. 2014).

Another interesting product with antioxidant properties, derived from grape pomace, is the grape seed oils. Grape seed oil is rich in tocopherols (α-, β-, and γ-tocopherols and α- and γ-tocotrienols) and phenolics which contribute to its high antioxidant capacity (Table 10.1) (Fernandes et al. 2013). Recently, grape seed oil was proposed as a candidate of complementary therapy for leukemia because of its positive effect in reducing oxidative stress caused by methotrexate treatment (Sahin et al. 2012). However, it should be pointed out that the content of available tocopherols in grape seed oils and thus their antioxidant capacity can widely vary depending on the extraction protocol used and the oil storage method (Passos et al. 2010). A comparative analyses of total phenolics and antioxidant activities of cold-pressed muscadine seed oils (varieties Carlos and Noble), commercially available red grape seed oil (variety Cabernet Sauvignon) and extra-virgin olive oil performed in our Lab, showed that muscadine grape seed oils had significantly higher antioxidant capacity than the extra-virgin olive oil and red grape seed oil [unpublished data]. Respectively, the cold-pressed seed oils from muscadines grapes have higher polyphenol contents and better antioxidant scores than the seed oils from European grapes (Lutterodt et al. 2011). This observation can be due to the different mixture and composition of accompanying antioxidants extracted from the seeds of American native and common European grape varieties. However, irrespective of the existing evidence indicating the health benefits of grape seed oils (Table 10.1), more research should be performed to identify the nature of active compounds, and their role as antioxidants in vivo.

10.3.2 Anti-inflammatory

Recent advances in the study of pathogenesis in relation to chronic diseases have helped us to understand their close connections with the processes of chronic inflammation. Currently, it is known that several inflammatory transcription factors such as nuclear factor-kB (NF-kB), signal transducer and activator of transcription-3 (STAT3),

activator protein-1 (AP-1), nuclear factor erythroid 2-related factor (Nrf2), and hypoxia inducible factor-1 (HIF-1) have played a major role in the pathogenesis of disease as cancer, cardiovascular diseases, obesity, diabetes, arthritis, and neurodegenerative disorders (Sung et al. 2012). Grape phenolics are plant-derived anti-inflammatory agents able to modulate the expression of these transcription factors (Georgiev et al. 2014a). Moreover, as natural compounds, they have gained particular attention because they can fight inflammation on multi-targeted way and are generally considered as safe (Georgiev et al. 2014a). Grape polyphenols have been found to reduce inflammation by acting as antioxidant, increasing antioxidant gene expression, blocking pro-inflammatory cytokines and transcription factors, attenuating endoplasmic reticulum stress signaling, suppressing inflammatory gene expression, and activating anti-inflammation transcription factors (Table 10.1) (Chuang and McIntosh 2011). Experimental research performed on rats showed that the consumption of freeze-dried grape powder can decrease the levels of inflammation markers in blood serum with 20–50 % (Chuang et al. 2012). It was also demonstrated that quercetin 3-O-glucoside and quercetin 3-O-glucuronide were the bioavailable compounds that attenuated with 30–40 % the gene expression of inflammatory cytokine tumor necrosis factor alpha TNFα in human adipocyte cells (Chuang et al. 2012). Another study carried out in rats showed that extracts from white and red grape pomaces were able to suppress lipopolysaccharide and galactosamine induced chronic inflammation (Table 10.1) (Nishiumi et al. 2012). Moreover, the authors found that the extract from red grape pomace was more effective to suppress the expression of inducible nitric oxide synthase and cyclooxygenase-2 proteins and it was able to inhibit the activation of NF-κB at a dose-dependent manner (Nishiumi et al. 2012).

10.3.3 Antimicrobial

Phenolic compounds are well-known antimicrobial agents. Therefore, it is possible for grape-derived nutraceuticals to have pronounced antimicrobial activities. A study of antimicrobial properties of extracts from different varieties *V. vinifera* (Merlot, Syrah, Cabernet franc, and Cabernet Sauvignon) showed that they were more effective against Gram-positive (*Listeria monocytogenes*) than Gram-negative (*Escherichia coli* and *Salmonella arizonae*) and yeast (*Candida albicans*) strains (Nada et al. 2012). The extracts of grape berry, seeds, pomace, and stems from red (varieties Mandilaria and Voidomato) and white (varieties Asyrtiko and Aidani) *V. vinifera* grapes have been reported to possess strong antilisteria activity (Anastasiadi et al. 2008). It was found that the extracts from seeds, skins, and pomace from Pinot noir were more effective against *S. aureus* and *C. albicans* than the extracts from Pinot meunier (Cheng et al. 2012). Recently it was also demonstrated that the liquid grape seed extracts have strong antimicrobial activity against vegetative cells and spores of *Alicyclobacillus acidoterrestris*, which make it suitable for application in fruit juices and beverages as natural antimicrobial agent (Molva and

Baysal 2015). The high content of oleanolic acid and proanthocyanidins in grape skin and seed extracts suppressed the formation of biofilm of cariogenic *Streptococcus mutants* and can be used for prevention of oral diseases such as dental caries, periodontal disease, and tooth loss (Wu 2009).

10.4 Studies of Intake of Grape-Derived Nutraceuticals in Humans

To understand the potential effects of grape-derived nutraceuticals in the humans, it is essential to considerer the mechanisms involved in the bioavailability of different grape polyphenols and their relevance for human health. Polyphenols undergo substantial metabolism after ingestion and their plasma concentrations usually range from 0 to 4 μmol irrespective of their high concentration in the diet (Manach et al. 2005). A recent study with rats demonstrated that the nonextractable proanthocyanidins in grape dietary fibers are progressively depolymerized into epicatechin monomers in intestinal tract (Mateos-Martín et al. 2012). The later were metabolized by the intestinal microbiota into phenolic acids which was found to be a bioavailable source of antioxidants for a period of 24 h after dietary fiber ingestion (Mateos-Martín et al. 2012). Therefore, it is essential that we understand the mechanisms involved in the bioavailability of different grape polyphenols and their relevance for human health. The daily intake of polyphenols administrated by person with food may vary in wide range (from 100 mg to 2 g). However, most of the consumed polyphenols (over 95 %) passes through the colon and have been degraded by the gut microflora (Shivashankara and Acharya 2010).

The study of biological effects of grape polyphenols on human body is important and of great interest to many scientists. González-Flores et al. (2012) investigated changes in urinary 6-sulfatoxymelatonin and total antioxidant capacity in young, middle-aged, and elderly individuals, in response to consumption of high hydrostatic pressure stabilized grape juice. It was found that consumption of grape juice (200 mL, two times per day) induced a significant increase in urinary 6-sulfatoxymelatonin and total antioxidant capacity in all analyzed groups independently by the age. The authors concluded that stabilized by high hydrostatic pressure grape juice from *V. vinifera* cv. Tempranillo is a high-quality nutritional product with valuable nutraceutical properties (González-Flores et al. 2012). In another study (Table 10.1), a triple-blind, randomized, placebo-controlled trial was conducted with seventy-five patients daily consumed one capsule (350 mg) containing grape extract (GE), resveratrol-enriched grape extract (StilVid®), or placebo (maltodextrin) (Tomé-Carneiro et al. 2012). After 6 months, only LDL cholesterol (LDLc) was significantly decreased in GE consuming group, whereas all atherogenic markers (LDL cholesterol, apolipoprotein-B, oxidized LDL, and oxidized LDL/apolipoprotein-B ratio) were decreased in StilVid® consuming group. Moreover, the consumption of GE or StilVid® had no adverse effects on patients and may exert additional cardioprotection (Tomé-Carneiro et al. 2012). Therefore, these experiments

can verify antioxidant and cardioprotective properties of grape polyphenols in vivo. However, more efforts should be focused on the study of grape-derived nutraceuticals/polyphenols bioavailability and understanding their molecular mechanisms of action in humans.

10.5 Modern Biotechnology in Production of Grape-Derived Nutraceuticals

The demand for grape-derived nutraceuticals will continue to grow as consumers continue to purchase natural health-promoting products. To increase the interest in consumer demand, new products with high quality have to be developed and released to the market. Nowadays, most of grape-derived nutraceuticals available in the market are derived from winery by-products. Despite the undeniable economic and ecologic benefits, the use of winery wastes for nutraceuticals production has some serious disadvantages. One major disadvantage is that the composition of phytochemicals can vary every year depending on seasonal variations in grape growing such as an average temperature, number of sunny days, and atmosphere humidity. This can cause a serious challenge in standardization of final product. The logistic of storage and transportation of winery wastes is another serious challenge. Grape pomace is rich in sugars and is characterized with high water content. When kept in non-aseptic environment, pomace is a perfect medium for supporting microbial growth. Contamination with yeasts and/or bacteria could destroy the active compounds and develop some undesired changes in organoleptics and consistency. Moreover, contamination with molds may result in the release of toxins which are inadmissible in food products. The major advancement made in plant biotechnology in the last few decades can help to overcome these issues (Steingroewer et al. 2013). Cultivation of plant cells, tissue, and organ cultures under controlled in vitro microenvironment is considered as a promising alternative for continuous eco-friendly production of valuable plant-derived secondary metabolites or as a sustainable source of conditioned raw plant biomass (Steingroewer et al. 2013; Georgiev et al. 2014b). Grape biomass obtained by this technology can be used in formulations of "new generation" grape-derived nutraceuticals. In other words, "New generation" grape-derived nutraceuticals could be classified as high quality, standardized, contamination-free grape products, obtained by combined biotechnological approaches including plant cell, organ and tissue in vitro culture technology, bioprocess engineering, optimization procedures, feeding and elicitation strategies, advanced product release and recovery techniques, and/or genetic or metabolic engineering strategies. The new generation grape-derived nutraceuticals are easily standardized, high value-added products with improved pharmaceutical properties in comparison to the bulk grape nutraceuticals. In the last few years, the scientific attention has been focused on development of such grape products. In our lab a red callus culture has been initiated from subepidermal berry skin cells of *M. rotundifolia* (Michx.) Small.var. Noble (Colova et al. 2011). The callus accumulates

significantly higher amount of anthocyanins compared to mature fresh berry skins and fermented freeze-dried skin powder. The culture has been adapted to cultivation in submerged conditions and stable homogenous cell suspension has been generated and supported for more than 3 years. Our experiments show that simple modifications in nutrient media composition can generate significant changes in accumulated dry biomass, color, and phenotype of cultured grape cells. Moreover, those changes are closely accompanied with significant improvements in metabolite profile and biological activities of polar and nonpolar extracts from the cell biomass (unpublished data). We are suggesting that simple media manipulations can be effectively applied to produce high-quality grape biomass with improved metabolite composition and desired biological activity. Similar changes in secondary metabolite profiles triggered by changes in nutrient medium composition (nitrogen and phosphate sources) have been recently reported for grape cell suspensions of *V. vinifera* cv. Pok Dum and the hybrid Bailey Alicante A (*V. lincecumii* × *V. labrusca* × *V. vinifera*) × (*V. vinifera* × *V. vinifera*) (Sae-Lee et al. 2014; Yin et al. 2012).

Hairy root cultures are able to produce remarkably high levels of secondary metabolites when cultured in phytohormone-free medium and can be easily obtained after genetic transformation of plant cells with the soil bacteria *Agrobacterium rhizogenes* (Georgiev et al. 2008). Hairy roots are considered as prospective systems for phytochemicals production, and various bioreactors as well as temporary immersion systems have been developed to provide effective scale-up of this technology (Steingroewer et al. 2013). Recently, hairy root culture was obtained by muscadine grape (*M. rotundifolia* var. Fry) and was suggested as a sustainable source of stilbenoids (resveratrol, piceid, ε-viniferin, and piceatannol) (Nopo-Olazabal et al. 2013).

Several yield-enhancing strategies could be applied to improve the productivity of different grape-derived in vitro systems. For example, elicitation with methyl jasmonate resulted in 7.36-fold increase in the content of resveratrol in muscadine hairy root biomass (up to 466 nmol g/DW) 12 h after treatment (Nopo-Olazabal et al. 2014). The elicitor was also found to increase the antioxidant capacity of hairy root extracts up to 62 μmol TE/g DW (80). Methyl jasmonate has also been used to increase stilbenoids content in cell cultures from *V. vinifera* (varieties MAL, ITA, and A. Lavallee) (Giovannelli et al. 2014). The obtained stilbenoids-enriched extracts showed high cytotoxicity to breast cancer (MCF7 line) and hepatocellular carcinoma cancer (HEPG2 line) cells without affecting the normal human fibroblast cell (MRC5 lines), and thus they have been proposed as new sources of biologically active stilbenoid compounds (Table 10.1). Polysaccharides are also powerful elicitors used to improve stilbenoids production by grape cell suspensions. Chitosan has been used to elicit mono-glucosylated stilbene production by *V. vinifera* var. Barbera cultivated in 1 l stirred-tank bioreactor operated at batch and fed-batch mode (Ferri et al. 2011). It was found that elicitation with chitosan in combination with fed-batch mode of cultivation resulted in maximal increase in mono-glucosylated stilbenoids and resveratrol (23- and 104-fold, respectively). Moreover, the authors reported that chitosan had stimulating effect on mono-glucosylated stilbenoids secretion and increased their release 2- to 18-fold (Ferri et al. 2011). Recently, the same group demonstrated

that chitosan treatment decreased the accumulation of proteins from various pathways of primary metabolites, and thus downregulated the energy, sugar, and amino acid metabolisms in grape cell culture (Ferri et al. 2014). The induced metabolomic modifications generated changes in the metabolite profiles of grape cells and could have direct effects on taste, flavor, organoleptic, and nutraceutical properties of resulted biomass. The use of different elicitors in combination with two-phase cultivation is a powerful strategy for recovery of secreted metabolites. For example, a combined use of methyl jasmonate and β-glucan with adsorbent resin Amberlite XAD7 was used to improve the resveratrol production by *V. vinifera* L. var. Gamay Fréaux cell suspension (Vuong et al. 2014). The authors used combination of elicitation, product release, and in situ recovery of resveratrol to achieve the final yield of 2400 mg/L, which is feasible for an industrial-scale production. However, recent analysis of available data showed that the effective elicitation of stilbenoids production by grape cell suspension cultures depends on the elicitor types, elicitation strategy, and the potential of selected cell line (Jeandet et al. 2014). The data showed that when the right elicitation strategy was applied on high producing cell lines a yield of 5000 mg/L resveratrol could be viable.

Another powerful approach for increasing resveratrol content in grape cell culture is the genetic engineering. Recently, cell suspension of *V. amurensis* Rupr was transformed by overexpressing the gene encoding for a calcium-dependent protein kinase (*VaCPK20*) under the control of an enhanced double CaMV 35S promoter (Aleynova-Shumakova et al. 2014). The resveratrol accumulation in transformed lines was increased between 9- and 68-folds when compared to the nontransformed control. The authors suggested that *VaCPK20* probably plays an essential role in the regulation of resveratrol biosynthesis.

It is clear that grape in vitro cultures are perspective systems for sustainable bioproduction of uniform, high-quality contamination-free grape biomass, as well as for valuable biologically active compounds with grape origin. Nowadays, grape cell technology has been commercialized only for the needs of health-care cosmetics in the form of inclusions in nanoliposomes (Georgiev et al. 2014a; Ananga et al. 2013a, b). However, to step-up on food nutraceuticals market, some important technological aspects, concerning maintenance of commercially viable cell lines, process scale-up, achieving of time-stable and economically feasible yields, nutraceutical products development, and market positioning strategy, are yet to be addressed.

10.6 Conclusions

In the last decade thousands of commercial products have been developed and released on the market in response to the continuously growing demand of natural additives with health beneficial properties. However, the increasing assortment of plant-derived products raises the needs for the development of strict definition and regulation of terminologies to be used, which may help consumers to understand

the differences between nutraceuticals, functional foods, botanicals and dietary supplements, and thus to be careful when making their choice. In the last few years, nutraceuticals made from grapes or grape products are of particular interest due to their specific combination of nutritional and medicinal properties. Regardless of the few existing examples for nonalcoholic and powdered wines, these products are still facing serious technological concerns complicating their commercialization. Low-alcoholic wines (0.3–5.0 vol.% alcohol) indeed seem to be more perspective products, since they have similar organoleptic properties and shelf-life close to the regular wines. However, the true nutraceutical value from grapes could be found in pomace. Utilization of grape pomace in the form of various nutraceutical products is a great example for transforming winery waste to high added-value products. We strongly recommend integration of grape pomace processing units in winemaking, since that low-cost investment technology for production of bulk nutraceuticals or food supplements could have significant economic and ecological effects, especially for small wineries. In order to produce highly standardized contamination-free grape-derived nutraceuticals with improved pharmaceutical properties, plant in vitro techniques may be used. We have classified these high-quality products, obtained by the combined methods of modern plant biotechnology as "New generation grape-derived nutraceuticals," and we have suggested their increased popularity among the most consumers. Moreover, by using the advanced techniques of genetic and metabolic engineering of in vitro grape cells, almost unlimited options for production of target biologically active metabolite or group of metabolites with direct pharmaceutical application could be realized. However, many technological questions, such as cell line development, process optimization, product isolation, formulation, bioavailability in humans, and safety, have to be resolved before commercial realization of this technology.

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Part III
Wine and Health

Chapter 11
Mechanism of the Protective Effects of Wine Intake on Cardiovascular Disease

Rosa M. Lamuela-Raventós and Ramón Estruch

11.1 Introduction

Wine, an alcoholic beverage obtained by the fermentation of grape must, contains a diverse range of compounds (see Table 11.1) that derive mainly from the raw material, grape berries, including sugars, vitamins, tartaric and malic acids, and almost all the polyphenols. Other components of wine, such as ethanol and secondary by-products like the polyphenol tyrosol, are formed during the fermentation process. Besides ethanol, wine polyphenols seem to be the main compounds responsible for the health effects attributed to moderate wine consumption. Although the phenolic profile of wine varies according to variety (Romero-Pérez et al. 1996a, b; Lamuela-Raventós et al. 1995), cultural conditions (De Andrés-de Prado et al. 2007), the wine-making process (Betés-Saura et al. 1996), and storage time, similar beneficial effects have been observed among different types of red wine. White wine seems to have less impact on the cardiovascular (CV) system, since a unit of white wine contains approximately 48 mg of polyphenols, while a unit of red wine has approximately 300 mg (see Table 11.1). This greater effect seems, therefore, to be related to the higher phenolic content, a consequence of maceration with pomace contact

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Table 11.1 Dry wine composition

Components	Percentage (g/100 mL)
Water	87
Ethanol	11
Sugars, acids	<1
Volatiles	0.5
Polyphenols in red wines	0.2
Polyphenols in white wines	0.02

during the wine-making process, which favors phenolic extraction from the solid parts of the grape berry. In this chapter, we have summarized the mechanisms by which main wine components (ethanol and polyphenols) might exert protective effects on CV diseases.

11.2 Mechanistic Effects of Ethanol Consumption on Health

Although excessive alcohol consumption is very harmful on several levels, epidemiological evidence consistently points to an inverse association between moderate alcohol consumption and CV events and risk factors (Mukamal et al. 2003; Brien et al. 2011; Ronksley et al. 2011). Responsible habitual alcohol use also appears to be linked to lower risks of diabetes mellitus, stroke, heart failure, and total mortality (O'Keefe et al. 2007). People consuming one-to-two standard drinks per day showed a lower rate of CV events than subjects abstaining from alcohol and those with a high alcohol intake, a relationship represented by a J-shaped (or U-shaped) curve (Ruidavets et al. 2010). Increase of plasma HDL cholesterol and Apo-AI is the most described effect of moderate alcohol consumption, and until recently, this was considered as the main protective effect of alcohol intake on the CV system.

Light-to-moderate alcohol intake has also been shown to improve outcomes in patients with established CV disease. In a meta-analysis of eight prospective studies involving 16,351 patients with a history of CV disease, the familiar J-shaped curve was observed, with a maximal protective effect of alcohol at approximately 26 g/day (or about two drinks daily) (Costanzo et al. 2010).

This effect is observed after the consumption of any alcoholic beverage in a dose-dependent manner, and accordingly, it is considered to be due to ethanol itself (Betés-Saura et al. 1996; Chiva-Blanch et al. 2013a, b). Accumulating scientific evidence also suggests that light-to-moderate alcohol intake may enhance insulin sensitivity, increase adiponectin levels, and improve endothelial function (Estruch and Lamuela-Raventós 2014; Crozier et al. 2009; Neveu et al. 2010). However, the protective effect of moderate alcohol consumption also depends on the pattern of alcohol use, whether the intake is concentrated at certain times, usually at the weekends, or is regularly spread over the week during meals. Binge drinking, usually defined as episodic excessive alcohol intake (≥5 drinks within a few hours), often

with intent to become intoxicated, is associated with a twofold higher risk of mortality (Ruidavets et al. 2010; Mukamal et al. 2005). Even occasional binges attenuate the protection offered by otherwise light-to-moderate consumption (O'Keefe et al. 2014).

Epidemiological studies and the meta-analysis of their results clearly show a statistically significant association between moderate alcohol consumption and CV events, an effect attributed to ethanol itself, since there is still no conclusive epidemiological evidence that wine polyphenols exert different effects from those of ethanol (Poli et al. 2013). For this reason, in order to evaluate the different effects of ethanol and polyphenols, randomized intervention clinical trials that evaluate ethanolic drinks with and without polyphenols are necessary. To date, the studies performed have only focused on intermediate markers of CV risk (Chiva-Blanch et al. 2012a, b, 2013a, b; Estruch et al. 2011), and studies on hard endpoints as final variables are still lacking. This fact may be explained since the duration of these studies is limited by the personal difficulties in accomplishing long interventions (Estruch and Lamuela-Raventós 2014).

