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Chapter 11
Wine Spoilage by Fungal Metabolites

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Contents

11.1 Introduction

This chapter is devoted to the description of toxic and spoiling secondary metabolites produced by moulds and yeasts in grapes and wines. The toxic compounds affect human health being a food safety issue while spoilage concerns wine organoleptical quality being, therefore, a technological matter. Amongst several compounds with detrimental effects on human health reported as occurring in wine – lead, pesticides, ethyl carbamate, biogenic amines, and more recently ochratoxin A (OTA) – only the last is of fungal origin and the most preoccupying of them. The occurrence

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of OTA in grapes and wines has a special meaning in enology, because wine has been known, since remote antiquity, as "the safest beverage". In fact, the enormous success of wine (and beer) for millennia in the Mediterranean basin is due to the bad sanitary quality of water, frequently contaminated by salmonella or other faecal pathogens around urban agglomerates. In the second half of the nineteenth century, Louis Pasteur stated that "Wine is the most hygienic of the beverages", implying that any pathogenic bacteria had capacity to grow or survive in wine. The occurrence of OTA in wine changes the paradigm of "the safest beverage", thus imposing a food safety concern to the wine industry.

Microorganisms have long been known as one of the main sources of wine spoilage. Earlier enology treatises mention the spoiling activities of lactic and acetic acid bacteria and yeasts, concerns being preferentially directed to the deleterious effects of acetic and lactic acid bacteria (Ribéreau-Gayon et al. 2006). More recently, yeasts have become a source of serious trouble for winemakers, especially after the confirmation of *Dekkera/Brettanomyces bruxellensis* as the producers of unwanted levels of 4-ethylphenol (4-EP) leading to phenolic or "horse-sweat" taints. These yeast species and lactic acid bacteria are also responsible for the production of tetrahydropyridines (THP), connected with the taint described as "mousiness".

This chapter outlines the most recent advances in the awareness of the problems raised by ochratoxin A, volatile phenols and tetrahydropyridines. We will focus on the factors leading to their occurrence, the relevance to wine quality and safety and the respective preventive measures suitable under vineyard and winery practices.

11.2 Ochratoxin A

Mycotoxins are metabolites produced by fungi and affecting human health, causing mycotoxicoses. These diseases have been known for a long time but only after the early 1960s have they been appropriately studied (Bennett and Klich 2003; van Egmond and Schothorst 2007). The first mycotoxin to be studied was aflatoxin. Since then the number of known mycotoxins have increased to 300 or 400 compounds of variable toxicity and occurrence. The main food commodities affected are cereals used for direct human consumption, for processing, or as animal feeds and dried fruits (van Egmond and Schothorst 2007). Some mycotoxins have been mentioned in grape products (patulin, aflatoxins, trichothecenes) but OTA is the main toxin of concern in the wine industry (Hocking et al. 2007). The occurrence of OTA in wine was reported for the first time in 1995 (Zimmerli and Dick 1996) and much data has been generated since then for wine, grape juice and raisins, especially in the last seven years.

11.2.1 Chemical Structure

Ochratoxin A was originally described as a metabolite of the mould *Aspergillus ochraceus* in a laboratory screening for toxinogenic fungi (van der Merwe et al. 1965).

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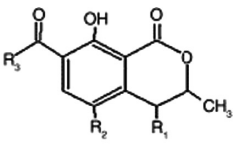
It is the most abundant and hence the most commonly detected member of the family of ochratoxins produced as secondary metabolites by moulds. Apart from ochratoxin α (OTα), the ochratoxins comprise a polyketide-derived dihydroisocoumarin moiety linked via the 7-carboxy group to l-β-phenylalanine by an amide bond. Ochratoxins consist of ochratoxin A (OTA), its methyl ester and its ethyl ester, also known as ochratoxin C (OTC), 4-hydroxyochratoxin A (4-OH OTA), ochratoxin B (OTB) and its methyl and ethyl esters and ochratoxin α (OTα), where the phenylalanine moiety is missing (Fig. 11.1). All of them behave like weak organic acids and the differences in the chemical structures have marked effects on their respective toxic potentials, OTA being the most toxic of the group (Ringot et al. 2006). The presence of chlorine in the OTA structure makes it unique among mycotoxins (Murphy et al. 2006). The empirical formula is $C_{20}H_{18}O_6NCl$ and the molecular weight is 403.82. The IUPAC developed formula of OTA is l-phenylalanine-*N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyran-7-yl)carbonyl]-(*R*)-isocoumarin (Ringot et al. 2006). The present knowledge of the biosynthetic pathway of OTA, which has not yet been fully established, has been reviewed by Ringot et al. (2006).

11.2.2 Toxicity and Public Health Safety

OTA is a mycotoxin considered to be a possible carcinogen (Class 2B) for humans (IARC 1993) and it has been shown to be nephrotoxic, hepatotoxic, teratogenic, carcinogenic and immunotoxic to several species of animals and to cause kidney and liver tumours in mice and rats (JECFA 2001). In humans it is accumulated in body tissues because it appears to be slowly eliminated, but the effects are not well established (Ringot et al. 2006). Ruminant animals, such as cows and ewes, are generally resistant to the effects of OTA due to its hydrolysis to non-toxic metabolites by protozoa in the stomachs before absorption into the blood (Kiessling et al. 1984). Recent studies on the toxic mechanisms are focused on the OTA ability to disturb cellular signalling and regulation and to modulate physiological signals and thereby



Ochratoxins	R1	R2	R3
OTA	H	Cl	-NH-CH(COOH)-CH_2- Phenyl
OTB	H	H	-NH-CH(COOH)-CH_2- Phenyl
OTC	H	Cl	-NH-CH($COOC_2H_5$)-CH_2-Phenyl
4-hydroxyochratoxin A	OH	Cl	-NH-CH(COOH)-C_2H- Phenyl
OTα	H	Cl	-OH

Fig. 11.1 Chemical structure of ochratoxins (reprinted from Ringot et al. 2006, permission to be obtained)

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to influence cells viability and proliferation, but its modes of toxicity remain "a continuing enigma" (see review of Ringot et al. 2006).

Given their toxicity, mycotoxins are subjected to regulations determining the maximum allowable levels. As a rule food products have mycotoxin levels lower than the limits (Jorgensen 2005) but about 40% of the notifications received in 2005 by an European rapid alert system for food and feed were related to risks to human health by mycotoxins (van Egmond and Schothorst 2007). These authors further mentioned that almost 90% of these notifications were related to aflatoxin in nuts and nut products imported to the EU. Cases related to OTA in wine were not referred to in this report. In addition, an indirect measure of the toxicity and risk to human health may be given by the standards of international legislation or guidelines of advisory boards. Concerning OTA there are no regulations in the USA or in Codex Alimentarius Commission, in contrast to patulin, fumonisin, aflatoxin and DON (Murphy et al. 2006), and so it seems admissible that these toxins represent a bigger threat than OTA.

