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,

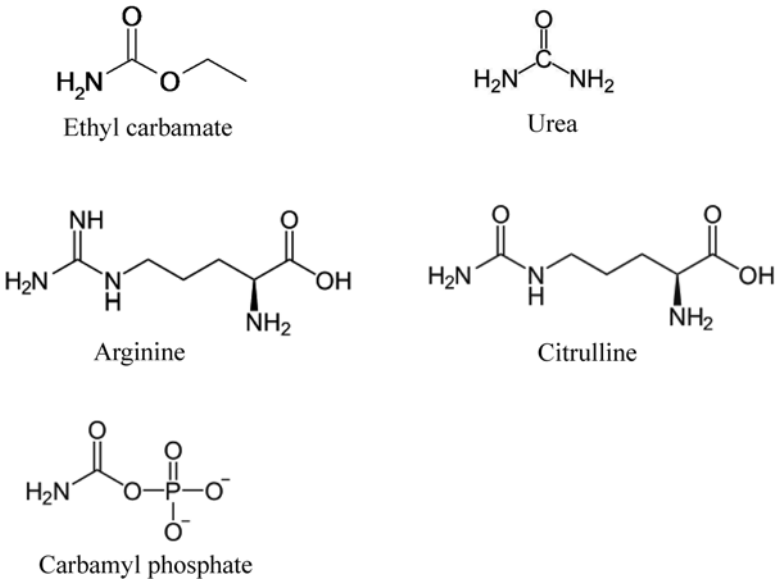

Fig. 1.1 Molecular structure of ethyl carbamate and its precursors in wine

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

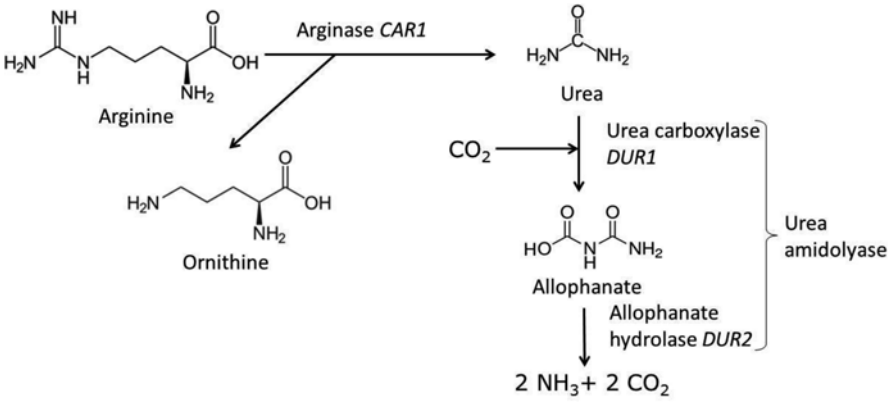

Fig. 1.2 Metabolic pathway of arginine by yeast

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).

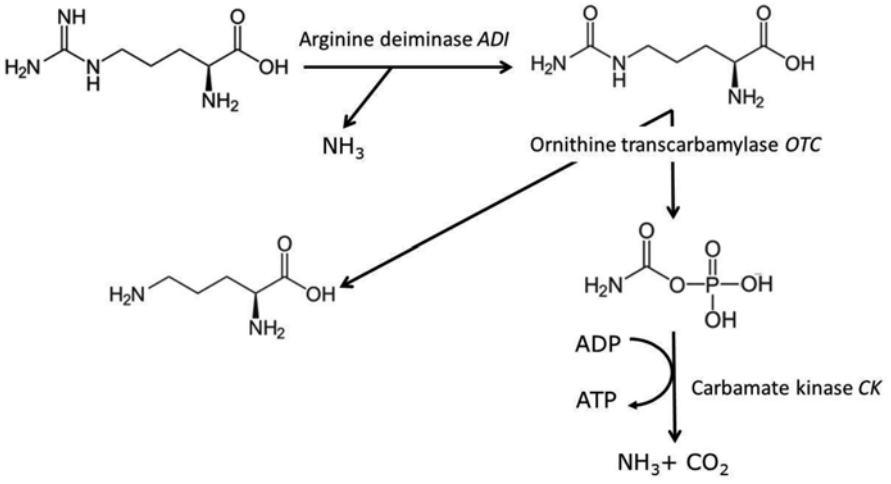

Fig. 1.3 Metabolic pathway of arginine by lactic acid bacteria

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

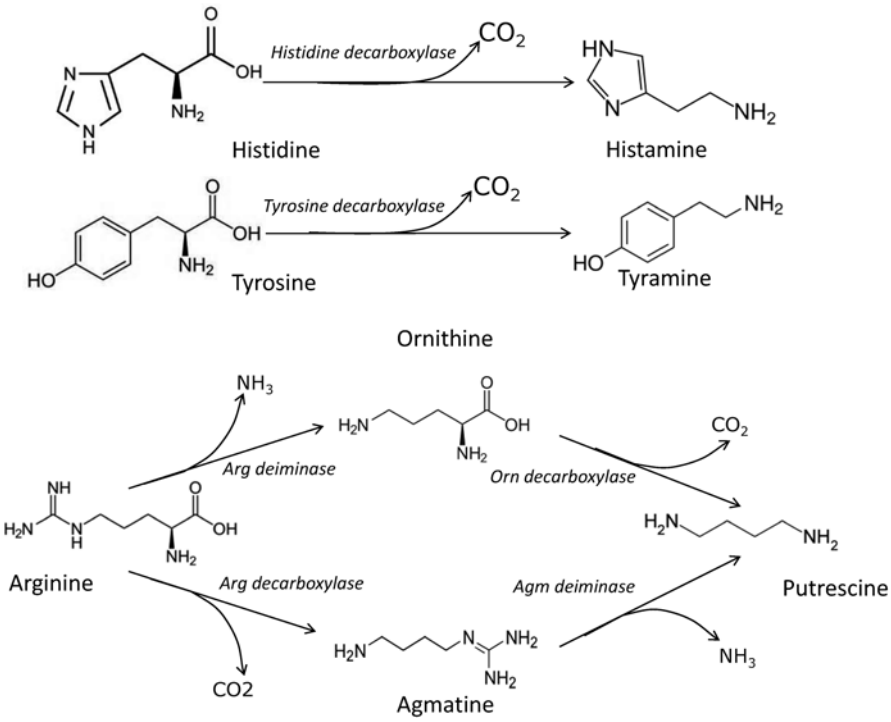

Fig. 1.4 Biogenic amines production pathways by lactic acid bacteria

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