11.3 Mechanistic Effects of Polyphenols on Health

Although present mainly in plant foods and beverages, polyphenols are not considered nutrients, since they are not essential for the human body. Instead, they are defined as a very heterogeneous group of bioactive compounds, with over 8000 different molecules identified (Crozier et al. 2009). All polyphenols are characterized by the presence of one or several phenolic groups in their structure, and are divided into five main groups accordingly: phenolic acids, flavonoids, stilbenes, lignans, and others such as secoiridoids (Neveu et al. 2010). This chemical structural diversity should be considered when studying the CV effects of these compounds.

Polyphenols are the most abundant antioxidants in our diet (Scalbert et al. 2005). Total dietary extractable polyphenol consumption is between 800 mg/day (Tresserra-Rimbau et al. 2013) and 1 g/day (Pérez-Jiménez et al. 2011), which is around 10 times higher than intake of vitamin C and 100 times higher than vitamin E and carotenoid intakes (Scalbert et al. 2005). However, not all polyphenols have the same role, since their diverse structures confer very different physiological properties. The mechanism by which these bioactive compounds may exert protective effects on the CV system is still unclear. Initially, all hypotheses have centered on their antioxidant capacity, since they show high antioxidant activity in vitro; nevertheless, their antioxidant capacity in the human body remains unclear (Rubio et al. 2014).

It is well known that the protective effects of polyphenols in vivo depend on their chemical structure (Crozier et al. 2009), accessibility and extractability from food (Tulipani et al. 2012), their intestinal absorption and interaction with microbiota, their metabolism and final biological action in the human body, and potential interaction with target tissues (Rubio et al. 2014; Hollman et al. 2011).

11.3.1 Wine Polyphenols and Oxidative Status

While alcohol by itself is known to induce oxidative stress, wine polyphenols seem to counteract this effect (Chiva-Blanch et al. 2013a; Estruch and Lamuela-Raventós 2014). Several clinical trials have shown the antioxidant effect of moderate red wine consumption. The intake of wine polyphenols was found to increase plasma antioxidant capacity (Micallef et al. 2007). However, the total antioxidant capacity assay is inconclusive because it detects urate as the main contributor, and the clinical significance of the increase in total antioxidant capacity due to urate concentrations is unclear. On the other hand, wine polyphenols apparently decrease plasma malondialdehyde (Estruch et al. 2011; Micallef et al. 2007), a measurement of oxidative stress status, as well as inhibit LDL cholesterol particle oxidation and increase activity of several antioxidant enzymes (Estruch et al. 2011). Despite a general consensus attributing antioxidant benefits in healthy volunteers to sustained wine consumption, there is not enough scientific evidence, at present, that sustained wine consumption provides antioxidant benefits other than to counteract a possible pro-oxidative effect of the alcohol (Covas et al. 2010). On the contrary, data on the antioxidant protective effects of red wine in oxidative stress situations are promising. Thus, postprandial oxidative stress seems to be counteracted by the ingestion of red wine, effect being attributed to wine polyphenols, despite the diversity of biomarkers used for its evaluation. It has been proposed that polyphenols, including the non-absorbable ones, could exert antioxidant and other cytoprotective effects in the gastrointestinal tract, where they are present in high quantities (Covas et al. 2010; Kanner et al. 2012). On this basis, the Mediterranean diet pattern that includes moderate wine consumption with meals would counteract the pro-oxidant effect of food digestion.

11.3.2 Wine Polyphenols and Inflammation

There is growing evidence for the anti-inflammatory effects of sustained wine consumption (Sacanella et al. 2007; Estruch et al. 2004; Chiva-Blanch et al. 2012b), even in low-cardiovascular-risk individuals (Sacanella et al. 2007; Estruch et al. 2004). Oxidative stress and inflammation are intertwined processes (Covas et al. 2010): inflammation promotes oxidative stress and oxidative damage, and vice versa (Bigarella et al. 2014; Crowley 2014). Red wine, but not gin, diminished C-reactive protein in plasma in healthy subjects (Estruch et al. 2004) and favored anti-inflammatory interleukins (Chiva-Blanch et al. 2012b). Similar results were observed for cell adhesion and cytokines (Estruch et al. 2004; Chiva-Blanch et al. 2012b), molecules that participate in the recruitment of circulating leukocytes to the vascular endothelium, initiating the atherosclerotic process. However, when dealcoholized red wine was compared with the same alcoholized wine, the phenolic content was found to be responsible for modulating leukocyte adhesion molecules, whereas both ethanol and polyphenols of red wine may modulate soluble inflammatory mediators in high-risk patients (Chiva-Blanch et al. 2012b).

11.3.3 Wine Polyphenols and Blood Pressure

A linear correlation between alcohol intake and increased blood pressure has been reported in normal subjects and hypertensive patients (Fuchs et al. 2001). However, in moderate amounts it appears to exert neutral or even beneficial effects on blood pressure, and red wine seems be superior to other alcoholic beverages in this respect (Poli et al. 2013; Estruch and Lamuela-Raventós 2014). Thus, in a crossover feeding trial, moderate doses of dealcoholized red wine decreased systolic and diastolic blood pressure while increasing plasma nitric oxide (NO) concentration (Chiva-Blanch et al. 2012a). Red wine tended to induce a similar pattern to dealcoholized red wine, although the changes did not achieve statistical significance, while moderate gin consumption had no effect. Thus, these blood pressure-lowering and NO-raising effects should be attributed to red wine polyphenols and not to alcohol. Polyphenol effects on blood pressure have also been demonstrated in the PREDIMED trial. The decrease in blood pressure was correlated with an increase in plasma NO concentration, and wine was one of the main sources of polyphenols in this Mediterranean population (Medina-Remón et al. 2015).

11.3.4 Wine Polyphenols and Lipid Profile

Compared to other alcoholic beverages red wine consumption may exert an additional protection effect against cardiovascular disease. Thus, in a recent feeding trial comparing the effects on wine, dealcoholized wine and gin, plasma LDL cholesterol, and apolipoprotein B concentrations were significantly reduced after wine and dealcoholized wine interventions, but not after gin, suggesting that this effect should be related to nonalcoholic content of red wine, mainly polyphenols (Chiva-Blanch et al. 2013b). In addition, both cross-sectional and intervention studies have shown that moderate wine intake reduces the plasma concentrations of in vivo-oxidized LDL, which has also been related to the polyphenolic content of red wine (Schroder et al. 2006). Thus, according to these results, polyphenols in red wine also help to improve lipid profile.

11.3.5 Wine Polyphenols and Diabetes Mellitus

In some prospective studies, the inverse association between moderate alcohol intake and low diabetes risk was most apparent in wine and beer drinkers compared to those who reported liquor intake (Wannamethee et al. 2003). The results of the few randomized clinical studies that examined the effects of moderate alcohol consumption on insulin sensitivity, an accurate measurement of the glucose metabolism, have been inconsistent. Although some have reported null results (Beulens

et al. 2006; Sierksma et al. 2004), another study has concluded that red wine exerts higher protective effects than other alcoholic beverages (Napoli et al. 2005). Thus, in a recent randomized clinical trial that examined the effects of three interventions (red wine, dealcoholized red wine, and gin), both red wine and dealcoholized wine decreased HOMA-insulin resistance index, a measurement of insulin sensitivity, by 30 % and 22 %, respectively (Chiva-Blanch et al. 2013b), suggesting that polyphenols contained in wine exert a positive effect on glucose metabolism. However, in another trial that included 51 postmenopausal women, consumption of 30 g/day of alcohol (ethanol in orange juice) was associated with a 7.2 % improvement in insulin sensitivity compared with 0 g/day, while consumption of 15 g/day of alcohol had no effect. Therefore, considering all these results together, it seems that both ethanol and polyphenols contained in alcoholic beverages are responsible for beneficial effect observed on glucose metabolism.

Interestingly, adiponectin has been proposed as an important link between moderate alcohol consumption and lower incidence of type 2 diabetes. Thus, in an open randomized crossover intervention study, plasma adiponectin concentration significantly increased after moderate consumption of wine, beer, and ethanol, but the changes after wine consumption (30 % increase) were higher than after ethanol (17 %) or beer (16 %). In this case, the additional effect of wine on adiponectin concentration could not be attributed to their polyphenolic content since dealcoholized wine did not exert any significant effect. Changes in sex hormones or dietary pattern on adiponectin concentrations may be other potential explanations for these findings (Imhof et al. 2009).

11.4 Conclusions

Results from randomized clinical intervention trials indicate that the health benefits attributed to moderate wine consumption on cardiovascular system may be due not only to ethanol, which increases HDL cholesterol and Apo A-1, but also to polyphenols contained in some alcoholic beverages (see Fig. 11.1). Although wine polyphenol content may vary, according to several factors, it seems that all wines, especially red wines, have a protective cardiovascular activity. Wine polyphenols counteract the pro-oxidant effect of meal digestion, decrease blood pressure, improve lipid profile and glucose metabolism, and exert anti-inflammatory and antioxidant effects. Therefore, a moderate consumption of wine during meals, following the Mediterranean life style, should be recommended. Despite the difficulties of carrying out long-term clinical intervention trials, with their concomitant ethical considerations, more research is needed to confirm the preventive effects of moderate wine consumption on "hard cardiovascular endpoints" such as myocardial infarction, stroke, and cardiovascular deaths, and elucidate whether other mechanisms are involved.

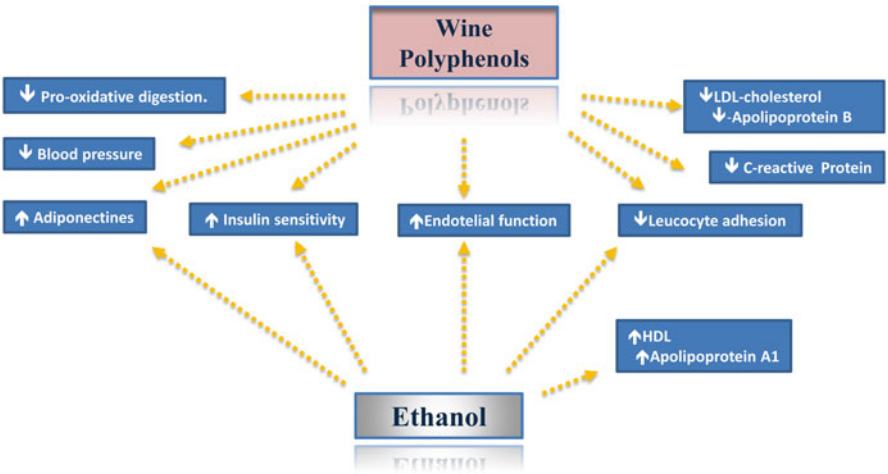

Fig. 11.1 Mechanistic effects of wine components, from intervention clinical trials

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Chapter 12
Role of Wine Components in Inflammation and Chronic Diseases

Creina S. Stockley

12.1 Introduction

It is well documented in peer-reviewed published data over more than three decades that the moderate consumption of alcoholic beverages such as wine is associated with a reduced risk of developing and dying from cardiovascular diseases (CVD), certain cancers, diabetes, and neurodegenerative disorders such as dementia (Grønbæk et al. 2000). This equates to a reduced risk of dying from all or any causes and is in comparison to abstainers and heavy consumers, where moderate consumption is generally considered as not more than two standard drinks/day for both men and women. These relationships have best been described as j-shaped and most relevant for individuals aged over 40–45 years, that is, in particular for those who are at greater risk of CVD (Di Castelnuovo et al. 2004; Mukamal et al. 2010; Jayasekara et al. 2014). A meta-analysis by Klatsky and Udaltsova (2007) suggests that the benefit extends to approximately four standard drinks/day [40 g alcohol/day], as does that of Doll et al. (2005) and Mukamal et al. (2006). The risk of adverse health effects increases, however, when alcohol consumption increases from light-to-moderate to heavy (Costanzo et al. 2010; Bagnardi et al. 2013).

Inflammation is a critical component of CVD, neurodegenerative diseases, diabetes, and some cancers which are diseases associated with increasing age. Ageing is accompanied by chronic low-grade inflammation state clearly showed by two- to fourfold increase in serum levels of inflammatory mediators. A range of factors has been claimed to contribute to this state where the most important role seems to be played by the chronic antigenic stress, which affects the immune system throughout life with a progressive activation of macrophages and related cells (de Martinis et al. 2005). This pro-inflammatory status, interacting with the genetic background,

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potentially triggers the onset of age-related inflammatory diseases such as atherosclerosis (Licastro et al. 2005).

After a brief description of the main topics involved in the inflammatory response, this chapter reviews the literature concerning the effects of wine consumption on inflammation related to atherosclerosis, cancer, and dementias, as well as the mechanisms behind these effects.

12.2 The Inflammatory Response

In response to cell injury elicited by trauma or infection, the inflammatory response sets in, constituting a complex network of molecular and cellular interactions directed to facilitate a return to physiological homeostasis and tissue repair as shown in Fig. 12.1. The response is composed of both local events and a systemic activation mediated by cytokines (Licastro et al. 2005). If tissue health is not restored or in response to stable low grade irritation, inflammation becomes a chronic condition that continuously damages the surrounding tissues (Mitchell and Cotran 2003). While acute inflammation is important to the immune response, chronic inappropriate inflammation can cause tissue destruction such as neurodegenerative and CV diseases.

Cytokines are largely secreted molecules that act on the surrounding microenvironment by providing cell to cell signalling. Cytokines are components of a large, complex signalling network. The effects of cytokines on target cells may be inhibited or enhanced by other cytokines, hormones, and cytokine-receptor antagonists and circulating receptors. Tumour necrosis factor α (TNF-α), Interleukin-1 (IL-1), and Interleukin-6 (IL-6) are the classical pro-inflammatory cytokines which activate both local and systemic effects (Licastro et al. 2005). Locally, they contribute to the activation of the inflammatory cells and together with chemokines, which induce the expression of adhesion molecules, cause their local recruitment. When the causes of the inflammatory reaction are of high intensity, the production of cytokines is increased and they are released in the circulation provoking an acute phase response.

Conversely, cytokines such as IL-10 damp down the activation of some effector functions of T-lymphocytes and mononuclear phagocytes, by inhibiting the release of pro-inflammatory cytokines and therefore turning off the inflammatory processes (Lio et al. 2004). The acute phase response which includes the hepatic synthesis of acute phase proteins such as C-reactive protein (CRP) is a highly conserved inflammatory response which is rapidly activated by infections or trauma via pattern recognition molecules. Acute phase protein concentration rapidly increases after infection, and their production is controlled primarily by IL-6- and IL-1-type cytokines. The acute phase proteins provide enhanced protection against microorganisms and modify inflammatory responses by affecting cell trafficking and mediator release. Some acute phase proteins have anti-inflammatory effects while others have important effects on leukocyte activation and trafficking (Gabay and Kushner 1999).

CRP is a sensitive systemic marker of inflammation and tissue damage. In most, but not all diseases, the circulating value of CRP reflects on-going inflammation much

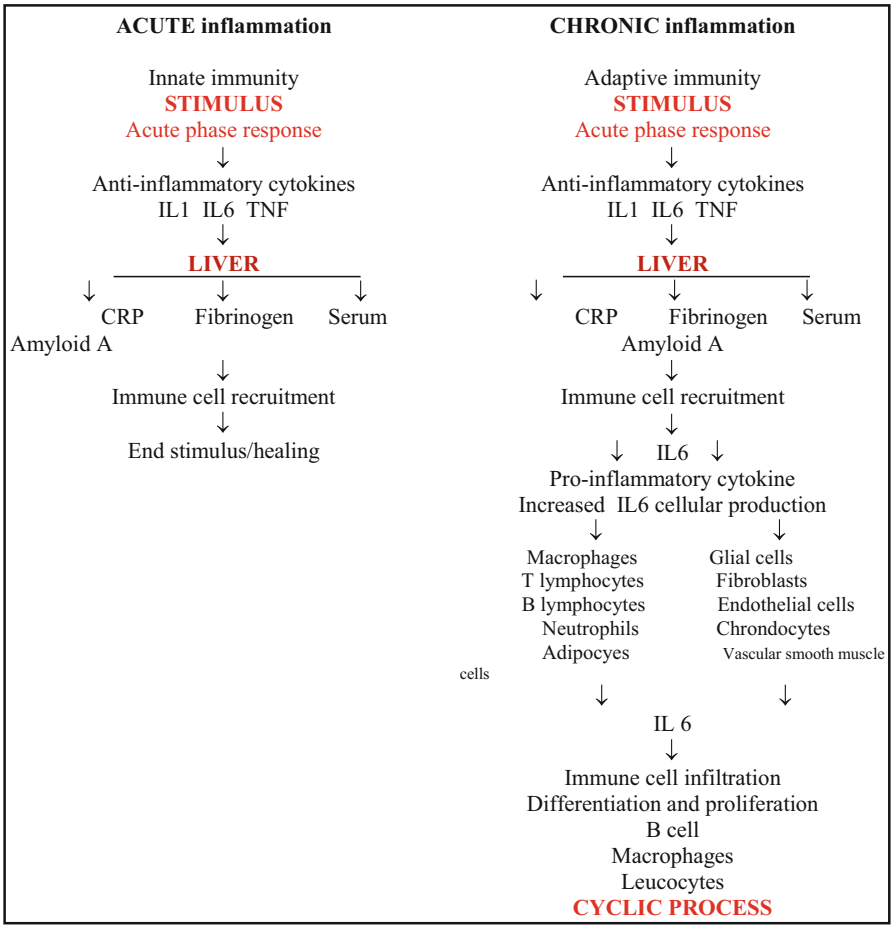

Fig. 12.1 Proposed pathways of inflammation

more accurately than do other biochemical parameters of inflammation, such as plasma viscosity or the erythrocyte sedimentation rate (Pepys and Hirschfield 2003).

12.3 Inflammation and Chronic Diseases Such as Atherosclerosis

Anti-inflammatory mechanisms have been suggested to contribute to the beneficial effect of moderate alcohol consumption on CVD risk beyond favourable changes in lipids and haemostatic factors. Studies on potential anti-inflammatory effects of moderate alcohol consumption in the general population are relatively few and many have not differentiated between types of alcoholic beverages.

Atherosclerosis is implicated in CVD (Witztum and Steinberg 1991). It is an inflammatory disease, characterised by local inflammation in the blood vessel wall (Libby 2002), but also shows a systemic, low-grade response as indicated by elevated C-reactive protein (CRP), total white blood cell count, fibrinogen and plasma viscosity, and decreased albumin which are all independently associated with an increased risk of CVD (Djoussé et al. 2002; Koenig et al. 2003; Imhof et al. 2008). Indeed, the atherosclerotic processes underlying CVD are also intimately connected with a state of chronic inflammation involving a variety of pathological changes including endothelial cell activation, low density lipoprotein (LDL) modification, macrophage chemotaxis, and vessel smooth muscle cell migration (Ross 1999).

It has been shown that as atherosclerosis develops vascular smooth muscle cells are released by platelets and endothelial cells, which proliferate and accumulate within the intima of the blood vessel wall, to further develop the atherosclerotic lesion or plaque. The primary mitogenic and chemotactic compound for the release of the vascular smooth muscle cells is platelet-derived growth factor, which exerts its effects via activation of two subtypes of trans-membrane receptor tyrosine kinases, α and β platelet-derived growth factor receptors (Heldin and Westermark 1999). In vitro research suggests that wine-derived phenolic compounds, and in particular flavonoids such as catechin, may inhibit the activation of the β platelet-derived growth factor receptors, and hence platelet-derived growth factor and the subsequent proliferation and migration of vascular smooth muscle cells (Rosenkranz et al. 2002). The postprandial suppression of smooth muscle cell proliferation by ethanol has also been observed, but the mechanism of action is independent to that of the wine-derived phenolic compounds (Locher et al. 1998).

Imhof et al. (2004) and Albert et al. (2003) both observed that in individuals consuming alcoholic beverages moderately, a lower concentration of C-reactive protein and other pro-inflammatory markers is observed than in abstainers or heavy consumers, an effect that was independent of effects on lipoproteins and on fibrinogen. An examination of 7887 men and women by Imhof et al. (2004) suggested that either wine or beer was associated with lower levels of systemic inflammatory markers, which implied that the alcohol component common to the wine and beer might be primarily responsible for the potential anti-inflammatory effects.

Thus it has been suggested that favourable changes in inflammation as well as in blood lipids and in haemostatic profile, in addition to reduced insulin resistance, might mediate the atheroprotective effect of moderate alcohol consumption (Lazarus et al. 1997).

12.3.1 Potential Interaction of Alcohol with Inflammation in Atherosclerosis

Alcohol has been shown to suppress the synthesis of pro-inflammatory cytokines and chemokines, such as TNF-α, IL-1β, IL-6, IL-8, and MCP-1, both in vivo and in vitro, in human blood monocytes (Szabo et al. 1995). This suggests that there is

a decrease of acute phase proteins or reactants after the consumption of alcoholic beverages given IL-6 is the primary regulator of genes encoding for acute phase reactants (Baumann and Gauldie 1994).

Moderate alcohol consumption may exert anti-inflammatory effects locally in the vascular bed as well as by influencing the concentration of circulating inflammatory mediators (Imhof et al. 2004). Dual anti-inflammatory effects have been observed that involve augmentation of anti-inflammatory cytokines such as TGF-β and IL-10 produced by macrophages and T lymphocytes, and attenuation of monocyte inflammatory responses involving inhibition of NF-κB (Szabo et al. 1992; Mandrekar et al. 2006).