At European Union level the Council Regulation (EEC) 315/93 of 8th February 1993 provided the legal framework for establishing maximum levels for food contaminants at Community level. In 1995, the European Commission (EC) initiated the activity SCOOP (scientific cooperation on questions related to food), which included a project to provide data on the occurrence of OTA in food commodities on the European market and on the dietary exposure to OTA in the EU member states (Jorgensen 2005). As a consequence, many data on the occurrence of OTA in human food and human blood plasma have become available since 1995. After the first SCOOP report, known as SCOOP-1 (European Commission 1997) a second SCOOP task was performed, extended to other commodities and processed foods, including wine and other grape products, to evaluate if the additional studies changed the conclusions of first SCOOP report. Not surprisingly, due to the detection of OTA in wine, much occurrence data has been produced since then, not only for wine but also for dried vine fruits (currants, raisins, and sultanas) and grape juice, particularly after 2000. The most relevant conclusion from those data was that the overall mean level of OTA in wine was 0.36 μg/kg (mean of 1470 samples), representing the second source, after cereals, to the OTA exposure in the European diet (European Commission 2002), raising relevant concern and electing OTA as a threat to the European wine industry. In particular, JECFA (2001) calculated that the human OTA exposure was of 58%, 21%, 7%, 5% and 3% of total OTA intake for cereals, wine, grape juice, coffee and pork, respectively (Murphy et al. 2006). However, the calculation of these figures was based on the controversial assumption that the mean intake is represented by the arithmetic mean value. If the median value was used, the value of OTA in red wine would have been only 0.02 μg/kg and the contribution of wine consumption for OTA intake-rate would drop to 2% (Otteneder and Majerus 2000). The maximum allowable limits for OTA in several food products have been established recently in the EU, being the adult's strictest value applied to wine (Table 11.1). In addition, a provisional tolerable weekly intake (PTWI) of 100–120 ng/kg body weight (bw) is advised (JECFA 2001) that was set based on a safety factor of 450 related with the renal function deterioration of pigs,

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Table 11.1 European Union and Swiss regulations for ochratoxin A (μg/kg)

Product	Maximum allowable concentration	Reference
Unprocessed cereals	5	Commission Regulation (2006)
All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption with the exception of foodstuffs	3	
Dried vine fruit (currants, raisins and sultanas)	10	
Roasted coffee beans and ground roasted coffee, excluding soluble coffee	5	
Soluble coffee (instant coffee)	10	
Wine (including sparkling wine, excluding liqueur wine and wine with an alcoholic strength of not less than 15 vol.%) and fruit wine	2	
Aromatised wine, aromatised wine-based drinks and aromatised wine-product cocktails	2	
Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must and concentrated grape must as reconstituted, intended for direct human consumption	2	
Processed cereal-based foods and baby foods for infants and young children	0.5	
Dietary foods for special medical purposes intended specifically for infants	0.5	
Coffee products (Switzerland)	8	Taniwaki (2006)

for which the lowest observed effect level (LOEL) was 0.008 mg/kg bw per day (Bakker and Pieters 2002). Current levels of weekly exposure to OTA in adults of EU member states vary between 15 and 60 ng/kg bw which is, at most, half quantity of the PTWY (CONTAM 2006).

Further evaluation of OTA occurrence in wines worldwide has revealed that variable proportions of wines are contaminated but only a rather small number has levels over the maximum allowable limit of 2.0 μg/kg (Ng et al. 2004; Mateo et al. 2007). Higher concentrations are more frequent in wines produced from dried grapes and in raisins, but are less consumed than table wines (Burdaspal and Legarda 2007; Valero et al. 2007b). Burdaspal and Legarda (2007) showed that sweet wines only contributed with 3.1–3.8% (regular consumers) or 0.3–0.4% (whole adult population) to the PTWI. Therefore, wines do not seem to have a significant contribution to human exposure to OTA.

11.2.3 Occurrence

Ochratoxin A is among the most important mycotoxins affecting foods being described in food commodities together with aflatoxin, deoxyvalenol, T-2 toxins

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and trichotecenes, although the relative weight of each other in public health has not been mentioned (see reviews of Frisvad et al. 2006 and Richard 2007). As in other mycotoxins, OTA can contaminate a wide variety of food commodities as a result of fungal infection in crops, in the field during growth, at harvest, in storage, and in shipment under favourable environmental conditions, especially when the foods are not properly dried. Then, if used in animal's feed it may contaminate their products (Murphy et al. 2006). OTA occurs mainly in the storage of cereals and grains under conditions that favour mould growth and toxin production. Therefore it is associated with cereal and cereal products, soy products, coffee and cocoa products (CAST 2003; Jorgensen 2005; Frisvad et al. 2006). Meat and meat products such as salami and hams, hard cheeses, spices and beer have also been related with OTA (Jorgensen 2005; Frisvad et al. 2006). Dried fruits (dates, plums, apricots, figs, sultanas) are typical affected commodities (Iamanaka et al. 2006). OTA may also occur in house dust and other airborne particulates (Richard 2007).

Concerning the wine industry, OTA has been detected in wines, grape juice, vinegar and raisins (Jorgensen 2005; Varga and Kozakiewicz 2006). These products were the last to be associated with OTA but studies in wines have greatly increased since the first report in 1995, being the OTA related food commodity most studied in the last decade (Jorgensen 2005), demonstrating the effort put into the solution of the problem by the wine sector. Table 11.2 lists the results of recent relatively large surveys worldwide demonstrating, as already mentioned, that wines with levels higher than the maximum allowable concentration of 2.0 μg/L are not frequent. As a rule, the OTA concentration increases from white and rosé to red wines, from cold to warmer regions, and dessert or sweet wines have higher mean levels (Varga and Kozakiewicz 2006 and Table 11.2). We are not aware of large surveys of OTA levels in grapes but raisins and sultanas have higher mean OTA levels than fresh grapes (Varga and Kozakiewicz 2006; Iamanaka et al. 2006).

11.2.4 Ochratoxin A Production on Grapes

11.2.4.1 OTA Producing Species

Ochratoxin A of food commodities is produced by a small number of fungal species in the genera *Penicillium*,*Aspergillus* and the *Aspergillus* teleomorphs*Petromyces* and *Neopetromyces* (Frisvad and Samson 2000; Frisvad et al. 2004).

The main species that occur in grapes and, consequently, in grape juices, raisins, wine and wine derivatives belong to the so-called black aspergilli, taxonomically included in the *Aspergillus* section *Nigri*. Unfortunately, the taxonomy of this section is not completelyknown, creating many difficulties on the identification of strains, originating a proliferation of taxa, including species, subspecies, and varieties (for a discussion see Samson et al. 2004 and Frisvad et al. 2006). Based on phenotypic comparisons of a broad collection of black aspergilli, Samson et al. (2004) considered 15 species provisionally accepted in *Aspergillus* section *Nigri*, four of those producing OTA and only two occurring on grapes, raisins and in

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Table 11.2 Occurrence and concentration of OTA (μg/L) in wines obtained in recent large scale surveys

Wine type	Number of samples	Percentage of positive samples	Percentage of samples > μg/L	Range	Mean	Median	Reference
White	60	25	0	<0.010–1.36	0.108	0.01	Otteneder and Majerus (2000)
Rosé	55	40	–	<0.010–2.38	0.119	0.01	
Red	305	54	–	<0.010–3.31	0.201	0.02	
Red (North Italy)	17	70	0	<0.010–0.54	0.12	0.05	Perrone et al. (2007)
Red (Central Italy)	46	59	0	<0.010–0.80	0.07	0.02	
Red (South Italy)	49	100	18	0.02–4.93	1.36	1.03	
Sweeta	290	96.9	6.8	<0.010–4.63	0.499	0.14	Burdaspal and Legarda (2007)
Sweet (A, B, CI)[b]	20	0	0	<0.024	<0.024	–	Valero et al. (2007b)
Sweet (CII)	26	54	15.3	<0.024–27.79	2.01	–	
Sweet (CIII)	75	60	26.7	<0.024–15.62	1.71	–	

[a]Maximum allowable levels for sweet and fortified wines are not legislated (see Table 11.1)

[b]European regions of wine production based on production conditions, soil, region and climate (Valero et al. 2007b)

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wine – *A. carbonarius* and to a lesser extent *A. niger*. Recently, a new species of section *Nigri* isolated from grapes and OTA non-producer – *A. ibericus* – was proposed (Serra et al. 2006), being likely that new species can enlarge that section in the near future. In fact, with recent work on the molecular characterization of a Southern Europe population of black aspergilli isolated from grapes it was concluded that these represent a complex of species, where some of them are peculiar to grapes (Perrone et al. 2006b). Another study performed with strains isolated from grapes in Italy indicated that *A. tubingensis* is able to produce OTA and that, together with *A. carbonarius* and *A. niger*, it may be responsible for the OTA contamination of Italian wines (Perrone et al. 2006a). Some references in the literature also describe the occurrence of the yellowish*A. ochraceus* (belonging to the *Aspergillus* section *Circumdati*) and the blue-green *Penicillium* species on grapes (Frisvad et al. 2004), but its importance seems to be minor when compared with black aspergilli.

The evaluation of OTA production by fungi on infected grapes is essential to establish the real producing ability by the different species because the results of tests on synthetic culture media are not always coincident with in vivo determinations (Bellí et al. 2007). Overall, most *A. carbonarius* strains have the ability to produce OTA in grapes whereas the proportion is lower in the other toxigenic species (Perrone et al. 2006a, 2006b).

From a scientific point of view, precise strain identification of OTA fungi producers is important to establish the phylogenetic relationships among species, to recognize the mycota of foodstuffs, and to understand the peculiarities and ecological needs of the species. However, from a viticultural and enological point of view, it will be much more important to establish the environmental conditions that are ideal for OTA fungi producers on grapes than their identification.

11.2.4.2 Factors Affecting Fungal Growth and OTA Production

The primary sources of *A. carbonarius* and *A. niger* are soil, bunch remnants or vine trash on soil vineyards, which are transported by wind from soil onto berry surfaces (Leong et al. 2006a; Hocking et al. 2007). Generally, the colonisation of grape bunches by black aspergilli and other fungi occurs when berry skin damage allows the entry into fruit tissues, where the low pH and high sugar content under aerobic conditions provide a competitive advantage for moulds. However, fungal invasion may occur without visible symptoms (Bellí et al. 2007). As a rule, the competition among contaminant microorganisms is more favourable to *Botrytis cinerea*, the agent responsible for common grey rot. However, other rot processes, such as sour rot and brown rot, can occur together with black aspergilli. The population dynamics of fungi outside or inside grape berries with skin damage is still poorly known, as it is difficult to establish which environmental conditions promote the dominance of each fungal species. Nevertheless, it has been shown that high temperatures (30 °C) and high relative humidity, between 80% and 100%, give rise to higher amounts of OTA produced by *A. carbonarius* on grapes (Bellí et al. 2007), suggesting that such conditions give competitive advantages to the black aspergilli population. Medina (2007) also showed that *A. carbonarius* growth was favoured

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by high a_w (0.98) and temperature (28 °C), whereas OTA production was increased at mild temperature (20 °C) and 0.96–0.98 a_w. Inaddition, Astoreca et al. (2007) found that optimum conditions of a_w and temperature are more restrictive for OTA production than for *Aspergillus* growth. The influence of relative humidity, rain or high a_w is less important than the influence of high temperature (Bellí et al. 2005; Medina 2007), explaining the occurrence of these fungi in hot and dry climate regions (Hocking et al. 2007). The influence of a_w appears to be more important in dried grapes, where black sultanas ($a_w = 0.629$) showed levels of OTA higher than 10 μg/kg when white sultanas ($a_w = 0.567$) did not (Iamanaka et al. 2006).

The frequency of the occurrence of OTA producing species on grapes is then essentially limited to conditions of high humidity and temperature, typical of sub-tropical and Mediterranean climates. In fact, OTA has been detected on grapes produced in France, South America, Spain, Italy, Portugal, Greece and Australia (as revised by Leong et al. 2006a), that have some wine regions with climatic conditions favouring black aspergilli species. In general, *A. carbonarius* is highly dominant, particularly in warmer regions, because their black spores are resistant to UV light and sun-drying (Leong et al. 2006a). However, in colder regions such as Germany, Northern Hungary, the Czech Republic or Northern parts of Portugal, France and Italy, black aspergilli have not been isolated from grape berries in spite of the presence of OTA in wines (for references see Blesa et al. 2006 and Varga and Kozakiewicz 2006) suggesting that other species, mainly *Penicillium*, adapted to cool temperatures, should be involved.