Any beneficial anti-inflammatory effects of moderate alcohol consumption may, however, be reversed with heavier alcohol consumption. Heavy alcohol consumers, both with and without alcoholic liver disease, have been observed to have an increased concentration of pro-inflammatory markers with concomitant tissue damage including the liver (Tilg and Diehl 2000; Imhof et al. 2004).

12.3.2 Potential Anti-inflammatory Effects of the Phenolic Compounds

In addition to the alcohol component of wine, wine-derived phenolic compounds may also modulate soluble inflammatory mediators. In healthy and high CVD risk individuals both alcohol and wine-derived phenolic compounds, for example, have been observed to downregulate serum concentrations of CD40 antigen, CD40 ligand, IL-16, monocyte chemotactic protein-1, and vascular cell adhesion molecule-1 (Chiva-Blanch et al. 2012). Independently, alcohol increased IL-10 and decreased macrophage-derived chemokine concentrations, whereas the wine-derived phenolic compounds decreased serum concentrations of intercellular adhesion molecule-1, E-selectin, and IL-6 and inhibited the expression of lymphocyte function-associated antigen 1 in T lymphocytes and macrophage-1 receptor, Sialyl-Lewis X, and C-C chemokine receptor type 2 expression in monocytes.

In addition, inhibition of NF-κβ activity has been observed in human monocytes post-consumption of moderate amounts of red wine but not after vodka (Blanco-Colio et al. 2000). Consistent with the results of this and other studies, Estruch et al. (2004) observed in healthy individuals significant reductions of vascular cellular adhesion molecule-1 (VCAM-1), intercellular CAM-1 (ICAM-1), IL-1a, and very late antigen-4 (VLA-4) lymphocyte expression and lymphocyte function-associated antigen-1 (LFA-1), macrophage-1 antigen (Mac-1), VLA-4 and monocyte chemoprotein-1 (MCP-1) monocyte expression after red wine, but not after the consumption of gin.

Furthermore, these results agree with in vitro or animal models that analysed the effect of wine-derived phenolic compounds in the regulation of these molecules. Resveratrol, for example, reduced ICAM-1 expression in human umbilical vein

endothelial cells (Ferrero et al. 1998) and IL-6 in vascular smooth-muscle cells (Inanaga et al. 2009). The pre-incubation of polymorphonuclear leukocytes with resveratrol resulted in a concentration-dependent inhibition of fMLP (formyl methionyl leucyl phenylalanine)-induced Mac-1 expression (Rotondo et al. 1998). A decrease in CCR2 expression was also observed in the arterial wall of apolipoprotein E2/2 mice after oral administration of resveratrol (Norata et al. 2007), while CCR2 expression was inhibited in a time- and dose-dependent manner by resveratrol in human THP-1 monocytes (Cullen et al. 2005).

Giovannini et al. (2002) observed in vitro that the hydroxycinnamate, caffeic acid modulated the expression and release of the cytokine TNF-α from human monocytes, even at low doses. TNF-α-induced adhesion of monocytes to human endothelial cells was also observed to be virtually abolished after red wine consumption which had a high phenolic content, but was only partially reduced after gin consumption. This effect may be due to the downregulation of adhesion molecules on the monocyte surface (Badía et al. 2004). In addition, comparison of a medium phenolic content cava sparkling wine to gin showed that the effects of cava on circulating CD40L, ICAM-1, and MCP-1, and monocyte surface expression of CD40, LFA-1, and VLA-4 were greater than those of gin (all $P < 0.05$) (Vázquez-Agell et al. 2007).

It may be concluded from these studies that wine-derived phenolic compounds have additive as well as different anti-inflammatory effects to those of alcohol. Accordingly wine potentially may exert a greater beneficial effect against early stages and progression of atherosclerosis than other alcoholic beverages.

12.4 Cancer

An examination of recent systematic reviews and meta-analyses suggests that the occurrence of cancers of the oral cavity, pharynx, larynx, oesophagus, liver, and breast may be causally related to the consumption of wine, and especially heavier wine consumption (IARC 1998, 2010; Bagnardi et al. 2015). In addition, it suggests that wine consumption may be less associated than other alcoholic beverages to the risk of certain cancers, which may reflect differences in chemical composition between the different alcoholic beverages, namely phenolic compounds (Benedetti et al. 2009; Ferrari et al. 2014). It was also suggested in the examination that light to moderate wine consumption may lower the risk of some other cancers such as lung (Chao et al. 2008), colorectal (Chao et al. 2010), endometrial (Friedenreich et al. 2013), ovarian (Yan-Hong et al. 2015), and lymphomas (Briggs et al. 2002), where wine-derived phenolic compounds may counter the cellular and other damage caused by alcohol and its primary breakdown product acetaldehyde.

12.4.1 Inflammation and Cancer

The majority of cancer occurs in individuals aged over 65 years. Therefore, it has been suggested that ageing could be considered as a surrogate marker of duration of exposure to relevant carcinogenic factors (Caruso et al. 2004). The link between inflammation and cancer appears to stem from two pathways (Mantovani et al. 2008), one intrinsic and the other extrinsic as shown in Fig. 12.2. Intrinsic inflammation is initiated by mutations that lead to activation of oncogenes and inactivation of tumour suppressors (tumour-promoter role) (Mantovani et al. 2010). Conversely, in the extrinsic pathway, infection or inflammation precedes cancer and increases the risk of cancer (tumour-initiator role) (Mantovani et al. 2010). The similarity between cancer tissue and inflamed tissue involves angiogenesis and tissue infiltrating leukocytes, such as lymphocytes, macrophages, and mast cells (Hanahan and Weinberg 2011; Trinchieri 2011).

Several recent studies have suggested that inflammation has an important role in all phases of tumour development, including tumour initiation, tumour promotion, invasion, metastatic dissemination, and evading the immune system (Hanahan and Weinberg 2011; Trinchieri 2011, 2012). Inflammation causes cellular stress and may trigger DNA damage or genetic instability, and chronic inflammation can contribute to primary genetic mutations and epigenetic mechanisms that initiate malignant cell transformation (Chang 2010; Trinchieri 2012). Tumour-promoting effects of inflammation alter tissue homeostasis, predisposing individuals to cancer

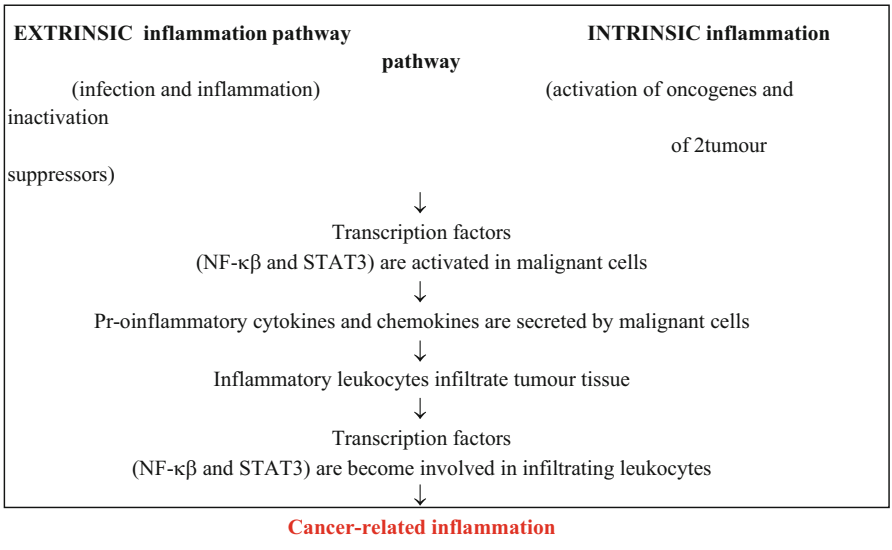

Fig. 12.2 The intrinsic and extrinsic inflammation pathways associated with the development of cancer (adapted from Guven Maiorov et al. 2013)

(Grivennikov et al. 2012). Inflammation establishes a tissue microenvironment, which tolerates tumour growth and metastasis by setting immunosuppressive mechanisms. Therefore, inflammation not only induces carcinogenesis but also makes immune cells incapable of destroying tumour cells.

Inflammatory cells supply growth factors to maintain proliferation, survival factors to escape from apoptosis, pro-angiogenic factors and extracellular matrix modifying enzymes that enable angiogenesis, invasion, and metastasis (Trinchieri 2011). Inflammatory cells can also secrete reactive oxygen species (ROS) that induce mutations, lead to failure of DNA repair, activation of oncogenes, and ultimately cancer (Chang 2010; Trinchieri 2011, 2012). ROS further activates inflammatory genes and takes part in tumourigenesis which is regulated by c-MYC, K-Ras, and Wnt signalling pathways (Trinchieri 2011).

12.4.2 Potential Anti-inflammatory Effects of Wine and Wine-Derived Phenolic Compounds in Cancer

Given that the pro-inflammatory cytokines IL-6, TNF-α, and IL-10, which are associated with atherosclerosis, also appear to be implicated in the pathogenesis of cancer, it can be assumed that the phenolic compounds described in Sect. 12.3.2 modifying the risk of atherosclerosis might also modify the risk of certain cancers. Phenolic compounds such as resveratrol appear to block the multistep process of carcinogenesis at various stages of tumour initiation, promotion, and progression although the actual mechanisms of action are still to be fully elucidated.

Resveratrol's anti-inflammatory anticarcinogenic effects may be partially ascribed to the inhibition of activation of NF-κβ and AP-1 and the associated kinases. For example, resveratrol may inhibit the synthesis and release of pro-inflammatory mediators such as TNF-α, which are also required for tumour promotion (Suganuma et al. 1999), modify eicosanoid synthesis, inhibit activated immune cells, or inhibit inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) via its inhibitory effects on the transcription factor NF-κβ or the activator protein-1 (AP-1) (Donnelly et al. 2004).

There have been innumerable in vitro, animal, and ex vivo studies in the past two decades which have partially determined resveratrol's mechanisms of action. Manna et al. (2000) initially observed in vitro that resveratrol blocked TNF-α-induced activation of NF-κβ in a dose- and time-dependent manner. Resveratrol also suppressed TNF-α-induced phosphorylation and nuclear translocation of the p65 subunit of NF-κβ, and NFκβ-dependent reporter gene transcription. Suppression of TNF-α-induced NF-κβ activation by resveratrol was observed in several cell lines such as myeloid (U-937), lymphoid (Jurkat), and epithelial (HeLa and H4) cells. Resveratrol was also observed by Manna et al. (2000) to block NF-κβ activation induced by PMA, LPS, H_2O_2, okadaic acid, and ceramide. The suppression of NF-κβ coincided with suppression of AP-1; most chemical compounds that activate NF-κβ also activate AP-1 (Karin et al. 1997). Resveratrol also inhibited the TNF-α-induced activation of MAPK kinase and c-Jun N-terminal kinase, and abrogated TNF-α-

induced cytotoxicity and caspase activation. Both ROM generation and lipid peroxidation induced by TNF-α were suppressed by resveratrol.

Subsequent animal studies have observed that resveratrol exhibits anti-inflammatory activity via pathways centred on COX-1 and COX-2 (Sengottuvelan et al. 2009). COX is the enzyme of the rate-limiting step of the pathway that produces mediators of inflammation, and there is also an inverse relationship between COX-2 enzymes and tumour growth (Banerjee et al. 2002). In addition to inhibition of COX-1 and COX-2 expression, through upstream suppression of the activity of NF-kB and I-kB kinase (Kundu et al. 2006; Golkar et al. 2007), resveratrol reduced the production of prostaglandin E2 (PGE2) and the formation of ROS in lipopolysaccharide (LPS)-activated microglial cells (Kim et al. 2007; Candelario-Jalil et al. 2007). Candelario-Jalil et al. (2007) reported that this activity of resveratrol may be based on the inhibition of the expression of microsomal PGE2 synthase-1 (mPGES-1) and not COX-2 in rat microglia; mPGES-1 is directly involved in the synthesis of pro-inflammatory PGE2. Kim et al. (2007) reported, however, that the expression of COX-2 and nitric oxide synthase was inhibited by resveratrol in LPS-activated microglia. Moreover, resveratrol has been observed to suppress the activity of T- and B-cells, and macrophages by decreasing the production of these pro-inflammatory proteins (Sharma et al. 2007) and also suppressing inflammatory processes in rat renal injury (de Jesus Soares et al. 2007).

12.5 Dementias

Moderate wine consumption rather than alcohol consumption per se has been specifically associated with a lower risk of developing dementia and specifically Alzheimer's disease (Deng et al. 2006; Simons et al. 2006; Mehlig et al. 2008; Nooyens et al. 2014). Both current and cumulative lifetime moderate wine consumption have been associated with a reduced risk of dementias compared to abstainers (Weyerer et al. 2010). For example, after controlling for potential confounders, current wine consumption of between 20 and 29 g/day was associated with a 29 % decrease in the incidence of overall dementias and a 49 % decrease specifically in the incidence of Alzheimer's disease. These wine consumers also had better physical as well as mental health.

12.5.1 Inflammation, Amyloid-β Factors, and Alzheimer's Disease

Amyloid-β (Aβ) plaques are characteristic hallmarks of Alzheimer's disease where Aβ is a core component of the plaque or lesion found in the neocortex and hippocampus of brains affected by Alzheimer's disease. It is formed after sequential proteolytic cleavage of the amyloid precursor protein (APP), a transmembrane glycoprotein. APP can be processed by α-, β-, and γ-secretases. Unlike α-secretase

which cleaves APP into non-toxic amyloid-α, the toxic amyloid-β protein is generated by successive action of the β and γ secretases. The γ secretase, which produces the C-terminal end of the amyloid-β peptide, cleaves within the transmembrane region of APP and can generate a number of isoforms of 39–43 amino acid residues in length. The most common isoforms are $A\beta_{40}$ and $A\beta_{42}$; the shorter form is typically produced by cleavage that occurs in the endoplasmic reticulum, while the longer form is produced by cleavage in the trans-Golgi network. The $A\beta_{40}$ form is the more common of the two, but $A\beta_{42}$ is the more fibrillogenic or polymeric and is thus associated with disease states, promoting pro-inflammatory responses and activating neurotoxic pathways leading to neuronal dysfunction, and the death and loss of neurons. For example, $A\beta_{42}$ has been shown to activate microglia (small non-neural cells forming part of the supporting structure of the central nervous system (CNS)) and stimulate their production of inflammatory cytokines such as interleukin-6 (IL-6). In brains affected by Alzheimer's disease, the continued presence of Aβ fibrils/plaques may keep microglia persistently activated, leading to chronic inflammation in the CNS (Rogers et al. 2002).

Inhibition of the accumulation of Aβ-peptides and the formation of Aβ fibrils/plaques from Aβ-peptides, as well as the destabilisation of preformed Aβ fibrils/plaques in the brain, would, therefore, be attractive therapeutic targets for the treatment of Alzheimer's disease and other related neurodegenerative diseases.

As mutations in APP associated with early-onset alzheimer's disease have been noted to increase the relative production of $A\beta_{42}$, a potential therapy may involve modulating the activity of β and γ secretases to produce mainly $A\beta_{40}$. Administration of red wine equivalent to two standard drinks to Tg2576 mice, which model Alzheimer's disease Aβ neuropathology and corresponding cognitive deterioration, has been shown to promote the non-amyloidogenic processing of the APP, which acts to prevent the generation of the amyloid-β peptide (Wang et al. 2006). For example, administration of red wine reduced amyloidogenic $A\beta_{1–40}$ and $A\beta_{1–42}$ peptides in the neocortex and hippocampus of Tg2576 mice and correspondingly decreased the neocortical Alzheimer's disease associated amyloid fibrils/plaque. Subsequent examination of APP processing and amyloid-β peptide generation increased the concentration of membrane-bound α-carboxyl terminal fragments of APP in the neocortex and α-secretase activity was also increased, while there was no significant change in the neocortical concentration of β and γ carboxyl terminal fragments of APP or in β and γ secretases activity.

12.5.2 Role of Wine and Wine-Derived Phenolic Compounds in the Development of Dementias

The typical red wine-derived phenolic compounds catechin, quercetin, epicatechin, myricetin, and tannic acid have, however, been shown in vitro to dose-dependently inhibit the formation of Aβ fibrils from fresh $A\beta_{(1–40)}$ and $A\beta_{(1–42)}$, as well as their

extension, and also to dose-dependently destabilise preformed Aβ fibrils (Ono et al. 2003, 2008). Only resveratrol, however, has been shown to decrease the level of intracellular Aβ produced by different cell lines expressing the wild type of Swedish mutant amyloid-β precursor protein (APP_{695}) by promoting its intracellular degradation (Marambaud et al. 2005). This mechanism was proteasome-dependent, that is, resveratrol appears to activate the proteasome involved in the degradation of Aβ, as the resveratrol-induced decrease of Aβ could be prevented by several selective proteasome inhibitors and by siRNA-directed silencing of the proteasome subunit β5. Resveratrol does not inhibit the production of Aβ because it has no effect on β and γ secretase activity.

12.6 Parkinson's Disease

Parkinson's disease (PD) is a common neurodegenerative disease that is characterised by the degeneration and progressive loss of dopaminergic neurons in the substantia nigra pars compacta region of the brain and the appearance of Lewy bodies and neurites which comprise insoluble amyloid-like fibrils that contain the protein α-synuclein (Hirsch et al. 2003). Oxidative stress apparently promotes the aggregation of α-synuclein (Maguire-Zeiss et al. 2005).

Inflammatory responses manifested by glial reactions, T cell infiltration, and increased expression of pro-inflammatory cytokines, as well as other toxic mediators derived from activated glial cells, are currently recognised as prominent features of PD. Consistent findings from certain animal models of PD suggest that neuro-inflammation is an important contributor to the pathogenesis of the disease and may facilitate the progressive loss of nigral dopaminergic neurons. While it may not be the primary cause of PD, additional epidemiological, genetic, pharmacological, and imaging evidence suggests that inflammatory processes in this specific brain region are critical for disease progression (Tufekci et al. 2012). Recent in vitro studies, however, have suggested that activation of microglia and subsequently astrocytes via mediators released by injured dopaminergic neurons is involved.

It is probable that immune responses are triggered secondary to cellular damage and/or neuronal loss in the affected regions of the nervous system. When dopamine and norepinephrine containing neurons degenerate in the brain of PD patients, neuromelanin, a by-product of catecholamine catabolism, is detected outside of neurons and often engulfed by microglial cells, which are the brain macrophages involved in innate immunity. In vitro *and* in vivo studies have since shown that neuromelanin is a potent trigger of microglial cell activation, stimulating the production of pro-inflammatory mediators TNF-α, IL-6, and NO and the up-regulation of NFκβ and p38 mitogen-activated protein kinase (MAPK) signalling pathways (Wilms et al. 2007; Zecca et al. 2008).

12.6.1 Potential Anti-inflammatory Effects of Wine and Wine-Derived Phenolic Compounds in Parkinson's Disease

A potential role for wine in PD has been less well studied although it is the second most common neurodegenerative disorder after Alzheimer's disease. A reduced risk of PD has been observed for moderate alcohol consumers (Gao et al. 2007) which did not distinguish between alcoholic beverages. Such a reduced risk has, however, been specifically shown for wine consumers where an inverse relationship between amount of wine consumed and risk was observed; the lowest risk was observed for wine consumers of approximately 140–420 g/week (Fall et al. 1999). Wine-derived phenolic compounds such as catechin and epi-catechin have recently been observed in vitro to inhibit the formation of α-synuclein fibrils and to destabilise preformed fibrils (Ono et al. 2008). As the pro-inflammatory cytokines IL-6, TNF-α, and IL-10 and the neurotranscriptor factor NFκβ appear to be also implicated in the pathogenesis of PD, it can be assumed that the anti-inflammatory effects of phenolic compounds such as resveratrol observed on other neurodegenerative diseases can be extended to PD. Resveratrol, for example, has already been shown to inhibit the production of NO and TNFα by LPS-activated microglia (Bi et al. 2005; Meng et al. 2008). LPS-induced release of PGE2 and IL-1β from microglia is also suppressed by resveratrol (Kim et al. 2007; Candelario-Jalil et al. 2007; Bureau et al. 2008). In primary midbrain neuron-glia cultures, resveratrol reduces LPS-induced overexpression of TNFα, IL-1β, IL-6, COX-2, and iNOS (Zhang et al. 2010). Resveratrol actually appears to target activated microglia through the modulation of signal transduction pathways such as suppression of MAPK signal transduction pathways (Bi et al. 2005; Zhang et al. 2010) and activation of the Sirt1 pathway, which in turn suppresses the activation of the NF-κB signalling pathway (Bi et al. 2005; Meng et al. 2008). The overall effects are to reduce pro-inflammatory mediators, eventually producing neuroprotection.

12.7 Conclusions

Population ageing is occurring on a global scale, with faster ageing projected for the coming decades than has occurred in the past (Candore et al. 2006). Globally, the population aged 60 years and over is projected to nearly triple by 2050, while the population aged 80 years and over is projected to experience a more than five-fold increase. Increased numbers of older individuals may have implications for associated expenditure on income support, housing, and health services, although a healthy, independent older population can also form a valued social resource, for example in providing care for others, sharing skills and knowledge, and engaging in volunteer activities.

The protective effect of moderate wine consumption against CVD, cancer, cognitive dysfunction, including dementia and Alzheimer's disease, and Parkinson's disease has been consistently observed over past decades. Sufficient epidemiological evidence and plausible biological mechanisms such as anti-inflammatory effects have accumulated to support a J-shaped relationship between moderate wine consumption and risk degenerative diseases that are often inter-related (Simons et al. 2006). Consequently, simple dietary measures such as moderate wine consumption to supplement a healthy exercise and nutrition routine, or as an adjunct to prescription medicines when appropriate, are thus needed to maintain an ageing population. A high level of risk factors for any degenerative disease, however, can mitigate any protective effects of moderate wine consumption, where effects of the excessive consumption of wine may be additive to any other risk factors for disease, increasing risk by two to three times.

While there is accumulating knowledge of the biological mechanisms of wine and the wine-derived phenolic compounds, much of this data is derived from in vitro and animal studies and not human clinical and ex vivo studies. Given that the bioavailability of wine-derived compounds such as resveratrol is relatively low compared to other components (Stockley et al. 2012), considerably more research is required to fully elucidate wine's mechanism of action in inflammation and degenerative diseases of ageing and hence its significance as part of a healthy diet and lifestyle.

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Chapter 13
Interactions Between Wine Polyphenols and Gut Microbiota

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13.1 Introduction

In the context of a diet and healthy lifestyle, it is generally accepted that, in spite of its ethanol content, the moderate consumption of wine has beneficial health effects, as evidenced by numerous scientific studies (recently reviewed by Artero et al. 2015). These effects include protection against cardiovascular diseases, such as atherosclerosis and coronary heart disease (Droste et al. 2013), diabetes type 2 (Chiva-Blanch et al. 2013), and neurodegenerative diseases (Li et al. 2012), among others. To date, most of these protective effects have been linked to the presence of phenolic compounds in wine.

Polyphenols are secondary plant metabolites that in the case of grapes are located on the solid parts of the fruit, mainly in the skins, seeds, and scrapes. During the winemaking process, the phenolic compounds pass into the wine, constituting one of the major groups of compounds in this fermented food (Monagas et al. 2005). From the chemical point of view, the term "polyphenols" encompasses a heterogeneous group of compounds that are characterized by possessing a benzenic ring substituted by one or several hydroxyl groups (–OH) and a functional side chain. According to their chemical structure, they are divided into two groups of compounds: flavonoids and non-flavonoids. The non-flavonoid compounds are characterized by a single ring of six carbons (C6), the most prominent in this group being hydroxybenzoic (C6–C1) and hydroxycinnamic (C6–C3) acids, phenolic alcohols (C6), and stilbenes (C6–C2–C6). The flavonoid compounds are characterized by two rings of six carbons joined by a central heterocycle of three carbons

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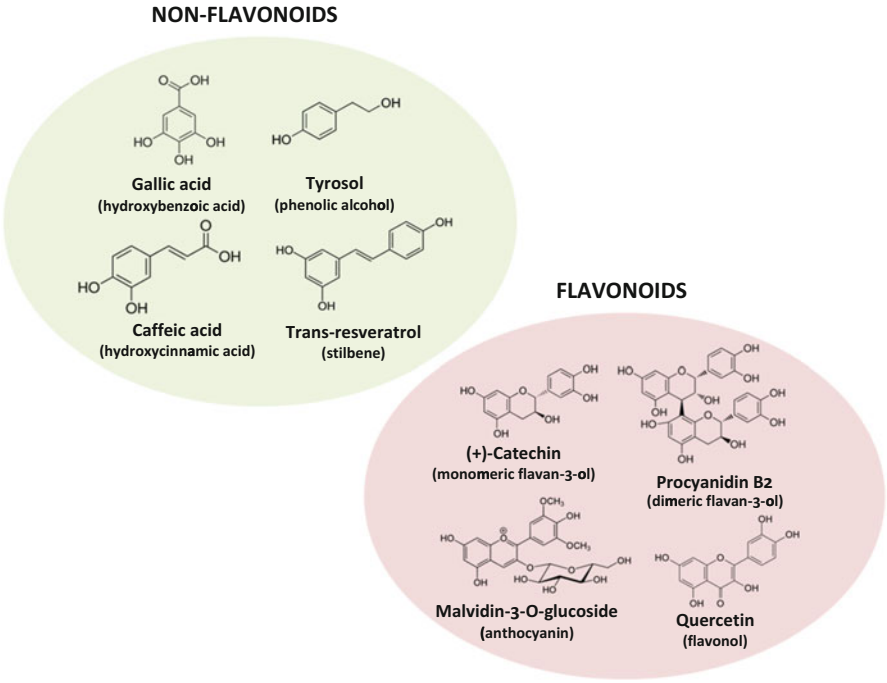

Fig. 13.1 Chemical structure of the major phenolic compounds present in wine

(C6–C3–C6), differing from each other in the degree of oxidation of heterocyclic oxygen and the saturation of the central ring. Among the flavonoids, the flavonols (quercetin, myricetin, kaempferol, and their glycosides) and flavan-3-ols (monomers and oligomeric and polymeric proanthocyanidins) stand out. In the case of red wine, anthocyanins are also included, which are the compounds responsible for the characteristic red color, highlighting in this group the malvidin-3-*O*-glucoside. As an example, Fig. 13.1 shows the chemical structures of the major phenolic compounds present in wine.

The total polyphenol content of phenolic compounds in wine is around 50–400 mg/L for white wines, and 900–1400 mg/L for young red wines, although their concentration is conditioned by several factors related to the grape (variety, soil, geography, climate, etc.) and by enological practices. Therefore, a moderate consumption of wine (250 mL/day) would provide an intake of 60 mg of polyphenols for white wines and 210 mg for young red wines.

The role of polyphenols in human health depends largely on their bioavailability, absorption, and metabolism of polyphenolic compounds. Once ingested, polyphenols are recognized by the human body as xenobiotics, which limit their bioavailability. Besides, depending on their degree of structural complexity and

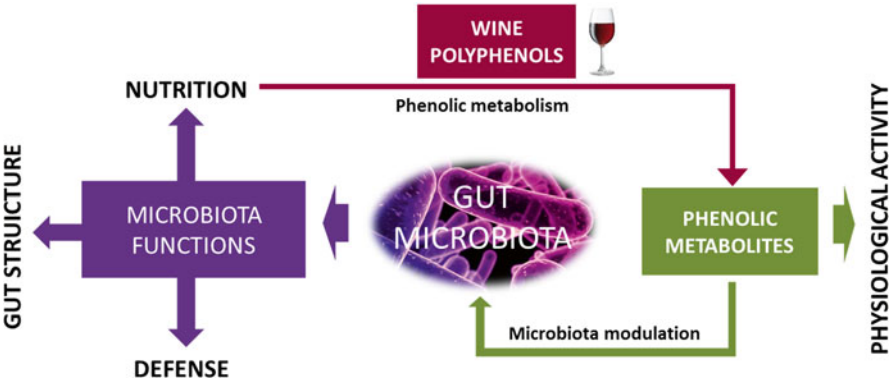

Fig. 13.2 Microbiota-polyphenols two-way interaction at intestinal level (adapted from Muñoz-González 2014)

polymerization, these compounds may be readily absorbed in the small intestine (i.e., low-molecular-weight polyphenols such as monomeric and dimeric structures) or reach the colon almost unchanged (oligomeric and polymeric polyphenols, such as condensed or hydrolyzable tannins) (Monagas et al. 2010). It has been estimated that 90–95 % of the total polyphenol intake may accumulate in the colon where they can be transformed by the resident microbiota into metabolites that could be even more bioactive than their precursors (Clifford 2004). Thus, the interindividual variability of microbial metabolism also impacts on the bioavailability and bioefficacy of polyphenols and their metabolites (Gross et al. 2010). In recent years, it has been reported that microbe-derived phenolic metabolites exert beneficial health effects, such as antioxidant activities (Biasi et al. 2014), antiproliferative actions and cytotoxicity (Tanaka et al. 1993), anti-inflammatory effects (Muñoz-González et al. 2014), and antithrombotic activities (Rechner and Kroner 2005), as well as having effects on the intestinal microbiota (Cueva et al. 2010). In relation to the latter, phenolic metabolites and nonabsorbed polyphenols could affect the growth of gut microbiota, thereby modifying their diversity and metabolic activity (Selma et al. 2009; Requena et al. 2010). Therefore, studies of wine polyphenols are expected to be carried out using a dual approach that includes the formation of bioactive polyphenol-derived metabolites and the modulation of colonic microbiota, at the framework of what has been called a two way "wine polyphenols-gut microbiota interaction (Requena et al. 2010; Dueñas et al. 2015) (Fig. 13.2). In this chapter, after describing some general aspects concerning gut microbiota, we have summarized the current knowledge about the modulation of gut microbiota by wine polyphenols as well as the intrinsic metabolism of wine polyphenols by intestinal bacteria, with special emphasis on the phenolic-metabolizer bacteria identified so far.

13.2 Gut Microbiota

The microbial content of the gastrointestinal tract changes along its length, ranging from a narrow diversity and low numbers of microbes in the stomach to a wide diversity and high numbers in the large intestine (Sekirov et al. 2010) (Fig. 13.3). The dominant bacterial phyla are the Firmicutes (including *Clostridium*, *Enterococcus*, *Lactobacillus*, and *Ruminococcus* genera) and Bacteroidetes (including *Bacteroides* and *Prevotella* genera). Other subdominant or minor phyla include Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia (Qin et al. 2010). It is assumed that several hundred species-level bacteria assemble in each individual in highly variable proportions, resulting in an individual microbial composition that remains stable in time (Rajilić-Stojanović et al. 2013). The temporal stability of the intestinal ecosystem is likely maintained by host-encoded mechanisms in parallel with colonization resistance, as a balanced climax community is not susceptible to new (invading) species. The temporal variation of the microbiota is mostly due to an altered abundance of existing species instead of a flux in the species composition (Rajilić-Stojanović et al. 2013).

The extensive development and use of molecular methodologies in recent years has led to breakthroughs in the gut microbiota composition. In this context, a pioneering study by Arumugam et al. (2011) has suggested that the microbiota of most individuals can be categorized into three predominant variants, or "enterotypes," dominated by three different genera: *Bacteroides*, *Prevotella*, and *Ruminococcus*, which are independent of age, sex, nationality, and body mass index (BMI) and allow the segmentation of subjects according to their intestinal microbiome. Nevertheless, this classification is not exempt from debate, as increasingly researchers are favoring the idea of a continuum or gradient of species functionality rather than a discontinuous variation with segregated types (Jeffery et al. 2012).

13.2.1 Factors Affecting Intestinal Microbiota

The composition of gut microbiota is strongly influenced by a range of factors that include, among others, age, diet, and environmental factors such as antibiotic therapy. With regard to age, individuals exhibit differences in terms of microbial diversity and variation at different life stages (Fig. 13.3) (O'Toole and Claesson 2010). Immediately after birth, babies are colonized by a population characterized by instability (Scholtens et al. 2012). Babies that are solely breast-fed until weaning have a microbiota dominated by *Bifidobacterium* and *Ruminococcus*, whilst those that are formula-fed tend to have a more diverse microbiota (Roger et al. 2010). Following the introduction of solid food to an infant's diet, a more stable community, similar to that of adult microbiota, becomes established after 2–3 years of age (Yatsunenko et al. 2012). However, this relative stability and diversity of the microbiota is

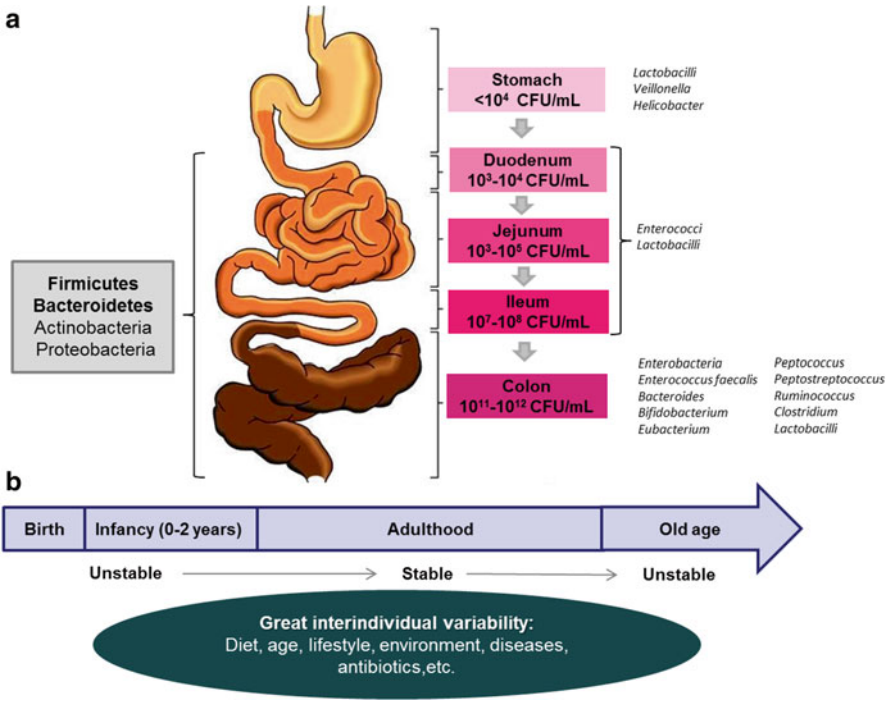

Fig. 13.3 (**a**) Variations in microbial number across the length of gastrointestinal tract. (**b**) Temporal aspects of microbiota establishment and factors influencing the composition of microbiota (adapted from Sekirov et al. 2010)

reduced in old age (Claesson et al. 2012); in particular, a decrease in the total number and species diversity of bifidobacteria and *Bacteroides* takes place. Interestingly, a recent study has shown for the first time that phenotypic effects can be vertically transmitted through the microbiome (Moon et al. 2015), suggesting that we should extend this new factor when it comes to understanding how the microbiome influences sickness and health.

Diet has long been considered one of the major external modulators of the human intestinal microbiota. A prominent example of the role of diet in the determination of the composition of the intestinal microbiota in humans is the study conducted by Filippo et al. (2010). The comparison of the gut microbiota of Italian (consuming a "Western" diet) and African (consuming a plant-rich "rural" diet, high in fiber content) children revealed that the latter were enriched with Bacteroidetes (mainly *Prevotella* and *Xylanibacter*), at the expense of Firmicutes, and a significantly lower amount of enterobacteria. Similar dietary associations have been found in a study linking the dietary patterns of American adults, belonging to the same geographic area, and of similar cultural backgrounds, with gut microbial enterotypes. Wu et al. (2011) found that the *Bacteroides* enterotype was positively associated with protein

and animal fat, whereas the *Prevotella* enterotype was associated with a diet high in carbohydrates. Hence, there is strong evidence that high levels of *Prevotella*, which contain genes for cellulose hydrolysis, characterize microbiomes that are exposed mainly to complex plant-derived carbohydrates.

Despite these findings, recent global analyses of sequence and HitChip data have indicated that interindividual variation played a more major role than dietary change in determining the overall species composition of the microbiota (Salonen et al. 2014). The explanation for this apparent contradiction is twofold. Firstly, many species (especially the less abundant ones) occur only in one or a few individuals. Secondly, it appears that within the microbiota only certain species are responsive to the particular dietary switches, in this case including those bacteria that are specialists.

Finally, antibiotic treatment has also been shown (Young and Schmidt 2004) to dramatically disturb the composition of the intestinal microbiota in humans. In general, antibiotic treatment leads to a decrease in the diversity of the microbiota (Jernberg et al. 2007) as well as to a change in metabolic activity. Nonetheless, the community is quite resilient and can resemble the pretreatment state in a matter of days or weeks (Dethlefsen et al. 2008).

13.2.2 Functions of the Intestinal Microbiota and Its Importance in Health

Understanding the long-range metabolic interdependence between human and gut microbial metabolism is of importance to health and nutrition status (Nicholson et al. 2012). Apart from the obvious role in digestion, the gut microbiota has been associated with trophic, metabolic, and protective functions (Fig. 13.2). In fact, some authors have suggested that the microbiota could act as an "organ" that interacts with the human host and performs many essential functions to maintain human health status (Tremaroli and Bäckhed 2012). Metabolic functions of the gut microbiota allow the human host to utilize many energetic sources. The breakdown of complex indigestible dietary carbohydrates and proteins is possible thanks to the metabolic activity of the gut microbiota. Moreover, the microbiota produces vitamins, synthesizes amino acids, influences ion absorption, and is involved in the conversion of dietary polyphenolic compounds and in the bile acid biotransformation process (DiBaise et al. 2008; Lefebvre et al. 2009). The main products of the substrate fermentation in the gut are short-chain fatty acids (SCFA), particularly acetate, propionate, and butyrate, which positively influence intestinal epithelial cell proliferation and differentiation and have different metabolic features (Lepage et al. 2013).

Another essential function of the intestinal microbiota is the maintenance of intestinal epithelium barrier integrity maintaining cell-to-cell junctions, promoting epithelial repair following injury, and playing a role in the regulation of enterocytes turnover (Cario et al. 2007).

But perhaps, along with the role of nutrition, the most important functions of the intestinal microbiota are the protection from external pathogenic microorganisms and the development of a functional immune system. In the first case, called "colonization resistance," the microbiota prevents pathogenic colonization by competing for attachment sites and nutrients (Sekirov et al. 2010), and through production and secretion of antimicrobials (Chung et al. 2012). Commensal bacteria are able to regulate the production of intestinal mucins by goblet cells, capable of inhibiting bacterial adhesion to intestinal epithelial cells (Wrzosek et al. 2013). The defense barrier of commensal microbiota could also be related to bacterial metabolic products. The production of SCFA causes a reduction of intestinal pH, which could prevent the growth of potentially pathogenic bacteria such as *Escherichia coli* and other members of the family Enterobacteriaceae (Zimmer et al. 2012). On the other hand, the microbiota is also essential for the development of a functional immune system, affecting both innate and adaptive immunities, and in promoting immune regulation at the intestinal surface. This can be readily appreciated from studies performed on germ-free (GF) animals (Sekirov et al. 2010), which generally are more susceptible to infection and have smaller Peyer's patches, reduced mesenteric lymph nodes, decreased cell numbers, and defects in antibody production compared to conventional animals (Lee and Mazmanian 2010). In turn, the composition of the microbiota influences individual variations in immunity, and the absence of beneficial host-specific bacteria may promote disease in genetically susceptible individuals (Blaser et al. 2013).

As the gut microbiota has a well-established role in host homeostasis, several highly prevalent gastrointestinal diseases have been associated with imbalances in microbiota composition (dysbiosis) (Robles-Alonso and Guarner 2013). These human diseases include autoimmune and autoinflammatory disorders, such as allergies, obesity, and inflammatory bowel disease (Schippa and Conte 2014). In this context, wine polyphenols and their microbial phenolic metabolites could play a key role since it has been demonstrated that they are able to exert, among others, anti-inflammatory properties through modulation of gut microbiota (Queipo-Ortuño et al. 2012), as described in Sect. 13.3.

13.2.3 Analytical Approaches

Until the 1990s, knowledge of the gut microbiota was limited to traditional culture-based techniques based on phenotypic identification. However, these techniques are very restrictive as there is a large number of species that are not cultivable (Eckburg et al. 2005). Recent developments in molecular biology have allowed more accurate investigation of microbial communities, as the new techniques are culture-independent. Molecular biological techniques are based on the differences in the sequence of nucleotides of the microbial genes. The majority of these techniques consist of the extraction of DNA from the sample, followed by amplification and

sequencing of 16S ribosomal RNA genes, which contain conserved and variable regions that allow taxonomic identification, ranging from the domain and phylum level to the species level (Robles-Alonso and Guarner 2013), thus providing information about microbial composition and diversity of species in a given sample. "Denaturing gradient gel electrophoresis (DGGE), fluorescent in situ hybridization (FISH), quantitative Polymerase Chain Reaction (qPCR) and capillary sequencing by using the Sanger method are among the most frequently used molecular techniques.

However, to perform a more complex analysis of the intestinal microbiota, emerging technologies, such as next-generation DNA sequencing (NGS) based on real-time monitoring of DNA synthesis, are of great interest. Due to these techniques the concept of "metagenomics" has emerged, defined as the study of metagenomes, i.e., the collective genetic content of the combined genomes of the constituents of an ecological community. In addition, the metagenomic approach also provides information about biological functions present in the community.

In the context of polyphenol–microbiota interactions, these emerging high-throughput-omic approaches can be adopted to identify genes and microorganisms involved in polyphenol (in)activation and conversion, to reconstruct metabolic pathways, and to monitor how microbial communities adjust their metabolic activities upon polyphenol exposure (Kemperman et al. 2010). Application of these technologies to human fecal samples requires further investigation to determine how these samples reflect metabolism inside the gut and, ultimately, to improve the understanding of the impact of polyphenols on host health (Hervert-Hernández and Goñi 2011; Kemperman et al. 2013).

Other potential molecular approaches include metatranscriptomics, metaproteomics, and metabolomics, which analyze the RNA, proteins, and metabolites, respectively, of complex communities. Environmental metatranscriptomics retrieves and sequences environmental mRNA from a microbial ecosystem to assess what genes may be expressed in that community. Metaproteomics allows us to link the abundance and activity of enzymes to their phylogenetic origin based on proteins. Lastly, the metabolome is the terminal downstream product of the genome and consists of the total complement of all the low-molecular-weight molecules in a cell, tissue, or organism.

13.3 Modulation of Gut Microbiota by Wine Polyphenols

As mentioned above, most of the polyphenols that are ingested in the diet reach the colon, where they can be converted by microbiota into bioactive metabolites that can affect the intestinal ecology and influence host health. In order to assess the modulating effect of wine polyphenols, several *in vitro* and *in vivo* animal and human

intervention studies have been carried out (Tables 13.1 and 13.2). For example, studies using batch culture fermentation, a model reflective of the distal region of the human large intestine, have evaluated both the polyphenols metabolism in the presence of human gut microbiota and the changes in microbial communities after incubation with pure phenolic compounds (Tzounis et al. 2008; Hidalgo et al. 2012), and with extracts rich in polyphenols, derived from grapes (Cueva et al. 2013) and wine (Barroso et al. 2013; Sánchez-Patán et al. 2012). In general, these studies have demonstrated the increase of some bacterial groups in the intestine, such as *Lactobacillus*, *Enterococcus*, and *Bifidobacterium*, and the decrease of others, mainly *Clostridium histolyticum* (Table 13.1). On the other hand, three recent studies conducted with gastrointestinal tract simulators (SHIME and SIMGI) found notable changes in certain intestinal bacterial groups after simulation with an extract of wine (Barroso et al. 2014; Kemperman et al. 2013) and with red wine (Cueva et al. 2015), the most affected bacterial groups being *Bacteroides* and *Bifidobacterium* (Table 13.1).