Zimmerli and Dick (1996) were the first authors to show that OTA content in southern wine-growing regions are higher than those of wines from northern areas, results also supported by the extensive work of Otteneder and Majerus (2000). More recently, OTA has been detected on grapes produced in many wine countries, where the highest amounts of OTA detected in each survey is generally correlated to vines growing in the warmest regions of each country (Blesa et al. 2006 and Table 11.2). However, the above-mentioned assumptions were not observed in Australia and South Africa, where no correlation was found between OTA incidence and wine region climate (Leong et al. 2006a). This fact was probably due to the rather low detected levels making these correlations imprecise. Further results in Australia showed apparently lower incidences in the cooler climate of Tasmania (Hocking et al. 2007). Canadian wines and grape juices have OTA levels comparable with data from cold climate wine growing areas in Europe (Ng et al. 2004 and Table 11.2). The fact that sweet wines from colder regions do not show high levels of OTA in contrast to those from warmer climates (Valero et al. 2007b) indicates that the main factor determining the OTA concentration in wines is the contamination of grapes by toxinogenic moulds.

11.2.5 Prevention and OTA Production Control on Grapes

Given the ubiquity of black aspergilli in vineyards of warm regions, all agents involved in the wine industry must learn how to live with them, minimising the

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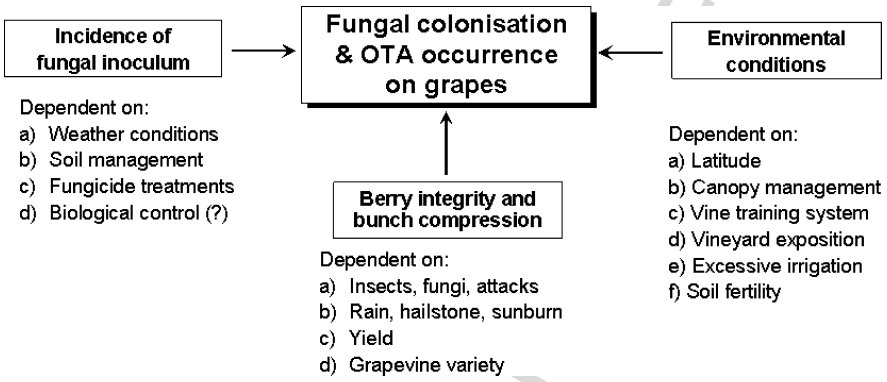

Fig. 11.2 Factors affecting grape colonisation by OTA producer fungi

effect of all factors – biotic and abiotic – that contribute to their infection and growth on grape berries (see Fig. 11.2). Tillage operations contribute to the spreading of fungal spores and should be minimised when possible (Leong et al. 2006a). Concerning fungicide treatments, although some compounds have shown its efficiency, others seem to stimulate OTA production (Magan 2006) and so the most significant function of agrichemicals is to avoid grape damage by phytopathogenic moulds or insects which provide an easy entry for spore infection. Recently, a Code of Good Viticultural Practices was recommended by the Office International de la Vigne et du Vin (OIV 2005) to be put in use mainly in critical areas of *A. carbonarius* occurrence. Prevention is especially important for dried grapes, either for direct consumption or for dessert wines, given their higher levels of OTA. The main measures to consider were the following, mainly directed to avoid berry damage:

- To avoid all the cropping practices that lead to an excessive vigour of vine plants and an exaggerated increase in the yield, which make bunches more compact and thereby susceptible to berry splitting (Mínguez et al. 2004; Leong et al. 2006a)
- To avoid the use of cultivars with thin skinned berries and too compact bunches (Bellí et al. 2007)
- To carry out a plant sanitary program with efficient agrichemicals against moulds, particularly powdery mildew and grey rot, and insects, mainly *Lobesia* spp., using the adequate dose and timing, and making sure that the active ingredients reach all parts of the bunch as well as penetrate its interior (Mínguez et al. 2004; Valero et al. 2007a)
- To control berry splitting due to rain just before harvest (JECFA 2001)

11.2.6 OTA Control Strategies in Wine

The factors affecting the levels of OTA in wines are systematised in Fig. 11.3. The presence of OTA in wines results exclusively from grape contamination. Thus, the

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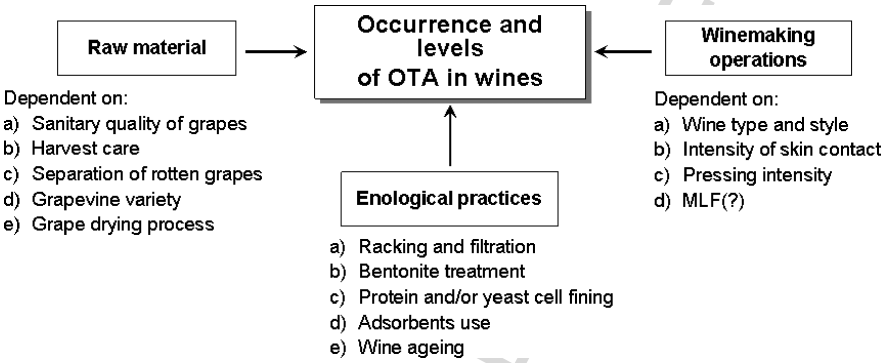

Fig. 11.3 Factors affecting the levels of OTA in wines

main measure to prevent the entry of grapes with high levels of OTA in wineries is to reject the rotten bunches and particularly those presenting brown or black rot. This is difficult to implement during harvest because rot may not be easily visible due to its development in the inner part of the bunch. In fact, bunch selection is only effective in small wineries, where the harvest is by hand and the personnel may be well trained. For large cooperatives or company wineries it is not so easy to separate the rot bunches, except if grape payment can be differentiated according to its health status. OTA determination is not an easy task at winery level (Cigič et al. 2006 and references cited therein). Rapid diagnostic tools are being developed but have not yet reached industrial laboratories (Magan 2006). This drawback can, however, be overtaken by the use of instruments that provide indicators of grape composition and health status, like the modern and costly FTIR (Fourier Transformed Infrared) spectroscopy. This method gives a measure of the grape rot that may be an indicator of the presence of OTA because gluconic acid and glycerol are correlated with *Botrytis*, sour rot and*Aspergillus* attack (Mínguez et al. 2004). When mechanical harvesting is used, it is impossible to reject rot bunches. In this case, the only measure is to control analytically the health quality of grapes in order to process the poor quality grapes separately.