With regard to animal experiments, several studies have been performed using red wine and grape seed extracts (Table 13.2). The fecal bacteria composition of rats fed with red wine polyphenols shifted from a predominance of *Bacteroides*, *Clostridium*, and *Propionibacterium* spp. to a predominance of *Lactobacillus* and *Bifidobacterium* spp. (Dolara et al. 2005). Another animal experiment was carried out to study the effect of the inclusion of grape seed extracts in the diet of broiler chicks (Viveros et al. 2011) on intestinal microbiota. It was observed that grape extracts modified the gut microbiota, increasing *E. coli*, and *Lactobacillus* and *Enterococcus* species populations. Recently, two animal studies performed in pigs have demonstrated that grape seed extract administration caused an ecological shift in the microbiome. Recently, two animal studies performed in pigs have demonstrated that grape seed extract administration caused an ecological shift in the microbiome, decreasing *Streptococcus* spp. and *Clostridium* cluster XIVa counts (Fiesel et al. 2014), and increasing *Lachnospiraceae, Clostridiales, Lactobacillus*, and *Ruminococcaceae* populations during the intervention period (Choy et al. 2014).

Investigations carried out with humans potentially provide the best models for studying the interactions of food components (e.g., polyphenols) with microbiota; however to date only a few studies have been conducted. Yamakoshi et al. (2001) reported that administration of a proanthocyanidin-rich extract significantly increased the fecal number of *Bifidobacterium* spp., whereas a reduction in the bacteria belonging to the *Enterobacteriaceae* family was observed. On the other hand, Queipo-Ortuño et al. (2012) assessed the effect of the moderate intake of red wine. A significant increase in the number of *Enterococcus*, *Prevotella*, *Bacteroides*, *Bifidobacterium*, *Bacteroides uniformis*, *Eggerthella lenta*, and *Blautia coccoides-E. rectale* was found. Specifically, an increase of *Bifidobacterium* spp. has recently been correlated with an increase in microbial metabolites derived from wine anthocyanins (Boto-Ordónez et al. 2014). In contrast, concentrations of *Clostridium* spp. and *C. histolyticum* group decreased after the red wine period. In summary, all these studies confirm the modulatory capacity of wine polyphenols on intestinal microbiota, which could have positive health effects or even prevent disease.

Table 13.1 Studies regarding modulation of gut microbiota by wine polyphenols using batch culture fermentations and gastrointestinal tract simulators

Studies using batch culture fermentation							
Reference	Fecal \concentration	Phenolic compound/ food	Dose	Time of incubation	Microbial technique	Growth enhancement	Growth inhibition
Tzounis et al. (2008)	10 %, w/v	(+)-Catechin	150 mg/L, 1000 mg/L	<48 h	FISH	*Bifidobacterium* spp. *C. coccoides-E. rectale* group *E. coli*	*C. histolyticum* group
Hidalgo et al. (2012)	10 %, w/v	Malvidin-3-*O*-glucoside Anthocyanidins mixture	20 and 200 mg/L 4850 and 48,500 mg/L	<24 h	FISH	*Lactobacillus-Enterococcus* spp. *Bifidobacterium* spp. *C. coccoides-E. rectale* group	
Cueva et al. (2013)	10 %, w/v	Grape seed extract fractions	300–450 mg/L	<48 h	FISH	*Lactobacillus-Enterococcus* spp.	*C. histolyticum* group
Sánchez-Patán et al. (2012)	1 % w/v	Red wine extract	600 mg/L	48 h	FISH		*C. histolyticum* group
Barroso et al. (2013)		Red wine extract	500 mg/L	48 h	qPCR	*Lactobacillus* spp. *Bifidobacterium* spp. *Bacteroides* spp. *Ruminococcus* spp.	
Gross et al. (2010)	20 % w/v	Red wine/grape extract	500–1000 mg/L	72 h	HITChip		

Studies using a gastrointestinal simulator							
Reference	Simulator	Phenolic compound/ food	Dose	Time	Microbial technique	Population increase	Population decrease
Cueva et al. (2015)	SIMGI	Red wine	3 × 75 ml one day (~405 mg polyphenols as total dose)	1 week	Plate count qPCR	*Lactobacillus* spp.	All bacteria group *Bacteroides* spp.
Kemperman et al. (2013)	Twin-SHIME	Red wine-grape extract	3× daily dosing (1000 mg polyphenols as total daily dose)	2 weeks	Plate count qPCR PCR-DGGE Pyrosequencing	*Klebsiella* spp. *Alistipes* spp. *Cloacibacillus* spp. *Victivallis* spp. *Akkermansia* spp.	*Bifidobacteria* *Blautia coccoides* group *Anaeroglobus* spp. *Subdoligranulum* spp. *Bacteroides* spp.
Barroso et al. (2014)	Twin-SHIME	Red wine extract	1× daily dosing (200 mg polyphenols as total daily dose)	2 weeks	Plate count qPCR PCR-DGGE		*Bacteroides* spp. *Bifidobacterium* spp. *C. coccoides-E. rectale* group

Table 13.2 Studies regarding modulation of gut microbiota by wine polyphenols in studies with animals and humans

Animal model studies							
Reference	Simulator	Phenolic compound/ food	Dose	Time	Microbial technique	Population increase	Population decrease
Dolara et al. (2005)	Rats	Red wine polyphenols powder	50 mg/kg	16 weeks	Plate count	*Lactobacillus* spp. *Bifidobacterium* spp.	*Propionibacterium* spp. *Bacteroides* spp. *Clostridium* spp.
Viveros et al. (2011)	Broiler chicks	Grape seed extract (GSE)	7.2 g/kg diet (GSE) (free access)	21 days	Plate count T-RFLP	*E. coli* *Enterococcus* spp. *Lactobacillus* spp.	
Fiesel et al. (2014)	Pigs	Grape seed extract	1 % (free access)	4 weeks	qPCR		*Streptococcus* spp. *Clostridium* Cluster XIVa
Choy et al. (2014)	Pigs	Grape seed extract	1 % w/w	6 days	Illumina MiSeq platform	*Lachnospiraceae, Clostridiales*, *Lactobacillus*, *Ruminococcaceae*	
Human intervention studies							
Reference	Volunteer numbers	Phenolic compound/ food	Dose	Treatment duration	Microbial technique	Population increase	Population decrease
Yamakoshi et al. (2001)	9	Proanthocyanidin-rich extract from grape seeds	0.5 g/day	6 weeks	Plate count	*Bifidobacterium* spp.	*Enterobacteriaceae*
Queipo-Ortuño et al. (2012)	10	Red wine	272 mL/day	20 days	qPCR	*Enterococcus* spp. *Prevotella* spp. *Bacteroides* *Bifidobacterium* spp. *Bacteroides uniformis* *Eggerthella lenta* *Blautia coccoides-E. rectale* group	*Clostridium* spp. *C. histolyticum group*

13.4 Catabolism of Wine Polyphenols by Intestinal Bacteria

Although polyphenol metabolism starts in the mouth due to β-glycosidase activity, the colon is seen as being the main important organ for the catabolism of wine polyphenols and is widely influenced by their chemical structure. Oligomers and polymers of flavan-3-ols are the major phenolic compounds present in the wine that reaches the colon (Monagas et al. 2010; Rodriguez-Mateos et al. 2014). The catabolism of dimeric procyanidins involves the C-ring opening, followed by lactonization, decarboxylation, dehydroxylation, and oxidation reactions, among others (Selma et al. 2009). In the case of galloylated monomeric flavan-3-ols, the microbial catabolism usually starts with the rapid cleavage of the gallic acid ester moiety by microbial esterases, giving rise to gallic acid, which is further decarboxylated into pyrogallol (Kohri et al. 2003; Meselhy et al. 1997). The C-ring is subsequently opened, giving rise to 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol, which is later converted into 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone in the case of (epi)catechin or 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone in the case of (epi)gallocatechin (Roowi et al. 2010). The valerolactone ring later breaks, giving rise to 5-(3′,4′-dihydroxyphenyl)valeric acid and/or 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid. Subsequent biotransformations of these valeric acids give rise to hydroxyphenylpropionic and hydroxybenzoic acids by successive loss of carbon atoms from the side chain through β-oxidation (Meselhy et al. 1997). With regard to the microbial catabolism of flavonols, they are directly transformed into 3,4- or 3,5-dihydroxylated phenylacetic acids (Aura 2008). In the case of anthocyanins, they are converted into 3,4-dihydroxy-, 4-hydroxy-, 3,4-dimethoxy-, or 3-methoxyl-4-hydroxyl benzoic acids according to the substitution pattern of the B-ring of the precursor anthocyanin molecule (Aura 2008; De Ferrars et al. 2014). However, in spite of the fact that anthocyanins are abundant in wine, their circulating levels in plasma are very low, which has been attributed to anthocyanin instability under neutral pH, their extensive metabolism *in vivo*, and their probable catabolism by intestinal microbiota (De Ferrars et al. 2014). On the other hand, non-flavonoid compounds present in wine, hydroxycinnamic esters (i.e., caffeic acid derivatives), are mainly transformed into 3-hydroxyphenylpropionic acid, benzoic acid, and 4-ethylcatechol (Gonthier et al. 2006).

Once absorbed, the microbial metabolites are mainly metabolized in the liver by phase II enzymes as conjugated metabolites (glucuronides and sulfates), which can reach the colon via enterohepatic circulation and are also susceptible to degradation by the intestinal microbiota. Finally, the phenolic metabolites are excreted via urine and feces (Jiménez-Girón et al. 2015; Muñoz-González et al. 2013).

13.4.1 Bacteria Identified as Metabolizers of Certain Phenolic Groups

Intestinal bacteria play a crucial role in the metabolism of wine polyphenols and may therefore contribute to health-promoting effects. Despite the advances recently made in the knowledge of the identification of phenolic metabolites, the specific bacterial species able to metabolize wine polyphenols in the gastrointestinal tract and the anaerobic degradation pathways remain largely unknown. One of the main factors limiting the isolation and subsequent identification of polyphenol catabolic bacteria is the difficulty in growing them in commercial culture media. The reasons for this cultivation anomaly include the unknown growth requirements of the bacteria, the selectivity of the media that are used, the stress imposed by the necessity of strictly anoxic conditions, and difficulties with simulating the interactions of bacteria with other microbes and host cells. Table 13.3 shows an overview of the intestinal bacteria involved in the metabolism of wine phenolic compounds as well as the metabolites produced. The main bacteria involved in flavonol (quercetin, quercetin-3-glucoside, and kaempferol), and flavan-3-ol (catechin and epicatechin) metabolism belong to the phyla Firmicutes, and Firmicutes and Actinobacteria, respectively. This metabolic activity has been also confirmed in *Lactobacillus plantarum* IFPL935, which has demonstrated its ability to favor the initial metabolism of red wine polyphenols (Barroso et al. 2013, 2014). This greater phenolic metabolic activity of members of Firmicutes phylum leads us to hypothesize that this group might possess a specific function to degrade polyphenols. On the other hand, it can be expected that the large individual differences generate differences in the microbial metabolite profiles, because human microbiota contains more than 1000 different species with high individual variation (Qin et al. 2010). However, different colonic communities share general metabolic activities, which convert food components to specific metabolite profiles (Jacobs et al. 2009).

Therefore, the identification of the bacteria responsible for polyphenol metabolism is of vital importance to map functional metabolic reactions and describe the interaction between host and microorganisms in order to understand metabolism. In turn, this knowledge would help in the development of potential functional foods and ingredients with health benefits for individuals who produce low levels of these bioactive metabolites.

13.5 Conclusions

The bioavailability and effects of polyphenols greatly depend on their transformation by gut microbiota. Several studies have demonstrated that metabolization of wine polyphenols by gut microbiota leads to the production of a wide variety of metabolites with potential positive effects on human health. Most of the wine polyphenol-metabolizing intestinal bacteria identified belong to the phylum Firmicutes. In turn,

Table 13.3 Intestinal bacteria involved in the metabolism of wine phenolic compounds (adapted from Moco et al. 2012)

Phenolic compounds	Metabolites	Gastrointestinal microbiota	References
Quercetin-3-glucoside	Quercetin; 3,4-dihydroxyphenylacetic acid; phloroglucinol	*Eubacterium ramulus*; *Enterococcus casseliflavus*	Blaut et al. (2003)
Quercetin	Taxifolin; alphitonin; 3,4-dihydroxyphenylacetic acid; phloroglucinol	*Eubacterium ramulus*; *Clostridium orbiscindens*	Blaut et al. (2003), Schoefer et al. (2003), Braune et al. (2001)
Kaempferol	4-Hydroxyphenylacetic acid	*Eubacterium ramulus*	Blaut et al. (2003)
(+)-Catechin	1-(3′,4′-Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol; 5-(3,4-dihydroxyphenyl)-γ-valerolactone; 4-hydroxy-5-(3,4-dihydroxyphenyl)valeric acid	*Eggerthella lenta* rK3; *Flavonifractor plautii* aK2; *Eubacterium* sp. SDG-2	Kutschera et al. (2011), Wang et al. (2001)
(+)-Epicatechin	1-(3′,4′-Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	*Eubacterium* sp. SDG-2	Wang et al. (2001)
(−)-Catechin	1-(3′,4′-Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 1-(3′-hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol	*Eubacterium* sp. SDG-2	Wang et al. (2001)
(−)-Epicatechin	1-(3′,4′-Dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol; 1-(3′-hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol; 5-(3,4-dihydroxyphenyl)-γ-valerolactone; 4-hydroxy-5-(3,4-dihydroxyphenyl)valeric acid	*Eggerthella lenta* rK3; *Flavonifractor plautii* aK2; *Eubacterium* sp. SDG-2	Kutschera et al. (2011), Wang et al. (2001)

wine polyphenols and their metabolites modulate the growth of selected bacterial groups, highlighting the importance of the two-way polyphenols–microbiota interaction in the maintenance of gut health. Even though it is well established that diet influences gut microbiota composition, recent findings suggest that interindividual variation plays a more major role than dietary change in determining the overall species composition of the microbiota. Therefore, further investigations using emerging molecular methods are necessary in order to achieve a better understanding of the underlying mechanisms in the polyphenols–microbiota–host triangle, and elucidate the implications of polyphenols for host health.

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Chapter 14
Neuroprotective Effects Associated with Wine and Its Phenolic Constituents

Adelaida Esteban-Fernández, Giulia Corona, David Vauzour, and Jeremy P.E. Spencer

14.1 Introduction

The development of cognitive impairment is a complex process that begins even in the absence of a symptomatic disease (Glisky 2007). Most of the neuronal disorders, which include all the diseases affecting the central and peripheral nervous system, produce a distress in cognitive function and memory. For instance, Alzheimer's (AD) disease is responsible for two of three cases of dementia, followed by Parkinson's disease (PD) which is the second common neurodegenerative disorder in society (Nussbaum and Ellis 2003).

Although the long-term high consumption of alcoholic drinks translates to increased cancer, cardiovascular diseases, cirrhosis, dementia, and depression (Letenneur 2004), a low-moderate wine intake is related to several health benefits, including improved brain function (Artero et al. 2015). Several studies have

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demonstrated the beneficial effects of moderate wine consumption against cardiovascular disease (Rehm et al. 1997; Klatsky et al. 2003), certain types of cancer (Bianchini and Vainio 2004), and/or diabetes (Napoli et al. 2005). Despite the well-established harmful effects of heavy alcohol intake (Hvidtfeldt et al. 2008), epidemiological studies have reported that a low-to-moderate intake of wine (150–300 mL) may reduce cognitive impairment (Lemeshow et al. 1998; Letenneur 2004; Orgogozo et al. 1997). In addition, data from the Personnes Agees Quid (Lemeshow et al. 1998) study demonstrated that people drinking three to four glasses of wine per day had an 80 % decreased incidence of dementia and Alzheimer's disease 3 years later, compared to those who drank less or did not drink at all (Orgogozo et al. 1997). Although wine is a complex matrix, such protection is believed to be in large part attributable to the intake of specific polyphenols present in great quantity in wine. Wine polyphenols derive mainly from the grape seeds and skin, and may be present at relatively high concentrations in wine, especially as flavonoids (flavan-3-ols, anthocyanins) and other polyphenols (resveratrol, gallic acid, or cinnamates). Red wines contain high levels of flavonoids and other phenolics relative to white wines (Manach et al. 2004); meanwhile Champagne wine is also relatively rich in phenolic compounds such as hydroxybenzoic acids, hydroxycinnamic acids (and their tartaric derivative esters), phenolic alcohols, and phenolic aldehydes (Vauzour et al. 2007; Chamkha et al. 2003). The increased levels of phenolic compounds in Champagne wine compared to other white wines derive predominantly from the two red grape varieties, Pinot Noir and Pinot Meunier, which are used in its production along with the white grape Chardonnay (Constant 1997). Generally, the phenolic composition varies with a wide range of factors, including species, variety, season, growing conditions, and processing practices (Jackson and Lombard 1993). In a similar manner wine benefits have been described in health, these chemical bioactive compounds have been also related to prevention of several chronic diseases such as cardiovascular disease, cancer, and neurodegenerative disorders (Vauzour et al. 2010a). In particular, flavonoids, a subclass of polyphenols, have been ascribed to exert anti-inflammatory properties (Rice-Evans and Miller 1996) and to modulate signalling pathways that regulate nitric oxide production (Bastianetto 2002) and neuronal survival (Schroeter et al. 2002). As such, there is a great interest in the potential of regular and moderate wine consumption to delay the onset of neurological disorders, such as Alzheimer's disease and dementia (Corder 2008; Chan et al. 2008; Commenges et al. 2000).

In this chapter, we review the effects of red wine and Champagne wine and their phenolic constituents on cognitive functions and we briefly describe their intracellular targets underlying their protective effects.

14.2 Studies of the Effects of Wine and Wine Constituents

Polyphenol-rich foods/beverages have received much attention with regard to their neuroprotective effects (Spencer 2009), including a potential to protect neurons against neurotoxin-induced injury (Rainey-Smith et al. 2008; Vauzour et al. 2008),

to suppress neuroinflammation (Vafeiadou et al. 2007), and to promote memory and learning (Shukitt-Hale et al. 2009; Liu et al. 2011; Rendeiro et al. 2012; Valls-Pedret et al. 2012). Despite the well-established harmful effects of heavy alcohol intake (Hvidtfeldt et al. 2008), epidemiological data suggest that moderate wine consumption may reduce the incidence of age-related dementia, including Alzheimer's disease (Orgogozo et al. 1997; Panza et al. 2009; Weyerer et al. 2011). As such, there is an interest in the potential of regular, moderate wine consumption to counteract normal brain ageing and to improve memory and learning, through its potential to deliver relatively high amounts of flavonoids and phenolic acids (Corder 2008; Chan et al. 2008).

14.2.1 Moderate Red Wine Consumption and Its Relation with Cognitive Function

Red wine has been described to have several benefits on cognition improvement and in prevention of neurodegenerative diseases, such as Alzheimer's disease (AD) or Parkinson's disease (PD). Even so the exact mechanisms by which red wine (or its polyphenols) may influence cognitive function are still unknown and there is no information on the specific bioactive polyphenolic compounds that are involved on these results. It is generally assumed that consumption of polyphenol-rich foods improves cognitive performance (Nurk et al. 2010; Polidori et al. 2009), and epidemiological evidences have indicated that moderate wine consumption, within the range recommended by the FDA dietary guidelines of one drink per day for women and two for men, may help to reduce the relative risk for clinical dementia (Orgogozo et al. 1997). Epidemiological studies reported that a moderate wine intake is positively correlated with the prevention of senile dementia and AD in elderly people (Orgogozo et al. 1997; Weyerer et al. 2011; Panza et al. 2009), and improves cognitive performance in both women and men, when compared to abstinence (Arntzen et al. 2010). These results are supported by other studies where a monthly and weekly intake of wine has been associated with a lower risk of dementia (Truelsen et al. 2002), and a decreased risk of cognitive decline (Stampfer et al. 2005). Altogether these results suggest that certain substances present in wine may reduce the incidence of dementia or cognitive impairment, and prevent neurodegenerative disorders.

Nevertheless, there are other rich-in-polyphenols food, whose neuroprotective properties have been described, such as apple (Cheng et al. 2014), blueberries (Papandreou et al. 2009), cocoa (Field et al. 2011), or green tea (Chen et al. 2009; Xu et al. 2010). A cross-sectional study has indicated that a diet rich in flavonoids (chocolate, wine, and tea) (Nurk et al. 2009) leads to improved cognitive performance in elderly people, with the most significant improvement obtained with wine intake, which again suggests that a moderate intake of this beverage could benefit cognitive functions.