Despite the fact that South African and Australian red and white wines did not show different OTA incidence (Leong et al. 2006a), most studies indicate that red wines, in the same region, contain higher OTA concentrations than rosés and white wines (for references see review of Varga and Kozakiewicz 2006). This may be explained by OTA release from grape skins (Otteneder and Majerus 2000) or by fungal spore release (Atoui et al. 2007) during red grape maceration. Therefore, winemaking processes should significantly influence its content. Several studies showed that OTA content in wines increased with the maceration time and decreased with solid-liquid separation steps, such as red wine racking or clarification of white juices (Fernandes et al. 2003; Leong et al. 2006b). The reported reductions may range from 50% to 80% of initial OTA concentrations (Hocking et al. 2007). In recent work performed with grapes from Northwest Portugal, Fernandes et al. (2007) showed

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that, in different red vinification trials, the mean carry-over of OTA from artificially infected grapes to wine was 8.1 wt% after malolactic fermentation, even without use of enological adjuvants (fining agents), corroborating the findings of Leong et al. (2006b). Reduction of OTA was associated with removal of spent fractions during winemaking, such as wine lees after fermentation or sediment after racking, in which OTA contents were higher than in the original grapes. Some studies were performed to assess the effect of enological adjuvants, such as bentonite, gelatin, charcoal, and yeast cell wall preparations on the removal of OTA from wines (Leong et al. 2006b; Mateo et al. 2007). Most of them reduce the OTA content of wines, but the necessary concentrations have a strong effect on the wine quality. In addition, up to 29% of OTA spontaneous reduction has been observed during wine storage over 10–14 months (Hocking et al. 2007).

Little is known about the eventual degradation or binding of OTA by yeasts or lactic acid bacteria during the fermenting process, though this has been demonstrated as possible (Angioni et al. 2007; Hocking et al. 2007). However, when compared with the physical removal of OTA during the vinification, this practice is quantitatively irrelevant. So, the most important measures for a Code of Good Enological Practices to prevent or reduce the OTA content in wines are:

- To train the harvesting staff to reject rotten bunches, particularly those affected by dark brown or black moulds
- To vinify separately the mechanical harvested grapes, when the sanitary quality of the crop is poor
- To monitor, in large wineries and cooperatives, the sanitary quality of the grapes by FTIR instruments in order to (i) favour with fair pricing the sanitary status of grapes, and (ii) process the grapes accordingly
- To avoid long periods of maceration and to use enological adsorbents, such as activated charcoal or yeast hulls, in red wine, and bentonite, in white wine, when the crop has a relevant percentage of rotten grapes
- To perform a rapid grape drying and avoid water condensation overnight for grapes used in the vinification of dessert wines
- To implement a complete HACCP plan, from the vine to the bottled wine or raisin, in the wine regions where the OTA occurrence is higher

11.3 Volatile Phenols

Volatiles phenols (VP) are secondary metabolites produced by yeasts, moulds and bacteria which affect the flavour of several fermented food commodities (Loureiro and Malfeito-Ferreira 2006). These molecules have been under study since the first detection in fermented grains (Steinke and Paulson, 1964). Later, Dubois and Brulé (1970) reported their presence in wines and presently the importance of VP is mainly due to their role in the mediatic "horse sweat" taint in red wines. Available toxicological data suggest that VPs do not warrant concerns about acute or long-term effects (Rayne and Eggers 2007).

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11.3.1 Chemical Structure and Occurrence

Volatile phenols are a group of molecules included in the non-anthocyanin phenolic compounds of white and red wines. The chemical structure of VP are characterised by a phenolic ring and a radical with different compositions (Fig. 11.4). The main volatile phenols of wines are 4-vinylphenol (4-VP), 4-vinylguaiacol (4-VG), 4-vinylcathecol (4-EC), 4-vinylsyringol (4-VS) and their respective reduced forms 4-ethylphenol (4-EP), 4-ethylguaiacol (4EG), 4-ethylcathecol (4-EC) and 4-ethylsyringol (4-ES). These molecules occur in wines with different concentrations, being vinylphenols typically associated with white wines and ethylphenols with red wines. Table 11.3 shows recent results on the average concentration range of these molecules in wines. In addition, vinylphenols may be also present in red wines as anthocyanin-vinylphenol adducts, like malvidin 3-glucoside-4-vinylphenol (Cameira-dos-Santos et al. 1996) and similar 4-VP, 4-VC and 4-VS derivatives (Schwarz et al. 2003; Suárez et al. 2007).

11.3.2 Origin

11.3.2.1 Availability of Precursors

The precursors of VPs are hydroxycinnamic acids which are enzymatically decarboxylated by a cinnamate decarboxylase, leading to vinylphenol derivatives, and

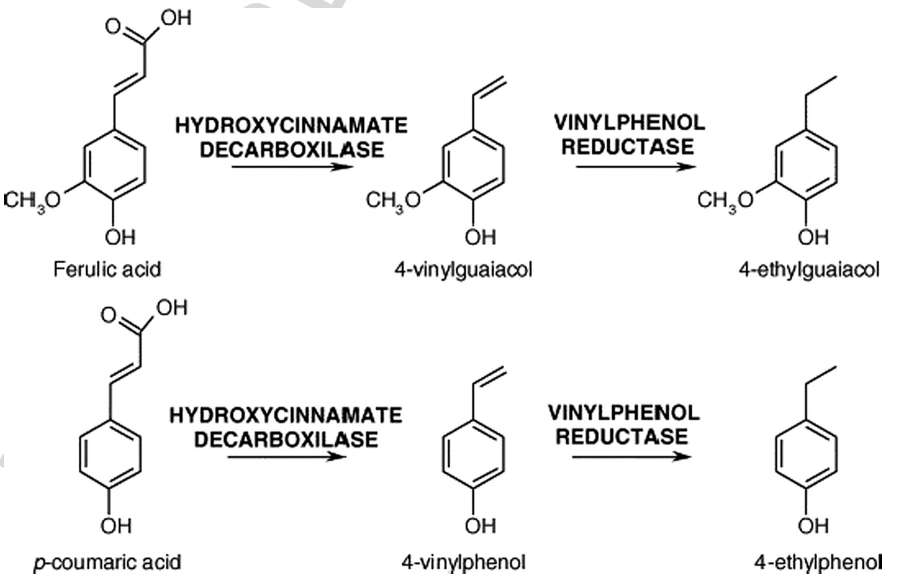

Fig. 11.4 Conversion of hydroxycinnamic acids to vinylphenols and ethylphenols (reprinted from Suárez et al. 2007, permission to be obtained)