This effect was also observed in animal studies where an attenuation of spatial memory impairment in an AD mouse model has been observed when mice were

orally fed with Cabernet Sauvignon (Wang et al. 2006) or Muscadine wine (Ho et al. 2009b). It also seems that wine exerts beneficial effects against AD by reducing β-amyloid-peptide (Aβ-peptide) aggregation (Wang et al. 2006; Ho et al. 2009b). This peptide is clearly related to memory deficits and, therefore, it has influences on memory function (Lesné et al. 2006). However different mechanisms of action have been described when mice were fed with different wines (and therefore different phenolic composition): on one hand, when Cabernet Sauvignon was employed, a reduction in the Aβ-peptide aggregation by modulating α-secretase activity (Wang et al. 2006) has been described. Whereas when Muscadine wine was employed, it seemed to interfere with the oligomerization of Aβ molecules to soluble high-molecular-weight Aβ oligomer species, responsible for initiating a cascade of cellular events resulting in cognitive decline (Ho et al. 2009b). Since different mechanisms of action were observed, the authors suggest that maybe a combination of polyphenols could be the key to prevent AD in the earlier stages. Red wine also reduced lipid peroxidation, increased antioxidant defenses (glutathione), and induced antioxidant enzyme activities in rats (Assunção et al. 2007), leading to an improvement in spatial learning and memory.

These observations suggest that distinct polyphenolic compounds from red wines could beneficially modulate cognitive function and prevent neurodegenerative disorders through multiple mechanisms, but it is necessary to go deep into the protective molecular mechanisms and possible synergistic interactions with other wine compounds, including aroma compounds, proteins, or carbohydrates which could be involved in these effects.

14.2.2 Champagne Wine and Cognitive Function

Champagne wine is a white wine relatively rich in phenolic compounds such as hydroxybenzoic acids, hydroxycinnamic acids (and their tartaric derivative esters), phenolic alcohols, and phenolic aldehydes (Vauzour et al. 2007; Chamkha et al. 2003). Animal studies have reported its neuroprotective effect. For instance, in a recent rodent study it was reported that Champagne wine is capable of enhancing spatial working memory (without altering motor performance) in aged animals (Corona et al. 2013). In contrast, moderate alcohol intake failed to induce spatial memory changes. These observations are in agreement with those observed following long-term red wine intake in a similar model of hippocampal-dependent spatial memory (Assunção et al. 2007). The effects of Champagne on spatial memory were paralleled by a number of changes in hippocampal and cortical protein expression, which may explain performance on spatial memory tasks. Targeted protein arrays indicated that Champagne induced the differential expression of a number of hippocampal and cortical proteins involved in signal transduction, neuroplasticity, apoptosis, and cell cycle regulation (Corona et al. 2013), including CNPase, a myelin-associated enzyme that constitutes around 4 % of total CNS myelin protein, and is thought to undergo significant age-associated changes (Hayakawa et al. 2007).

It is reduced in Alzheimer's disease and Down's syndrome patients (Vlkolinsky et al. 2001).

Furthermore, Champagne-induced hippocampal increases in the cytoskeletal associated protein, dystrophin, may be beneficial as a lack of this protein in the hippocampus has been associated with impaired cognitive function (Muntoni et al. 1991), spatial memory (Vaillend and Ungerer 1999), and long-term potentiation (Vaillend et al. 2004). Indeed, patients lacking dystrophin in the hippocampus and neocortex (due to mutation in the dystrophin gene) display a range of cognitive deficits (Anderson et al. 2002). Intervention with the phenolic rich Champagne also led to the increased expression of a range of "other" cytoskeletal proteins, including plakoglobin (γ-catenin), spectrin, calponin, cytokeratin pep4 and pep19, myosin Va, and focal adhesion kinase (Corona et al. 2013). Such proteins facilitate complex neuronal network formation in the brain and operate with neuronal membrane proteins (e.g., ion channels, scaffolding proteins, and adaptor proteins) at sites of synaptic contacts to regulate synaptogenesis and coordinate synaptic strength (Priel et al. 2009; Goda 2002). These results therefore suggest that smaller phenolics such as gallic acid, protocatechuic acid, tyrosol, caftaric acid, and caffeic acid, in addition to flavonoids, are capable of exerting improvements in spatial memory via the modulation in hippocampal signalling and protein expression.

14.2.3 Neuroprotective Effects of Grape-Derived Phenolic Extracts and Phenolic Compounds

The phenolic composition of wine mainly derived from that of grapes, although wine-making techniques may also affect it. The use of grape-derived phenolic extracts and pure phenolic compounds constitutes a good approach to study the effects of wine consumption in certain targets, such as the case of cognitive disorders.

When 200 mg/kg/day of grape polyphenolic extract (GPE) (equivalent to a human dose of 1 g/day) was orally administered to Tg2576 AD mice models, a reduction of high-molecular-weight soluble oligomeric β-amyloid-peptide in the brain was reported (Wang et al. 2008). In a similar manner, GPE intake significantly decreased the levels of Aβ-peptide (Liu et al. 2011), amyloid plaques, and microgliosis (Wang et al. 2009b) in mice brains.

In addition, oral administration of GPE (200 mg/kg/day of GPE equivalent to a human dosage of 1 g/day) in a mouse model of AD seems to significantly attenuate the development of AD-type Tau neuropathology in the brain (Wang et al. 2010). Tau is a brain protein directly related to AD due to its implication in neuronal viability and axonal transport (De-Paula et al. 2012). Taken together, these results suggest that grape-derived polyphenol extracts could protect against brain damage in neuronal diseases by modulating protein aggregation, including Tau and Aβ-peptide.

In addition to AD, there are other neurodegenerative disorders where beneficial GPE effect has been described. The neuroprotective role of GPE in an experimental mice model of autoimmune encephalomyelitis (Giacoppo et al. 2015) as in a gerbil

ischemia model, where it protected against ischemia/reperfusion (I/R) injuries (Wang et al. 2009a), has been reported.

When the effect of isolated phenolic compounds being part of GPE was analyzed, several evidences of neuroprotective role have been described. The non-flavonoid polyphenol resveratrol has been widely studied by its protective capacity in neurodegenerative disorders, such as epilepsy, AD, PD, Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), or nerve injury (Pasinetti et al. 2015; Rocha-González et al. 2008). It is suggested that resveratrol is able to diminish the amyloid plaques in mice, as reported by Karuppagounder and colleagues (2009), and exert a protective effect against neuroinflammation as confirmed in aged mice, by reducing both interleukin-1β (IL-1β) levels in plasma and IL-1β expression in brain (Abraham and Johnson 2009).

Resveratrol was also shown to reduce drug-induced neuronal death in male mice (50 or 100 mg/kg/day for 1 or 2 weeks) (Blanchet et al. 2008), having an antidepressant effect on rats by modulating the activation of hippocampal brain-derived neurotrophic factor (BDNF), a protein implicated in chronic effects of many antidepressants (Hurley et al. 2014). The intake of this non-flavonoid polyphenol also seems to improve cognitive function by increasing insulin-like growth factor-I (IGF-I) production in the mice hippocampus (Harada et al. 2011).

Among other phenolic compounds found in wine, epigallocatechin-3-gallate inhibits in vivo iNOS activity and improves mice memory deficiency (Lee et al. 2009), while quercetin has been described to decrease extracellular β-amyloidosis, tauopathy, astrogliosis, and microgliosis in the hippocampus and the amygdala, when applied to aged AD mice model (Sabogal-Guáqueta et al. 2015). It also induced improved performance on learning and spatial memory tasks.

Other wine polyphenols such as rutin have been studied by its implication on prevention of neuroinflammatory processes (Koda et al. 2009), whereas the knowledge of the effect of other polyphenols, such as phenolic acids, or metabolites derived from polyphenols food source intake, is scarce at this moment.

14.3 Mechanisms of Action

The potential health benefits of wine consumption are generally ascribed to the polyphenolic compounds that they contain in high abundance, particularly the red wines (900–1400 mg/L) (Scalbert et al. 2005; Ho et al. 2009a). Figure 14.1 shows the main mechanistic effects of wine components in cognitive function. Inside the several properties of natural polyphenols, the antioxidant one has been widely studied. In fact, once, it was believed that the ability of these compounds to scavenge reactive oxygen species (ROS) was the main mechanism by which they exert their protective effect against neuronal diseases (Ramassamy 2006; Li et al. 2004a). However antioxidant mechanism does not seem to be physiologically relevant in vivo, and there are several other ways by which polyphenols could interact against neuronal damage (via MAPK, NF-κB …) (Kim et al. 2010; Li et al. 2004a, b; Spencer et al. 2012). Most notably, differential modulation of a range of proteins

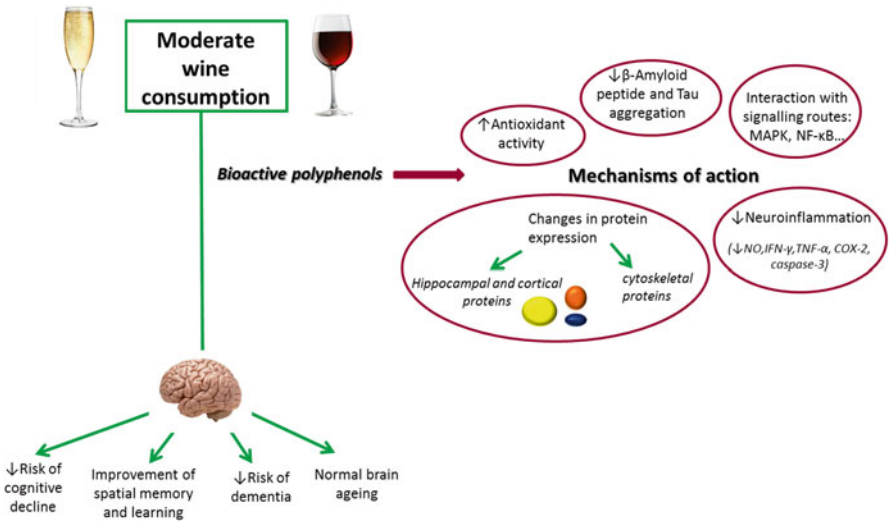

Fig. 14.1 Mechanistic effects of wine components in cognitive function

has been observed, such as brain-derived neurotrophic factor (BDNF), cAMP response element-binding protein (CREB), p38, dystrophin, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase), mammalian target of rapamycin (mTOR), B-cell lymphoma2-extra-large protein (Bcl-xL) in response to Champagne supplementation compared to the control group, and the modulation of mTOR, Bcl-xL, and CREB in response to alcohol supplementation (Corona et al. 2013).

GPE are able to prevent AD by inhibiting β-amyloid peptide aggregation into high-molecular-weight oligomers (Wang et al. 2008; Ono et al. 2008; Liu et al. 2011), with the resulting reduction of its cytotoxicity. In a similar manner, these phenolic extracts are suggested to attenuate the development of AD Tau-related neuropathology by modulating ERK 1/2 expression, a mitogen-activated protein kinase (MAPK), in mice brain (Wang et al. 2010). GPE can interfere with the assembly of Tau peptides into neurotoxic aggregates, affecting the misfolding of Tau protein to form Tau filamentary aggregates (in relation with the initiation and progression of neurodegeneration and cognitive dysfunctions in tauropathies) (Ho et al. 2009a; Wang et al. 2010). GPE not only inhibited the formation of Tau aggregates, but also promoted the dissociation of preformed Tau peptide aggregates. These results taken together suggest that grape-derived polyphenols could prevent cognitive decline by modulating Tau-mediated neuropathologic mechanisms, in a similar way they prevent β-amyloid peptide effects.

Resveratrol is believed to prevent AD by reducing Aβ levels, promoting the in vitro intracellular degradation of this peptide (Marambaud et al. 2005). Resveratrol could prevent AD by interacting with β-amyloid peptide via NF-κB activity or NF-κB/(sirtuin 1) (SIRT1) pathways (Jang and Surh 2003; Chen et al. 2005), even though the neuroprotective effect of resveratrol is not attributable to a direct activation of SIRT1 and the full mechanisms of action of resveratrol are not yet characterized (Choi et al. 2012; Tang 2010; Pasinetti et al. 2015). Resveratrol was also shown to

reduce nitric oxide (NO), IFN-γ, and TNF-α gene expression when applied to activated microglial cell cultures at 0.1 μM and 0.1–10 μg/ml, respectively (Bureau et al. 2008; Bi et al. 2005). These genes are directly related to inflammation-mediated apoptotic death routes of neuronal cells, one of the main underlying causes of neuronal disorders.

Tyrosol, caffeic acid, and gallic acid, phenolic compounds found at relatively high concentrations in Champagne and other types of wine, have been shown to potently inhibit peroxynitrite-induced cellular injury at physiologically relevant concentrations (0.1–10 μM) (Vauzour et al. 2007), whilst nanomolar levels of tyrosol, caffeic acid, and *p*-coumaric acid protect cortical neurons against 5-S-cysteinyl-dopamine-induced injury (Vauzour et al. 2010b). Indeed, the level of protection induced by these phenolics was equal to, if not greater than, that observed for similar concentration of the flavonoids, (+)-catechin, (−)-epicatechin, and quercetin (Vauzour et al. 2010b). The hydroxycinnamate, caffeic acid, has also been shown to be neuroprotective, counteracting inflammatory injury induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) by decreasing the production of a number of inflammatory cytokines, downregulating the expression of iNOS, COX-2, and glial fibrillary acidic protein, and lowering the production of NO and PGE_2 (Tsai et al. 2011). In addition, caffeic acid phenethyl ester may protect cerebellar granule neurons against glutamate-induced neuronal death via inhibition of p38 phosphorylation and caspase-3 activation (Wei et al. 2008) and significantly prevents hypoxic-ischaemic-induced neonatal rat brain damage in the cortex, hippocampus, and thalamus (Wei et al. 2004). Catechin is able to reduce DNA damage and inhibit tBHP-induced translocation of NF-κB to the nucleus in microglial cells (Huang et al. 2005), whereas the flavan-3-ol compound, epigallocatechin-3-gallate, inhibited iNOS activity (Kim et al. 2009) and diminished neuronal death.

Indeed, for any polyphenol to exert direct neuroprotective actions they must also undergo permeation of the blood–brain barrier (BBB), something that has been reported for both flavonoids and hydroxycinnamates (Janle et al. 2010; Youdim et al. 2003, 2004). However, whilst the ability of flavonoids to cross the BBB is believed to be dependent on lipophilicity, small phenolics are thought to transverse the BBB via amino acid transporters, such as has been reported for 4-ethylcatechol (Meiergerd and Schenk 1994). Furthermore, caffeic acid and other phenolics such as 3-hydroxyphenylacetic acid share structural similarities with L-DOPA and, as such, may undergo BBB transport via catecholamine transporter systems.

All together, these processes act to maintain the number and quality of synaptic connections in the brain, a factor known to be essential for efficient long-term potentiation (LTP), synaptic plasticity, and ultimately the efficient working of memory.

14.4 Conclusions

Human clinical trials and animal studies have identified polyphenol-rich foods and beverages as being capable of delaying the onset of age-related cognitive impairment. Through studies with grape-derived phenolic extracts and isolated

compounds, wine polyphenols have been postulated to evoke protection through the actions of absorbed flavonoids and their metabolites at the cellular level, enhancing neuronal function and/or stimulating cell regeneration. Although antioxidant mechanisms cannot be excluded, they are unlikely to be relevant in physiological conditions, and recent evidences suggest that such effects are mediated by their ability to modulate neuronal signalling, to stimulate neurotransmitter release, and to stimulate hippocampal neurogenesis.

Evidences that moderate wine consumption induces improvements in cognitive function, dependent on the potential of phenolic compounds to modulate neuronal cell signalling, have been reported. Concretely, in the case of one of the most prevalent neurodegenerative diseases, Alzheimer's disease, it seems that a moderate wine intake could be beneficial in the early stages of the disease. Wine polyphenols could prevent β-amyloid peptide and Tau protein aggregation, but also neuroinflammation process, an immune response that underpins neurodegenerative diseases, due to their interaction with several pathways involved in the process. In animal models of AD, a supplementation of red wine or its derivatives (isolated polyphenols or GSE) induces an improvement on cognitive functions, including memory or spatial memory, through changes on β-amyloid peptide levels in the brain. These results have been corroborated in human studies, where beneficial effects have been described.

It is important to mention that a key factor on the wine potential to prevent neuronal diseases is the bioavailability of the polyphenols after wine intake. Despite the scarce pharmacokinetic data of polyphenols, several studies demonstrated that polyphenols and their bioactive metabolites could readily cross BBB and exhibit pharmacological effects in the target regions of brain.

Altogether, these results suggest that wine consumption holds a potential to limit neurodegeneration and maintain healthy brain function. However, the pharmacological potential of these natural compounds still remains to be translated in humans in clinical conditions. The challenge ahead therefore is to proceed cautiously until rigorous randomized controlled clinical trials have been undertaken to determine whether wine polyphenols and/or their in vivo metabolites have efficacy in patients suffering from loss of neuronal function.

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Chapter 15
Metabolomic Approaches in the Study of Wine Benefits in Human Health

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15.1 Introduction

The past few decades were characterized by revolutionary developments in life science technologies. High-throughput and efficient, robust and rapid "-omics" technologies brought many advantages to research, allowing global analysis of samples on different system levels (Kussmann et al. 2008). They technically enabled the profiles of specific types of molecules (DNA, RNA, proteins, lipids, small compounds, etc.) to be studied and came to be used as holistic research approaches, such as transcriptomics, proteomics, lipidomics, glycomics and metabolomics, providing overall information about the status of the organism. Driven by constantly accumulating technical and methodological advances, the growth in "-omics" research has promoted its practical implementation in all the existing fields of life science, and nutrition is no exception (Kussmann et al. 2006, 2008). In fact, nutrition is probably one of the research fields where "-omics" approaches can bring many advantages, promote progression and open up new perspectives (Ordovas Munoz 2013). On the whole, the application of "-omics" technologies in nutrition is expected to stimulate exploration

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of the physiological processes involved in the interaction between nutrition and health and help to identify potential markers of this relationship. Basically, the principal goal of their application is to support the accomplishment of the main objective of nutrition research—to comprehensively understand the role of diet in human health. In this context, metabolomics is seen as one of the most powerful of the "-omics" approaches and can be widely applied in nutrition research (Kussmann et al. 2006, 2008; Ordovas Munoz 2013; Llorach et al. 2012; Scalbert et al. 2014; Primrose et al. 2011; Gibney et al. 2005; Brennan 2013).

Compared to other "-omics" sciences (i.e. genomics, transcriptomics, proteomics), a metabolomic approach has several potential advantages. Metabolomics involves the study of small molecules or metabolites present in biological samples (Brennan 2013). The metabolome, a whole set of metabolites, can be characterized by two components: (1) endogenous—metabolites defined by genetic background, age, sex, etc., and (2) exogenous—metabolites appearing in our organism from the environment, where the most important external factor is diet (Scalbert et al. 2014). On the whole, the metabolome is very complex and is constantly varying, as it is under continuous influence from numerous extrinsic and intrinsic factors (Gibney et al. 2005). In this sense, the study of metabolites reveals useful biological information since metabolites represent biological intermediates and/or end points of biological pathways and, therefore, are implicated in the health status of the organism (Brennan 2013; Llorach et al. 2012; Ordovas Munoz 2013). Thus, the characterization of metabolites in response to any kind of stimulus provides us with a snapshot of the metabolism or a molecular fingerprint of it, and this can serve as an index of the biological state of the organism (Astarita and Langridge 2013).

Currently nutritional research is experiencing a remarkable transformation driven by this new technological tool (Brennan 2013; Astarita and Langridge 2013; Gibney et al. 2005; Ismail et al. 2013; Jones et al. 2012; Llorach et al. 2012). Thus, a metabolomic approach has been increasingly applied in the analysis of food components, the identification of diet-derived metabolites, the evaluation of their bioavailability and metabolism, the analysis of gut microbiota metabolic activities, and the determination of physiological response to a particular diet regimen, food or nutraceuticals (Astarita and Langridge 2013; Scalbert et al. 2014). Finally, the application of metabolomics to nutrition research promotes the emergence and expansion of a novel "-omics" discipline, "nutrimetabolomics," devoted to the exploration of the complex metabolic relationship between nutrition and health at metabolome level. As a result, nutrimetabolomics forms an essential part of nutrition system biology (Panagiotou and Nielsen 2009; Jones et al. 2012), which is a new integrating approach to studying the impact of nutrition on biological systems by measuring and integrating genomic, proteomic and metabolic data.

In this chapter, we would like to focus on the achievements and perspectives to studying the impact of wine consumption on human well-being and health using this new methodological approach, metabolomics. Therefore, in the following sections we will consider the specificities of metabolomic analysis and we will offer an overview of the findings provided by applying a nutrimetabolomic approach in current wine research, emphasizing its relevance to understanding the complexity of the interaction between wine and health.

15.2 Specificity of Metabolomic Studies

Metabolomics in nutrition attempts to decipher metabolic alterations in response to a specific food ingredient, food product or diet (Kussmann et al. 2008; Scalbert et al. 2014). Depending on the research question, two analytical strategies are used in metabolomic studies: targeted and non-targeted (Patti et al. 2012; Zulyniak and Mutch 2011). Targeted analysis is a hypothesis-driven approach and is applied when a study is focused on the analysis of already known metabolites. It provides high-quality (quantitative) data on compounds using dedicated and optimized analytical methods. Historically targeted analysis was focused on studying a single or several resemble metabolites. Current technical progress has significantly improved the accuracy, sensitivity and capacity of analytical platforms, which has permitted the analysis of complex metabolite systems, such as pathways and classes, driven by both specific (targeted profiling) and exploratory (non-targeted profiling and fingerprinting) interests (Fernie et al. 2004). In contrast to targeted analysis, non-targeted analysis is a data-driven approach, aimed at analysing the metabolome as broadly as possible, providing a holistic overview of the metabolic changes occurring under different conditions. Such wide-ranging metabolome screening provides only qualitative data, which is used for the generation of new hypotheses. Both approaches can complement each other in respect of providing question data that is relevant to research; thus, certain information about metabolites and related pathways that could not be gained through a targeted approach due to its methodological restrictions could be explored by untargeted analysis; and vice versa, qualitative information obtained from a data-driven approach could be validated and confirmed by targeted analysis.