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Table 11.3 Range of volatile phenol concentrations in wines (μg/L)

Wine type	Volatile phenols					Observations	Reference
	4-Vinylguaiacol	4-Vinylphenol	4-Ethylguaiacol	4-Ethylphenol	4-Ethylcathecol		
White	200–324	1341–2802	Nd[a]-238	Nd-228	–	5 samples	Mejías et al. (2003)
Red	Nd-880	Nd-2174	72–255	97–782	–	5 samples	
	5.4–236	8.1–98	0.53–420	8.6–1500	–	57 aged red wines	López et al. (2002)
	–	–	46.6–169.3	399.5–2231.6	–	18 red wines	Tat et al. (2007)
	–	–	4.3–410.5	<0.5–586.2	–	54 red barrel aged wines	Rayne and Eggers (2007)
	–	–	Nd-60	127–494	42–160	9 tainted Pinot Noir wines	Hesford and Schneider (2004)
	–	–	Nd-329	298–3780	49–427	11 tainted red wines	
Sherry	820–1170	800–2000	1000–2000	300–550	–	4 wines	Domínguez et al. (2002)

[a] Not detected

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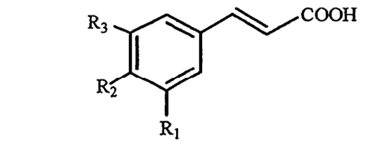
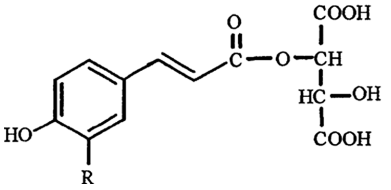


Hydroxycinnamic acid	R_1	R_2	R_3
p-Coumaric	H	OH	H
Caffeic	OH	OH	H
Ferulic	OCH_3	OH	H
Sinapic	OCH_3	OH	OCH_3



Hydroxycinnamic ester	R
Trans- caffeoyltartaric acid (caftaric acid)	OH
Trans-p-coumaroyltartaric acid (cutaric acid)	H
Trans- feruloyltartaric acid (fertaric acid)	OCH_3

Fig. 11.5 Hydroxycinnamic acids and their esters (obtained from Monagas et al. 2005, permission to be obtained)

reduced by a vinylphenol reductase, originating the ethylphenol derivatives (Hereszтyn 1986a and Fig. 11.4). In grape juices, hydroxycinnamic acids are esterified, mainly to tartaric acid (Fig. 11.5). In wines they may be present in the free or esterified form, either with tartaric acids or other polyphenols (Table 11.4). Recent work has shown that most of the free acids from tartaric esters appear after the malolactic fermentation (Hernández et al. 2006, 2007; Cabrita et al. 2007). The other esterified forms of hydroxycinnamic acids are cinnamoyl-glucoside anthocyanins (Romero and Bakker 2000; Monagas et al. 2005; Oliveira et al. 2007) and *trans-p*-coumaric acid hexoses (Monagas et al. 2005; Hernández et al. 2006, 2007). Boselli (2006) also mentioned the existence of hydroxycinnamic acid esters with ethanol, like ethyl caffeoate, in white wines.

The above-mentioned release of hydroxycinnamic acids from anthocyanin esters during wine maturation may be only due to chemical reactions but conversions of acid precursors to volatile phenols are typically dependent on enzyme or microbial activity.

In grapes or grape juices, the tartaric esters may be hydrolysed by enzymes from contaminant fungi or from commercial pectolytic preparations, both with cinnamoyl decarboxilase activity, releasing free hydroxycinnamic acid forms (Dugelay et al. 1993; Gerbaux et al. 2002). However, the tartaric esters are mostly hydrolysed after malolactic fermentation (Hernández et al. 2006, 2007), it being hypothesised that the hydrolytic activity of lactic acid bacteria follows the completion of malic conversion to lactic acid (Cabrita et al. 2007) (see Table 11.4).

It is accepted that yeasts only metabolise the free acid forms, although brewing *S. cerevisiae* was supposed to possess feruloyl esterasic activity (Coghe et al. 2004). Then the availability of free hydroxycinnamic acids appears to be crucial for the production of VPs either by yeasts or bacteria.

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Table 11.4 Range of concentrations of hydroxycinnamic acids and their esters (mg/L)

Hydroxycinnamic acids and their esters						Observations	Reference
trans-Caftaric	*trans*-Caffeic	*trans*-Coutaric		2-Glutathionylcaftaric	Malvidin-(6-coumaroyl)-3-glucoside		Ibern-Gómez et al. (2002)
12.9–34.3	5.5–11.8	6.1–22.1		3.1–6.6	6.7–43.1	6 red wines	
Caftaric	Caffeic	Coutaric	Coumaric	Fertaric	Syringic		Pérez-Magariño and González-San José (2005)
8.89–26.06	2.36—9.95	5.35–10.04	2.87–11.21	0.40–2.14	7.08–10.61	6 red wines not aged	
Caftaric	Caffeic acid	Coutaric	Coumaric acid				Makris et al. (2006)
4.3–122.3	0—31.2	2.8–39.0	0–7.1			41 young red wines	
Caftaric	Caffeic	*p*-Coutaric	*m*-Coutaric	Fertaric	Ferulic		Gómez-Míguez et al. (2007)
13.43–25.29	0.04—0.98	0.76–3.59	0.12–0.14	0.03–0.08	0.13–0.04	White musts	
27.13–57.14	0.78—4.11	3.08–4.94	1.03–0.48	1.34–4.20	0.58–1.44	White wines	
trans-Caftaric	*trans*-Caffeic	*trans*-Coutaric	*trans-p*-Coumaric	*trans*-Ferulic	*trans-p*-coumaric hexose		Gómez-Míguez et al. (2007)
14.98	2.68	13.75	2.06	-	1.23	Red wine before MLF[a]	
6.85	26.24	8.68	16.01	0.84	1.84	Red wine after MLF	
cis-Caftaric		*cis*-Coutaric	*cis-p*-Coumaric				
2.11		4.33	1.91			Red wine before MLF	
0.18		2.17	0.52			Red wine after MLF	
Caftaric	Caffeic	Coutaric	*p*-Coumaric	Fertaric	Ferulic		Cabrita et al. (2007)
4.10	0.47	0.85	0.24	0.40	–	Red wine before MLF	
0.25	6.90	0.12	2.31	0.06	0.95	Red wine after MLF	

[a]Malolactic fermentation

11.3.2.2 Conversion of Hydroxycinnamic Acids

Once hydroxycinnamic acids are released in the grape juice or in the wine, they may be converted into vinylphenol and ethylphenol derivatives depending on the presence of specific growing microbial populations.