Metabolome responsiveness to external and internal stimuli is the fundamental asset of nutrimetabolomic studies; however, on the other hand, it is a major challenge from a methodological standpoint. Multiple sources of metabolic variability, such as genetic polymorphisms, environmental conditions and feeding patterns (Kim et al. 2014; Nicholson et al. 2011; Floegel et al. 2013) determined by a variety of biological, cultural, socio-economic, psychological and behavioural factors (Kussmann et al. 2008), need to be carefully considered in the experimental design of any nutrimetabolomic study in order to reduce biological components in metabolic variations that are not linked to the dietary intervention itself (Zulyniak and Mutch 2011). On the other hand, the workflow procedure should be standardized as well to minimize the technical variability component in the global system of metabolic variation (Kim et al. 2014; Burton et al. 2008; Zulyniak and Mutch 2011; Issaq et al. 2009).

A five-step workflow is typically applied in metabolomic studies, including nutrimetabolomic ones (Llorach et al. 2012; Brennan 2013): (1) sample collection; (2) sample preparation; (3) data acquisition; (4) data analysis; and (5) biological interpretation of the obtained results.

The choice of sample type, preparation, separation platform and analytical instrumentation should be considered in respect of obtaining high-quality data used for analysis, especially in untargeted metabolomic analysis, and will be defined by the objectives of the study (Patti et al. 2012; Zulyniak and Mutch 2011; Tulipani et al.

2013; Issaq et al. 2009). Blood (serum and plasma), reflecting mainly instantaneous metabolic events and dynamics of nutrient and metabolite flow in the inter-organ metabolism (Kussmann et al. 2008) or information stemming from prolonged nutrient exposure (Zulyniak and Mutch 2011), and urine, providing a time-averaged representation of recent homeostatic metabolic changes and of gut microbial metabolic activities (Kussmann et al. 2008; Bouatra et al. 2013), are among the first-choice samples used in nutrition research. In addition to their relevant physiological meaning for nutrition, these biofluids are obtained in a non-invasive way using well-standardized sampling protocols, which make them widely used in human intervention and epidemiological studies and, consequently, in nutrimetabolomic studies. However, depending on the initial biological question, other types of samples could be used in nutrition research (Gibney et al. 2005; Jenab et al. 2009), including faecal samples, increasingly applied to study information on metabolic gut health, digestive efficiency and the activity of gut microbiota (Kussmann et al. 2008).

Any type of biosample is characterized by its inherent metabolome complexity. Sample preparation methods depend on the sensitivity and specificity of the selected analytical techniques and consist of extracting analytes from the biological matrix and bringing them into a format compatible with the analytical technique used (Fernández-Peralbo and Luque de Castro 2012; Tulipani et al. 2013; Issaq et al. 2009). Various technical platforms are applied in metabolome analysis, among which mass spectrometry (MS), coupled to separation techniques such as gas (GC) or liquid (LC) chromatography, and nuclear magnetic resonance spectrometry (NMR) are the most commonly used (Ismail et al. 2013; Kussmann et al. 2008; Zulyniak and Mutch 2011; Issaq et al. 2009). However, none of the currently existing analytical platforms are able to measure the whole metabolome (Brennan 2013; Issaq et al. 2009). Each technology has its own advantages and disadvantages, reflected in its capacity to detect specific types of compounds (Issaq et al. 2009), and therefore they provide different sets of information, some parts of which could be overlapped. To obtain optimal coverage of the metabolome, especially in non-targeted analysis, a combination of several platforms could be used in metabolomic studies (van Dorsten et al. 2010; Jacobs et al. 2012a).

Metabolomic studies, especially non-targeted ones, generate huge amounts of data, which should be carefully preprocessed prior to being analysed in order to transform the data in the data set into more comparable formats in order to ease and improve its analysis (Smolinska et al. 2012; Issaq et al. 2009). The quality of generated data is monitored by applying specific controls (Gika et al. 2008). While data obtained from targeted profiling normally could be treated by applying univariate statistical analysis, the complexity of metabolic profiling very often requires data mining techniques based on multivariate statistical methods (Issaq et al. 2009; Trygg et al. 2007). A set of unsupervised methods (pattern recognition and clustering analysis, without prior knowledge of sample class) and supervised methods (several discriminant analyses based on previously predefined classes of samples) could be applied to data in order to evaluate alteration in metabolic signatures or assess possible metabolic markers, respectively. Any supervised modelling used for identifying metabolites that differ between studied groups should be validated, in order to mini-

mize false positive discoveries. The differential signals recognized by multivariate analysis should be further identified, by applying a series of analytical and computational approaches, combined in a multistep identification process (Llorach-Asuncion et al. 2010; Smolinska et al. 2012). In many cases, metabolites can be positively identified if their orthogonal measures (retention time, mass spectrum, accurate mass, MS/MS fragmentation, isotope abundance pattern or chemical shifts, peak intensities and spin coupling patterns) match a known standard or literature values, otherwise they are marked as unknowns, which could be previously reported but not identified or de novo described (Wishart 2011). Identified altered metabolites should be investigated in terms of their correspondence to specific metabolic pathways in order to make a biological interpretation of the metabolic alteration and to draw conclusions about its relevance to human health.

15.3 Metabolomic Approach in Studying the Interaction between Wine and Health

Over the last decade, the metabolomic approach also started to be applied in the investigation of the metabolic impact of wine and its components (principally wine polyphenols) on the human organism. Several human metabolomic studies, as shown in Table 15.1, have so far been conducted in wine nutrition research targeting diverse subjects. For reviewing purposes we have combined them according to the methodological approaches applied, and research focus addressed, into: wine- and grape-derived polyphenols (PPh) metabolomic research (Sect. 15.3.1), discovery of wine consumption and effect biomarkers (Sect. 15.3.2), resveratrol metabolism profiling (Sect. 15.3.3) and targeted research on the metabolic interaction between microbiota and wine (Sect. 15.3.4).

15.3.1 Metabolic Imprints of Wine Polyphenol Consumption

A number of metabolomics studies were conducted by a group from the Netherlands in which the effect of red wine- and grape-derived PPh consumption on human host and gut bacterial metabolism was studied using ^{1}H-NMR spectroscopy on its own or in combination with GC-MS, LC-MS untargeted and targeted approaches in order to increase the coverage of the metabolome and to expand the interpretation of nutritional intervention (van Dorsten et al. 2010; Jacobs et al. 2008, 2012a, b). In their first work (Jacobs et al. 2008) (Table 15.1, N1), designed as a methodological study aimed at developing and validating the NMR-based untargeted metabolome profiling of faeces, the impact of wine and grape juice extracts on human faecal metabolome was evaluated in samples collected in a midterm randomized crossover intervention study. Methodologically, the study was performed by using differential polarity extraction procedures, which enabled the detection and analysis of a relatively wide

Table 15.1 Application of metabolomics in studies with wine and wine-derived products in humans

No.	Intervention and dosage	Study design and duration	Subjects and health status	Samples analysed	Metabolomic approach	Main findings	Ref.
1	MIX (GJX+WX) vs. GJX (800 mg GAE PPh/day)	PC, RM, CO (e.g. B/A) MTI (4 weeks)	31 ♂ 22 ♀ Mildly hypertensive non-smokers	Faeces	Untargeted ^{1}H-NMR	– Detection of wide range of compounds: SCFA, organic and amino Acs, PhAcs, lipid components, etc. – ↓ Isobutyrate (GJX+WX) – Interindividual variability in faeces related to concentration rather than to composition of detected metabolites	Jacobs et al. (2008)
2	MIX (GJX+WX) vs. GJX (800 mg GAE PPh/day)	PC, RM, CO (e.g. B/A) MTI (4 weeks)	33 ♂ 25 ♀ Mildly hypertensive non-smokers	24 h urine	Untargeted ^{1}H-NMR and NMR (HipAc)	– ↑ HipAc (35 %), ↑ 4-OHHipAc, betaine, ↑ trigonelline, and unidentified aromatic compounds (GJX+WX) – ↑ Citrate (GJX+WX)	van Dorsten et al. (2010)
					Targeted GC-MS-based PhAcs profiling	– ↑ 18 PhAcs (GJX+WX and GJX): SyrAc, 3- and 4-OHHipAc; 3-OHPhAtAc, 4-OH-mandelic and vanillylmandelic Acs – ↑ HipAc, 3-OHPhPrAc, 3,4-diOHPhPrAc and 1,2,3-triOHBenzen (GJX+WX) – ↑ 4-OHPhAtAc, homovanillic and diOHFer Acs and phenylglutamine (GJX) – ↑ TCA: isocitrate (GJX+WX), *cis*-aconitate and oxaloacetate (GJX)	

3	WGM (870 mg red wine + 540 mg grape juice extracts)	PC, RM, CO STI (4 days)	35 ♂ Healthy	24 h urine	Targeted GC-MS-based PhAcs profiling and NMR (HipAc)	– ↑ SyrAc, 3-OHHipAc; pyrogallol; 3-OHPhAtAc; 3-(3-OHPh)PrAc and FerAc; trans-CinAc; VanAc	Jacobs et al. (2012a)
			Subpopulation 21 ♂ Healthy	24 h urine and plasma	Untargeted GC-MS- and LC-MS/MS-based polar and non-polar fractions profiling Targeted SPE-LC-MS/MS-based catecholamines and steroids profiling	Urine metabolites ($n=17$): – Phenolic metabolites: ↑ 3-OHHipAc; pyrogallol; 3-OHPhAtAc; HipAc; catechol, 4-OHHipAc; 3,4-diOHHipAc; VanAc; trans-FerAc – Endogenous metabolites: ↑ indole-3-lactic acid; glucose-1-phosphate; sucrose; nicotinic acid; 1-methylhistidine; ↓ 3-indoxylsulphuric Ac; p-cresol Sulf; 3,4-diOHPhglycol Plasma metabolites ($n=12$): ↓ tyrosine; sphingomyelin; dehydroepiandrosterone Sulf; two lysophosphatidylcholines; two phosphatidylcholines; threonine; ↑ oleic acid; phosphatidylcholine, phosphate (lipid fraction)	

(continued)

Table 15.1 (continued)

No.	Intervention and dosage	Study design and duration	Subjects and health status	Samples analysed	Metabolomic approach	Main findings	Ref.
4	WGM (870 mg red wine + 540 mg grape juice extracts) in 200 mL water vs. WGM_Soy (870 mg red wine + 540 mg grape juice extracts) in 200 mL soy drink	PC, RM, CO (e.g. B/A) STI (4 days)	35 ♂ Healthy	24 h urine	Untargeted ^{1}H-NMR	Sub-profiles of SPE fractioned urine: – Fraction of aromatic compounds, e.g. microbiota PPh metabolites: ↑ 3-OHPhAtAc; homovanillate; 3-(3-OHPh)-3-OHPrAc; 4-OHHipAc, HipAc, 3-OHHipAc, caprate, 2-PhPrAc, tryptophan, several unknowns (WGM vs. PC independently of food matrix) – no impact of tested food matrix on WGX polyphenol metabolism – Fraction of semi-polar compounds: ↑ *cis*- and *trans*-aconitates (WGM_Soy vs. PC) ↑ *cis*-aconitate ↓-tryptophan (water vs. soy matrix in WGM treated) – Fraction of polar metabolites, e.g. endogenous origin: ↑-citrate, *cis*-aconitate, trigolline (WGM_Soy vs. PC) Non-fractioned urine profiles: ↑ 2-OHHipAc,HipAc and unknown (WGM vs. PC) ↑ 2-OHHipAc, HipAc, *cis*-aconitate; citrate (WGM_Soy vs. PC) ↑ *cis*-aconitate (water vs. soy matric in WGM treatments)	Jacobs et al. (2012b)

5	RWA (272 mL/day, 30 g EtOH/day, 2933 PPh in mEqGAE/L) vs. RWD (272 mL/day, 0 g EtOH/day, 2694 PPh in mEqGAE/L) vs. GIN (100 mL/day, 38 g EtOH/day, 0 PPh in mEqGAE/L)	RM, CO (e.g. B/A) MTI (4 weeks)	61♂ High-risk coronary heart disease	24 h urine	Untargeted ^{1}H-NMR	*Wine markers*: – food metabolome markers: tartrate (RWA and RWD), ethanol and mannitol (RWA) – endogenous markers: 3-methyl-oxovalerate (first-stage catabolite of branched-chain amino acid (BCAA)) – gut microbiota markers: HipAc, 4-OHPhAtAc	Vazquez-Fresno et al. (2012)
6	RWA (272 mL/day, 30 g EtOH/day, 2933 PPh in mEqGAE/L) and PREDIMED (epidemiological study)	RM, CO MTI (4 weeks) CS (free-living subjects): Non-consumers vs. consumers	56 ♂ High-risk coronary heart disease 91 (♂+♀) High-risk coronary heart disease	24 h urine	Untargeted ^{1}H-NMR	*Wine intake individual markers (by AUC%)*: Ethyl glucuronide (86.3 %); Tartrate (85.7 %); Ethanol (75.6 %); 2,3-butanediol (75.6 %); 3-methyl-2-oxovalerate (70.8 %); Mannitol (67.4 %); Three unknown compounds (range 73.8–76.5 %). *Wine intake combined markers (by AUC%)*: [Tartrate+Ethyl glucuronide] marker (90.7 %) in AI study [Tartrate+Ethyl glucuronide] marker (92.4 %) in cohort study. *Markers of wine effect*: 3-methyl-2-oxovalerate (both AI and cohort study)	Vázquez-Fresno et al. (2014)

(continued)

Table 15.1 (continued)

No.	Intervention and dosage	Study design and duration	Subjects and health status	Samples analysed	Metabolomic approach	Main findings	Ref.
7	RWA (375 mL) vs. GE (15 tablets/400 mL water)	PA (e.g. B/A) AI	10♂ (7♂ RW and 3♂ GE) Healthy non-smokers	Plasma kinetics Urine kinetics	Targeted LC-ESI-MS/MS resveratrol metabolism profiling	– 17 identified Resv and piceid metabolites: Resv- glucoside, phase II metabolites of *cis*- and *trans*-Resv, phase II metabolites of piceid and phase II metabolites of diH-Resv – Plasma and urinary pharmacokinetics of RW-derived piceid and Resv host and microbial metabolites	Rotches-Ribalta et al. (2012a)
8	RWA (272 mL, 30 g EtOH/day) vs. RWD (272 mL, 0 g EtOH/day)	RM, CO MTI (4 weeks)	59 (♂ + ♀) High cardiovascular risk	24 h urine	Targeted LC-ESI-MS/MS resveratrol metabolism profiling	– Improve detection of sulphated Resv metabolites – 21 identified host and microbiota-derived Resv metabolites – Bioavailability of Resv is not affected by alcohol	Rotches-Ribalta et al. (2012b)

9	RWA (272 mL/day, 30 g EtOH/day, 2933 PPh in mEqGAE/L) and RWD (272 mL/day, 0 g EtOH/day, 2694 PPh in mEqGAE/L) and GIN (100 mL/day, 38 g EtOH/day, 0 PPh in mEqGAE/L)	RM, CO MTI (4 weeks)	8 Healthy	Faeces	Targeted UPLC-ESI-MS/MS microbial PhAcs profiling	– ↑ 8 phenolic acids: 3,5-diOHBzAc, protocatechuic Ac, p-coumaric Ac; 3-O-MetGalAc, VanAc; SyrAc; 4-OH-5-PhValAc, PhPrAc – Alcohol does not influence the formation of RW phenolic metabolites by the gut microbiota. – Evaluated "basal" profiles of faeces phenolic compounds – Interindividual variability in faeces RW-derived phenolic content reflects variation in colonic microbiota	Jimenez-Giron et al. (2013)
10	RWA (250 mL/day, ≈450 mg total PPh/day, where ~270 mg/L monomeric and oligomeric flavan-3-ols)	PA, RM MTI (4 weeks)	41 (19♂+22♀) – 33 intervention – 8 control	Faeces	Targeted UPLC-ESI-MS/MS microbial PhAcs profiling	– 35 quantified metabolites – No wine original phenolics detected in faeces – High "basal" interindividual variability in faeces phenolic content – ↑ total phenolic metabolite content of faeces after RW – Subject grouping by total RW-derived PPh detected in faeces: (1) <500, (2) 500–1000, and (3) >1000 μg/g – ↑ 10 compounds: 3,5-diOHBzAc, protocatechuic Ac, 3-O-MetGalAc, VanAc; 3-OHPhAtAc; SyrAc; 4-OH-5-(3′,4′-diOHPh)ValAc; 4-OH-5-PhValAc, 5-(3′-OHPh)-γ-valerolactone, 3-PhPrAc	Munoz-Gonzalez et al. (2013)

(continued)

Table 15.1 (continued)

No.	Intervention and dosage	Study design and duration	Subjects and health status	Samples analysed	Metabolomic approach	Main findings	Ref.
11	RWD (272 mL, 0 g EtOH 2694 mEqGAE/L)	B/A MTI (4 weeks)	36 ♂ High cardiovascular risk	24 h urine	Targeted UPLC-ESI-MS/MS microbial-derived phenolic profiling	– Identified 37 deconjugated and 24 conjugated metabolites – ↑ 21 phase II metabolites: 11 flavan-3-ols (Sulfs, Glucs of epicatechin and Met-epicatechins); 4 OHBzAcs (Met-GalAc-Sulf and ethylgallate-Sulf; 2 OHBzAc-Glucs); 7 phase II OHPh-valerolactone metabolites (DHPV- and MHPV-Sulfs and -Glucs) – ↑ 28 microbial metabolites: 13 OHBzAc derivatives (2,4-, 2,6-, 2,5-3,5-diOHBzAcs; protocatechuic Ac; SyrAc; 4- and 3-OHBzAcs; 4- and 3-OHHipAcs; GalAc and Met-GalAc; ethylgallate); 5 OHPhAtAc derivatives (PhAtAc; 3- and 2-OHPhAtAcs; 3,4-diOHPhAtAc; homovanillic acid); 6 OH-cinnamic Acs (*m*-, *o*- and *p*-coumaric Acs; CafAc; FerAc; SinAc); 3 OHPhPrAc derivatives (3-(4-OHPh)- and 3-(3-OHPh)-PrAc; dihydro-CafAc); 2 glycines (vanillyl- and feruloyl-glycines); 2 DHPVs; enterolactone and pyrogallol	Boto-Ordonez et al. (2013)

AI acute intervention (single dose), *AtAc* acetic acid, *AUC* area under curve of receiver operating characteristics (ROC); *B/A* before to after comparison, *B/A* before/after design of the study, *BzAc* benzoic acid, *CafAc* caffeic acid, *CinAc* cinnamic acid, *CO* crossover design, *CS* cohort study, *DHPV* dihydroxyphenylvalerolactone, *EtOH* ethanol, *FerAc* ferulic acid, *GAE* gallic acid equivalents, *GalAc* gallic acid, *GE* grape extract, *GIN* gin, *GJX* grape juice extract, *Gluc* glucuronide, *H* hydro, *HipAc* hippuric acid, *OH* hydroxy, *LLE* liquid-liquid extraction, *Met* methyl, *MHPV* methylhydroxyphenylvalerolactone, *MIX* the wine (WX) and grape juice (GJX) extracts mix, *MTI* midterm intervention, *PA* parallel arm design, *PC* placebo-controlled study, *Ph* phenyl, *PhAc(s)* phenolic acid(s), *PPh* polyphenols, *PrAc* propionic acid, *Resv* resveratrol, *RM* randomized intervention, *RWA* red wine (containing alcohol), *RWD* dealcoholized red wine, *SCFA* short-chain fatty acids, *SinAc* sinapic acid, *SPE* solid-phase extraction, *STI* short-term intervention, *Sulf* sulphate, *SyrAc* syringic acid, *ValAc* valeric acid, *VanAc* vanillic acid, *WGM* mix of wine- and grape juice-derived extracts, *WX* wine extract

range of metabolites with different physicochemical properties, including polar compounds such as short-chain fatty acids (SCFA), organic acids, amino acids, trimethylamine, ethanol, glycerol and glucose, and less polar compounds such as cholate, lipids, bile acids and phenolic acids. As a result, more elaborate NMR-based profiling of faecal samples provided a holistic view on the fermentation products of dietary components and the activity of microbiota. The application of an advanced approach revealed that, in contrast to GJX (grape juice extract), a 4 weeks consumption of MIX (mix of grape juice and wine extracts) induced significant changes in NMR metabolome profiles of faeces. Some interesting results on the interaction between wine polyphenols and microbiota were reported. Thus, the reduction of isobutyrate was monitored after MIX consumption, which was related by the authors to the inhibition of protein fermentation by wine extract PPh. The wine part of the MIX extract contained roughly 100 times more catechin than the grape juice part. Since no such changes were observed after GJX consumption, the catechin content of the wine extract was linked to the isobutyrate effect, and thus it was suggested that it was able to modulate the microbial ecology of the gut. Other interesting observations were related to the variability of metabolomic profiles. Thus, interindividual variability was related to metabolite concentration rather than to metabolite composition, suggesting that different colonic microbiotas share general biochemical characteristics, whereas high intra-individual variability in faeces metabolome indicated that diet and lifestyle greatly affect the metabolome, suggesting that they should be considered carefully in the design of nutrimetabolomic studies.

A global metabolite profiling of 24 h urine was studied by the group in a subsequent study (van Dorsten et al. 2010) (Table 15.1, N2) in order to investigate the urinary excretion of phenolic acids of microbiota origin and to study changes in other urinary metabolites that could signal an impact of grape PPh intake on (metabolic) health status. As in the previous study, contrasting MIX with GJX intervention enabled researchers to set aside those effects provoked by the consumption of wine-derived PPh. The multivariate analysis of global ^{1}H-NMR profiling revealed that the consumption of wine PPh within complex extract resulted in significant increase of hippuric acid (HipAc) and 4-hydroxyhippuric acid (4-OHHipAc) in 24 h urine. Due to the limited sensitivity of the NMR technique in the detection of phenolic acids, a more sensitive GC-MS targeted approach (Grun et al. 2008) was used to complement the analysis. As a result, about 18 different phenolic acids were found to be significantly elevated due to both interventions. However, HipAc (already in agreement with the NMR findings), 3-hydroxyphenylpropionic acid (3-OHPhPrAc), 3,4-dihydroxyphenylpropionic acid (3,4-diOHPhPrAc) and 1,2,3-trihydroxybenzen (1,2,3-triOHBenzen) were associated only with the consumption of MIX (Table 15.1, N2), which was related to its higher wine-derived procyanidin and catechin content. In addition to giving insights into the metabolic degradation of grape and wine PPh in humans, this metabolomic study has also demonstrated changes in endogenous urinary metabolites. A slightly different effect between consumption of the two extracts on endogenous metabolite excretion was also related to differences in the PPh content of these interventions. Thus, NMR detected an increase in urinary excretion of citrate after MIX intake, suggesting that

wine PPh could have an impact on mitochondrial tricarboxylic acid (TCA) cycle turnover. The GC-MS targeted profiling further advocated changes in the TCA cycle by monitoring the elevation of other urinary TCA intermediates, such as isocitrate. In addition, NMR metabolome profiling showed that MIX supplementation was associated with raised excretion of betaine, an important methyl donor and a regulator of homocysteine homeostasis and to a lesser extent trigolline, a metabolite of niacin (vit B3). 4-Hydroxymandelic (4-OH-mandelic) and 3-methoxy-4-hydroxymandelic (3-MO-4-OH-mandelic, vanillylmandelic) acids, metabolites of catecholamines, detected by GC-MS, were also elevated due to the MIX consumption. Thus, the authors suggested that endogenous biological pathways, such as phenylalanine metabolism, biogenic amines and catecholamine metabolisms, could be modulated by the consumption of wine PPh.

In a subsequent study (Jacobs et al. 2012a) (Table 15.1, N3), the group tried to identify phenolic metabolites, known to originate from gut microbial fermentation, in both urine and plasma after 4-day consumption of a mix of wine and grape juice extracts (WGM) similar to the previous study. A combination of specific sample preparation with both targeted and untargeted mass-spectrometric approaches was used to achieve optimum profiling of interesting metabolites. Urine profiling by targeted GC-MS detected several phenolic acids as the most relevant for gut microbiota fermentation of the consumed PPh; however, the origin (wine or grape juice extract) of the detected phenolic acids remained unclear. A wide range of metabolites involved in many different metabolic pathways was analysed in this study with the aim of generating new hypotheses on the mechanism of action of ingested polyphenols. The most noticeable were changes in microbial fermentation products of aromatic amino acids (Table 15.1, N3), which were in line with changes observed in faeces (Jacobs et al. 2008). In addition, the reduction, detected in this study, in urinary 1-methylhistidine, a metabolite of dietary protein, and in tyrosine, threonine and lysine plasmatic levels, also supported the idea of the ability of PPh to influence protein digestibility. These data led the authors to the hypothesis that wine and grape PPh or their microbial metabolites may benefit human health by reducing colonic protein fermentation and/or changing microbial amino acid metabolism. These results encourage the follow-up and study of the cross-talk between the gut microbial metabolism and host metabolic response following PPh and protein consumption. A subtle alteration in the urinary levels of 3,4-dihydroxyphenylglycol (3,4-diOHPhglycol) was observed (Table 15.1, N3) that suggested that wine PPh, most probably due to the presence of tyramine, might influence, through norepinephrine, turnover of catecholamine metabolism. The reduction of plasmatic sphingomyelin, two lysophosphatidylcholines and two phosphatidylcholines was detected and this, according to the authors, might indicate that wine and grape PPh affect the inflammatory signalling cascade, possibly at the mucosal gastrointestinal barrier.

The main pitfalls of conventional NMR metabolome profiling, i.e. limited sensitivity and signal overlapping, could be partially overcome by the application of SPE sub-profiling of urine, which was demonstrated in the latest study of the group

(Jacobs et al. 2012b) (Table 15.1, N4). The methodological upgrade consisted in the application of different polarity fractions obtained by automated SPE to NMR analysis in order to enable more accurate quantification and less ambiguous identification of metabolites. This methodological improvement was tested on 24-h urine samples collected in a short-term (4 days) crossover placebo-controlled intervention trial in which volunteers consumed a PPh-rich mixture of red wine and grape juice extracts (WGM) in two different food matrixes: water and soy drink. As a result, the increase in urinary excretion of several microbiota-derived phenolic acids (Table 15.1, N4), normally undetectable by conventional NMR, was monitored. These changes were related to WGM consumption, independently of the food matrix applied. However, on the other hand, it was observed that soy drink induced an excretion of aconitate, citrate and trigonelline, suggesting that endogenous metabolism was affected possibly via the tricarboxylic cycle (TCA). This could be very important, since earlier studies (van Dorsten et al. 2010) suggested that the TCA cycle was a putative target for wine PPh consumption. Consequently, the issue of the suitability of soy drink as a potential carrier for wine and grape PPh should be studied further. In this respect, some metabolic issues should be additionally clarified, since even under recent upgrades, the methods had restricted sensitivity to some lower abundant metabolites of WGM origin, endogenous compounds and some soy-derived PPh metabolites. On the whole, the study opened encouraging prospects for the technical improvement of NMR-based compound detection and analysis, aimed at providing better access for the analysis of metabolites involved in the interaction between diet and health.

15.3.2 Impact of Wine Consumption on Human Metabolome

In parallel to nutrimetabolomic studies focused principally on the PPh component of wine and grape juice extracts, a couple of untargeted studies on complex wine were performed by our group (Vázquez-Fresno et al. 2014; Vazquez-Fresno et al. 2012) using a conventional ^{1}H-NMR-based untargeted approach (Table 15.1, N5 and N6). The individual and combined impacts of the two principal bioactive parts of wine, alcohol and PPh, on human urinary metabolome were evaluated in the first study (Vazquez-Fresno et al. 2012) (Table 15.1, N5) by analysing 24-h urine samples after midterm (4 weeks) intervention with red wine (RWA) at real-life doses, which was performed in parallel with dealcoholized red wine (RWD) (the same PPh content) and gin (ethanol control) treatments in a randomized crossover study. The detection of tartaric acid, a major acid in grapes and also a chief component of wine, after RWA and RWD intake was related to wine PPh consumption, whereas ethanol was related to alcohol component of wine, and was obviously also detected after gin consumption. Two recognized colonic microbiota PPh metabolites, hippuric acid (HipAc) and 4-hydroxyphenylacetic acid (4-OHPhAtAc), were related to the consumption of the PPh content of wine (Table 15.1, N5). However, in

contrast to 4-OHPhAtAc, which also increased after RWA consumption, HipAc did not change as a result of this treatment, suggesting that the alcohol content of wine might have some impact on the metabolic pathway related to the production of this compound. As a result, the study reported on the following markers of red wine consumption: tartaric acid, as a wine-derived compound, 4-OHPhAtAc related to the gut metabolism of wine PPh, and ethanol as the principal metabolite of wine alcohol. Shortly after this study, urinary tartaric acid was confirmed as a sensitive and specific dietary biomarker of wine consumption in a randomized crossover feeding trial (Regueiro et al. 2014). Finally, based on the obtained results, it was also projected that a combination of tartrate and ethanol markers could also be used in the monitoring of global compliance to wine dietary intervention. In fact, in our second study (Vázquez-Fresno et al. 2014), the combined biomarker model was successfully developed based on the previous intervention study. Ethanol glucuronide, a principal metabolite of ethanol, was identified and in combination with tartaric acid was shown to be a strong NMR-detected biomarker for wine consumption not only in a controlled intervention study with wine but also in a cohort study involving free-living subjects (Table 15.1, N6). Moreover, this "tartrate-ethyl glucuronide" model, being more sensitive than individual markers themselves, showed a promising performance since its sensitivity accounted for discrimination of up to 3 days post-consumption of one glass of wine in a free-living population. So far, only resveratrol and resveratrol metabolites fulfil the criteria to be considered as nutritional biomarkers of wine consumption for application in epidemiological studies (Zamora-Ros et al. 2009). These findings provided wine nutritional research with promising biomarkers, which could be used alone or in combination with classical dietary assessment methods in clinical and epidemiological studies in order to be able to estimate more precisely the relationship between wine and health.

An endogenous product of first-step catabolism of branch-chain amino acids (BCAA), 3-methyl-2-oxovalerate, was identified in urine after consumption of wine in an intervention study (Vazquez-Fresno et al. 2012; Vázquez-Fresno et al. 2014) (Table 15.1, N5 and N6). Interestingly, this metabolite was also elevated in the group of wine consumers from the epidemiological study (Vázquez-Fresno et al. 2014) (Table 15.1, N6), thus behaving really as a robust marker of the effects of wine consumption. In general, elevated levels of BCAA could be related to a decrease in their metabolic rate, which is associated with health-compromising statuses such as insulin resistance, diabetes, and cardiovascular disease (Batch et al. 2014). The increase of urinary 3-methyl-2-oxovalerate levels detected in both studies could suggest that the induction of BCAA catabolism by wine PPh components has benefits for health, which should be studied in further investigations. On the whole, the results of these two studies showed the capacity of a conventional NMR-based untargeted approach to obtain a comprehensive metabolome picture including food metabolome and endogenous biomarkers of moderate wine intake, thereby fully supporting the application of this approach in complex clinical or epidemiological studies.

15.3.3 Metabolomics in Resveratrol Research

Resveratrol is a natural stilbene with specific physicochemical properties responsible for its biological activity (Szekeres et al. 2010). Interest in resveratrol as a principal bioactive compound of wine lies in a reductionist approach, which investigates the beneficial health impacts of individual wine PPh as the main factors responsible for the "French paradox" effect (Renaud et al. 1998; Chiva-Blanch et al. 2013). In this respect, resveratrol has been and is extensively studied for its biological activity in relation to the observed benefits of wine consumption for health (Delmas et al. 2011). Although this PPh is also present in some other foods, wines are the most important source of resveratrol in people's diet (Zamora-Ros et al. 2008). Thus, resveratrol was shown to be a good marker of dietary intake of wine in both interventional (Zamora-Ros et al. 2006) and epidemiological studies (Zamora-Ros et al. 2009). In fact, the latter study (Zamora-Ros et al. 2009) showed that using resveratrol metabolites, instead of single resveratrol, could increase the ability to discriminate between wine consumers and non-wine consumers even in a free-living population.

Previous studies showed an extensive metabolic conversion of dietary resveratrol consumption (Delmas et al. 2011; Andres-Lacueva et al. 2009). Consequently, the role of resveratrol metabolism became recognized both in relation to its biological activity and human health interactions (Delmas et al. 2011) and as a good marker of wine consumption (Zamora-Ros et al. 2009). In contrast to other wine PPh, resveratrol metabolites could not be monitored by conventional non-targeted metabolomic approaches (NMR- and MS-based), principally due to the relatively low concentrations of bioavailable resveratrol metabolites, in addition to specific technical limitations of these approaches. In this respect, more sensitive targeted profiling of metabolites is a method that can provide a comprehensive overview of the complexity of resveratrol metabolism. Recently two LC-MS-based targeted studies evaluating complex profiles of metabolites provided updated information on resveratrol metabolism in humans (Rotches-Ribalta et al. 2012a, b). In the first study (Table 15.1, N7), the pharmacokinetics of plasmatic and urinary resveratrol metabolic profiles was studied after moderate consumption of red wine. Seventeen metabolites of resveratrol (12 metabolites) and piceid (5 metabolites) were identified (Table 15.1, N7). This was the first pharmacokinetics study to consider such a large metabolic profile of resveratrol and piceid in humans after an acute intake of RWA in moderate doses. Later, in a second study (Rotches-Ribalta et al. 2012b) (Table 15.1, N8), resveratrol metabolite profiling was extended by refining the sample preparation procedure. Thanks to the availability of some standards, an optimized methodology permitted more accurate identification and quantification of sulphoconjugated metabolites (e.g. disulphated and sulphoglucuronidated), which were previously underestimated, but whose impact could be significantly relevant to human health through the modification of resveratrol stability, chemical structure, transport and metabolism at the cellular level of the organism (Delmas et al. 2011; Patel et al. 2013). In total, 21 different resveratrol metabolites, including those formed by gut and microbiota metabolism, were included in the targeted resveratrol profiling of 24 h urine samples from the

crossover intervention study with a 4 weeks intake of red wine (RWA) and dealcoholized red wine (RWD) with the same PPh content. Targeted profiling of urinary resveratrol metabolites showed that the alcohol component of wine did not affect the resveratrol bioavailability, either in total or as a composition of its various metabolites. On the whole, these studies presented a focused resveratrol metabolism profiling approach, which is not only able to provide a global sum of the total metabolites, which is of important value for resveratrol bioavailability estimation, but also show the importance of each individual resveratrol metabolite in metabolism and the health effect of wine-derived resveratrol.

15.3.4 Application of Targeted Metabolomics in Exploring Wine-Microbiota Interaction

Recent interest in the role of colonic microbiota in the bioavailability and bioactivity of dietary PPh (Cardona et al. 2013; Etxeberria et al. 2013) has encouraged further investigation into the metabolic fate of grape- and wine-derived PPh in the human organism. Previously, studies tended to evaluate the microbiota-mediated interaction between wine and health mainly through a reductionist approach, focusing on single components of wine as its primary PPh, such as resveratrol (Bode et al. 2013), catechin and procyanidins (Gonthier et al. 2003), anthocyanin (Forester and Waterhouse 2008), etc., or vice versa on activities of specific strains of colonic microbes (Barroso et al. 2013). However, the high complexity of gut microbiome and its high interindividual variability challenge such evaluations; in addition, the impact of wine as a complex food could not be explained. The application of metabolomic approaches opens up the opportunity to have a more holistic look at the issue (Moco et al. 2012).

The composition of microbial phenolic metabolites in faecal samples collected after regular consumption of either red wine (RWA), dealcoholized red wine (RWD) or gin (GIN) was analysed using targeted LC-MS (Jimenez-Giron et al. 2013) in a pilot crossover study (Table 15.1, N9). For this purpose, the method for detecting and quantifying 60 different phenolic metabolites in faeces was earlier developed and validated (Sanchez-Patan et al. 2011). The analysis showed that the microbial metabolic profile of faeces was substantially modified after moderate intake of red wine, either dealcoholized or not, and eight phenolic acids were significantly increased. Metabolites derived from the catabolism of both flavan-3-ols and anthocyanins seemed to contribute to these changes. According to the results of the study, the bioavailability and biotransformation of red wine PPh by gut microbiota, and likewise resveratrol (Rotches-Ribalta et al. 2012b), were not affected by the alcoholic matrix of the wine. Large interindividual differences in the formation of microbial metabolites after each red wine intervention, but not after gin intervention, were related to variations in the microbiota composition among subjects.

The developed targeted analysis was applied by the same group in a larger study with 41 volunteers (Munoz-Gonzalez et al. 2013) (Table 15.1, N10). This time, changes in the microbial-derived phenolic metabolite profile of faeces due to moderate 4-week red wine consumption were monitored in a parallel-armed study. Of 60

targeted compounds, 35 were identified in all faeces samples. No detectable amounts of the phenolic compounds present in wine, such as anthocyanins, flavan-3-ols and stilbenes, were found in the faecal samples, suggesting their extensive catabolism by colonic microbiota. Ten compounds, mainly benzoic and 4-hydroxyvaleric (4-OH-valeric) acids (Table 15.1, N10), showed a statistically significant increase after the wine intake. The authors reported that red wine consumption increased the total phenolic metabolite content of faeces to varying degrees. According to their gut microbial capacity to metabolize wine PPh, individuals were tentatively classified into three "metabotypes" (Bolca et al. 2013) by microgram of wine-derived total PPh detected per g of faeces: (1) low (<500 μg/g), (2) moderate (500–1000 μg/g), and (3) high (>1000 μg/g) metabolizers. Thus, these results supported a hypothesis on the existence in the human population of different PPh-metabolizing phenotypes (van Velzen et al. 2009). Although these data should be confirmed and better explored in a larger intervention study, they already carry great significance when considering individual PPh-metabolizing phenotypes in wine-health interaction studies. In general, these two targeted metabolomic studies on faeces confirmed that gut microbiota is among the principal objects of wine PPh action.

The scientific community has become aware that the microbe-derived metabolites of PPh represent a large proportion of wine PPh intake, impacting on their bioavailability and potentially exhibiting some bioactive effects (Forester and Waterhouse 2009; Monagas et al. 2010). Analysing bioavailability through monitoring host and microbiota-derived metabolites together, we can reveal the main protagonists of corporative interaction among wine PPh and microbiota and human health, in addition to obtaining valuable information on the microbiota catabolic activity on wine PPh. Led by this idea, the host and microbiota metabolites of consumed red wine PPh formed within and excreted in urine from our organism were studied using a metabolomic targeted approach in our group (Boto-Ordonez et al. 2013) (Table 15.1, N11). The wide-ranging urinary metabolomic fingerprint of phenolics ($n=61$) was monitored by a specially developed LC-MS technique in enzymatically hydrolyzed and non-hydrolyzed 24 h urine samples collected before and after a regular 4 weeks intake of dealcoholized red wine (RWD). As a result, 37 enzymatically deconjugated and 24 conjugated metabolites were detected, of which 49 were increased after RWD consumption, pointing to a considerable complexity of wine PPh microbial catabolism (Table 15.1, N11). So far, this targeted profiling has provided the most complete urinary phenolic metabolic fingerprint after wine consumption. The highest percentage of increase corresponds to microbial metabolites derived from flavan-3-ol and anthocyanin degradation. Some of the detected wine PPh microbial catabolites were recently shown to exert specific microbiota-directed bioactivities in in vitro (Sanchez-Patan et al. 2012) and in vivo (Boto-Ordonez et al. 2014) studies. In this way, wine PPh can beneficially interplay with microbiota activity with respect to human health. Some such interplays were recently reported when substantial changes in the gut microbiota provoked by wine consumption were associated with certain health-relevant changes in the human organism (Queipo-Ortuno et al. 2012; Clemente-Postigo et al. 2013). Therefore, microbiota could be seen not only as a new metabolic organ of wine PPh but also as a target of their action with respect to health benefits for humans.

As a final point, these studies showed the potential of a targeted metabolomic approach in wine-microbiota research focused primarily on evaluating the complexity of wine PPh metabolism and on detecting wine colonic metabolites as potentially bioactive molecules within the human organism both systemically and at the level of colon.

15.4 Conclusions

The nutrimetabolomics studies, so far performed in wine nutrition research, showed that the consumption of wine and wine-derived PPh impacts strongly on our metabolism, provoking global changes in both exogenous and endogenous metabolites. Thus, alterations in homeostatic metabolism and in immediate metabolic events were observed. Moreover, the gut microbiota metabolic efficiency and activity were also affected. These findings provided information on the complex metabolic relationship between wine and the human organism. In addition, targeted metabolomic studies on wine PPh and their metabolites suggested the application of metabolic patterns of resveratrol and other wine-derived PPh as markers of wine dietary exposure, which is an important step forward toward more accurate assessment of wine effects in future clinical and epidemiological studies. The main findings thus far of wine metabolomic research are briefly summarized in Fig. 15.1. Finally, the outcomes of these studies showed the advantage and great potential of the application

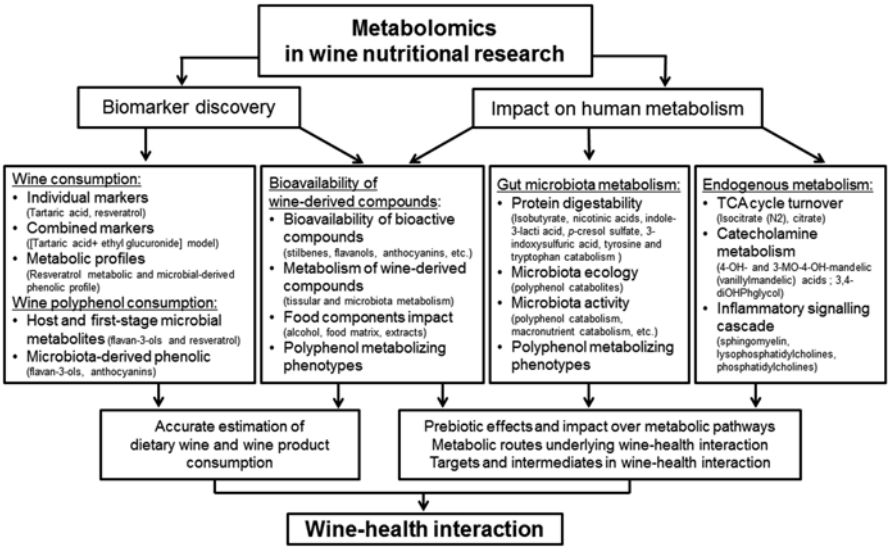

Fig. 15.1 Applications of a metabolomic approach to wine nutrition research and the relevance of its finding to understanding wine-health interaction

of a metabolomic approach in wine nutrition research, especially in understanding and revealing novel mechanisms underlying the health benefits of wine consumption and in searching for targets of wine benefits to human health.

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