During fermentation, *S. cerevisiae* may produce vinylphenol derivatives due to the presence of cinnamate decarboxylase enzymes (Chatonnet et al. 1992, 1993) which are inactive in red juices due to the polyphenol components of red wine (Chatonnet et al. 1997). Several grape juice contamination yeast species also have the ability to form vinylphenols (Dias et al. 2003a) but their contribution to the vinylphenol content of wines may only be relevant when are not inhibited by *S. cerevisisiae* (Barata et al. 2006).

After the decarboxylation step, vinylphenols may be reduced to ethylphenols but the sequential decarboxylase and reductase activities, regarding wine yeasts, have only been demonstrated in *D. bruxellensis* and in *P. guilliermondii* (Barata et al. 2006). The former species may also convert 4-VP into 4-EP in the absence of hydroxycinnamic acids (Dias et al. 2003b).

The production of 4-ethyphenol in wines is dependent on the presence of growing yeast populations. As *P. guilliermondii* does not grow in wines with average ethanol of 12 vol.% it is not likely to produce significant levels of 4-EP during wine maturation. Concerning *D. bruxellensis* it does not grow in white wines explaining the absence of phenolic taint in this type of wines (Malfeito-Ferreira et al. 2001). Several lactic acid bacteria (*Lactobacillus* spp., *Pediococcus* spp.) have also been characterised concerning the production of ethylphenols in synthetic media (Table 11.5) but in wines they are not regarded as significant 4-EP producers (Chatonnet et al. 1995, 1997). The main starter used in wines for malolactic conversion, *Oenococcus oeni*, does not seem to produce vinyl or ethylphenols even in synthetic media (Couto et al. 2006).

The conversion of *p*-coumaric acid into 4-EP only occurs when *D. bruxellensis* is growing on a carbon and energy source, the conversion rate being dependent on the substrate (Dias et al. 2003b). The conversion of the other hydroxycinnamic acids by yeasts has not been deeply studied. Most studies are related with *p*-coumaric acid metabolism but the conversion of ferulic, caffeic acids or sinapic acids may not be equally efficient, as demonstrated, in synthetic medium, for *D. bruxellensis* (Heresztyn 1986a), *S. cerevisiae* (Chatonnet et al. 1989) and *D. anomala* (Edlin et al. 1995). Knowing that caffeic acid is more concentrated than *p*-coumaric acid in wines (see Table 11.5) it would be expected that 4-EC would be present in higher concentration that 4-EP, but the few results published do not corroborate this hypothesis (see Table 11.4). Then, *D. bruxellensis*, although utilising caffeic acid (Heresztyn 1986), may not produce 4-EC with the same efficiency as 4-EP in wines. AQ6

11.3.2.3 Changes in Wine Composition

The above-mentioned metabolic activities of microorganisms should be taken into account when studying wine compositional alterations. In fact, the effect of

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Table 11.5 Metabolic activity of microorganisms related with production of volatile phenols in wine industry

Species	Function	Metabolic activity	Reference
Aspergillus niger	Mould for commercial enzyme production (pectinase, hemicellulase)	Active cinnamoyl esterase releasing free hydroxycinnamic acids in juices	Dugelay et al. (1993)
Saccharomyces cerevisiae	Fermenting yeast	Active hydroxycinnamate decarboxilase producing vinylphenols in fermenting white juices	Dugelay et al. (1993); Chatonnet et al. (1989); Shinohara et al. (2000); Barata et al. (2006)
Dekkera bruxellensis	Spoilage yeast	Active hydroxycinnamate decarboxylase and vinylphenol reductase producing ethylphenols in synthetic media, juices and wines	Heresztyn (1986); Chatonnet et al. (1995); Shinohara et al. (2000); Rodrigues et al. (2001); Dias et al. (2003a, 2003b)
Pichia guilliermondi	Contamination yeast	Active hydroxycinnamate decarboxylase and vinylphenol reductase producing ethylphenols in synthetic media and grape juices	Barata et al. (2006)
C. albidus, C. laurentii, C. stellata, C. wickerhamii, *D. hanseniiI, H. anomala*, *H. uvarum, K. apiculata*, *K. thermotolerans*, *M. pulcherrima*, *P. guilliermondii*, *P. membranifaciens*, *R. rubra, S. pombe*, *Z. bailii*	Contamination yeasts	Active hydroxycinnamate decarboxilase activity producing vinylphenols in synthetic media and grape juices	Chatonnet et al. (1992); Shinohara et al. (2000); Dias et al. (2003a)
Lactobacillus spp., *Pediococcus* spp.	Fermenting and spoilage lactic acid bacteria	Active hydroxycinnamate decarboxylase and vinylphenol reductase producing ethylphenols in synthetic media	Cavin et al. (1993); Couto et al. (2006)
Oenococcus oenii, *Lactobacillus plantarum*	Fermenting lactic acid bacteria	Active cinnamoyl esterase releasing free hydroxycinnamic acids in red wines	Hernández et al. (2007); Cabrita et al. (2007)

microorganisms on the polyphenolic composition does not seem to have been considered when establishing differentiation between grape varieties (Makris et al. 2006), between ripening stages (Pérez-Magariño and González-San José 2005) or bottle aging (Monagas et al. 2005). However, as observed in Table 11.4, changes in hydroxycinnamic acid compositions may be explained by microbial activity, which are higher than changes between grape varieties or ripening stages. These variations may explain, at least partially, the controversy on the evolution of hydroxycinnamates during wine processing (Monagas et al. 2005 and references cited therein).

The balance of VP and precursors is also influenced by non-microbial reactions. The esterified forms of hydroxycinnamic acids or vinylphenols form a pool of molecules which release or combine the acids during wine maturation, apparently without the influence of microorganisms (Hernández et al. 2006, 2007; Suárez et al. 2007). In addition, hydroxycinnamic acids may suffer oxidative condensation and browning during aging (Yokotsuka and Singleton 2001). Oak chips may also release 4-EG up to 0.15 μg/g, or 4-VG up to 7.76 μg/g, as influenced by higher toasting intensity (Natali et al. 2006). Overall, sources of VPs other than microbial should not account by more than 100 μg/L (Rayne and Eggers 2007). AQ7

11.3.3 Effect of Volatile Phenols on Product Quality

The sensorial effect of a volatile compound may be positive or negative to wine depending on its smell and concentration. In wines it is not easy to define beneficial or detrimental effect because the odours of mixtures of different compounds are perceived differently than those of single compounds and there is also a matrix effect on the perception. In addition, the rejection of an odour occurs at higher concentrations than the detection, leading to different detection and preference thresholds. The value of a detection/preference threshold may measure the spoilage effect of molecules with sensorial activity. These may be defined as the minimum concentration under which 50% of the tasters, in a 70 person jury, statistically detected/rejected the sample (Chatonnet et al. 1992). For instance, in Bordeaux red wines, the preference threshold for 4-EP is about 620 μg/L, and for the mixture (10:1) of 4-EP and 4-EG is 426 μg/L (Chatonnet et al. 1992). Below these concentrations, volatile phenols may contribute favourably to the complexity of wine aroma by imparting aromatic notes of spices, leather, smoke or game, appreciated by most consumers. Above those levels, wines are clearly substandard for some consumers but remain pleasant for others. To increase the difficulty in the definition of spoiling concentrations, these thresholds are dependent on grapevine variety and on the style of wine (Gato et al. 2001; Coulter et al. 2003).

In the case of vinylphenols, they contribute to the spicy, floral and pharmaceutic character of white wines. The 4-VG has been detected in high levels in the variety Gewürztraminer (Grando et al. 1993). The depreciation due to high levels of 4-VG plus 4-VP in white wines of the German variety Kerner was associated with hot regions (e.g. South Africa) or exposure of grapes to sunlight, but no explanation

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was given to the fact (Rapp 1998). The overall incidence of vinylphenols in white wines is not known and but it seems to have decreased after the improvement in the purity of commercial pectolytic enzymes used in juice clarification.

In the case of ethylphenols, concentrations of 4-EP and 4-EG above the preference thresholds dominate the flavour contributing to the phenolic character. The mouthfeel sensations are also altered by increasing the metallic notes (Coulter et al. 2003). Volatile phenols are currently determined by gas-liquid chromatography after wine extraction with organic solvents (Loureiro and Malfeito-Ferreira 2006). However, the main flaw of this technique is the absence of 4-EC quantification, which requires derivatisation (Hesford and Schneider 2004). Despite this fact, numerous recent improvements in volatile phenol analysis were only directed to extraction procedures (López et al. 2002; Mejías et al. 2003; Díez et al. 2004; Fariña et al. 2007; Pizarro et al. 2007; Rayne and Eggers 2007).In contrast, Carrillo and Tena (2007) presented an HS-SPME extraction followed by GC-MS of derivatised samples accounting for 4-EC. In addition, liquid chromatography has been present as an alternative to GC, having the advantage of avoiding sample extraction (Vanbeneden et al. 2006; Caboni et al. 2007; Larcher et al. 2007; Nicolini et al. 2007) but the proposed methods do not account for 4-EC.

The real incidence of VP in wines world wide is not known perhaps due to the difficulty in performing routine instrumental analysis in wineries. Normally, winemakers analyse samples suspected to have problems and so the reported proportion of affected wines is most probably biased. Some data from analytical laboratories have shown that from 6% to 74% of analysed samples may bear levels of 4-EP plus 4-EG higher than the preference threshold (426 μg/L) of a 10:1 mixture (Loureiro and Malfeito-Ferreira 2006). Although precise numbers are not available, we believe that the phenolic taint is the main microbiological problem leading to higher economical losses in winemaking industry. Moreover, as already mentioned, the sensory detection of VP depends on the type of wine (Gato et al. 2001; Coulter et al. 2003) and so a higher proportion of wines may be badly affected by these compounds.

The 4-EG is present in about one tenth of the 4-EP concentration (Chatonnet et al. 1992) but this rate is not always observed (Rodrigues et al. 2001; Coulter et al. 2003). The other odour active VP, 4-EC, has just begun to be studied. The fact that the precursors of 4-EC, caffeic acid and its esters, are present in relatively high concentrations in wines (see Table 11.4) and that the detection threshold for 4-EC (described as having a phenolic smell similar to that of 4-EP) is about 50 μg/L (Porret et al. 2004) suggests that its influence in phenolic taint should be not be neglected. Occasional discrepancies between sensorial detection and concentration of 4-EP and 4-EG may be explained by the hidden presence of 4-EC. Sinapic acid gives 4-VS and 4-ES but the syringols do not affect medium odour (Heresztyn 1986a).

The wines affected by ethylphenols are practically only red wines. All types are susceptible to the phenolic taint depending on the growth of *D. bruxellensis.* However, red wines matured in oak barriques are the typical wine product affected by

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this taint because these containers provide a highly favourable ecological niche for *D. bruxellensis* (Loureiro and Malfeito-Ferreira 2006).

11.3.4 Control Measures

When wines are affected by volatile phenols there is, at present, no effective curative process. In this situation, oenologists always weigh the possibility of blending tainted wine with "clean" wine. Although this measure may attenuate the defect of the tainted wine by dilution it cannot be seen as a curative measure. In fact, mixtures of wines with null or low levels of 4-ethylphenol are only effective for small proportions of tainted wines because large volumes of "clean" wine must be used to obtain a blend with 4-EP levels lower than the preference threshold. Then, effective curative measures would depend either (i) on the reduction or elimination of the sensorial effect or (ii) on the extraction of odour active molecules from the wine. These strategies have not yet been effectively tested in practical conditions. Guilloux-Benatier et al. (2001) hypothesised that yeast lees have the property to adsorb volatile phenol, which was later shown, at least partially, in laboratory conditions, by Chassagne et al. (2005). A reverse osmosis procedure to reduce volatile phenols is commercially available in New Zealand (http://www.armourtech.co.nz/memstarreverseos.html). Salameh et al. (2007) showed that *p*-coumaric acid may be adsorbed on *Brettanomyces* cells, decreasing its availability as substrate. Commonly, when adsorbents are added to wine, favourable aroma compounds are also removed and a balance must be drawn between benefits and losses of wine attributes.

If a curative approach is not effective, then prevention is, at present, the most reasonable way to deal with the problem. Bearing in mind that, to produce volatile phenols microorganisms needs the substrate to be available and active, the preventive measures may be directed either to minimise the release of free acid or to avoid microbial activity.

In juices, prevention should be based on (i) decreasing the release of free acids that is favoured by mould infections of grapes and by the decarboxylase activity of commercial enzyme preparations and (ii) avoiding the production of volatile phenols that is favoured by the uncontrolled activity of contamination yeasts growing in damaged grapes or in juices. Then, the main measures to be adopted are:

- Separation of sound grapes from damaged grapes
- Use of sulphur dioxide to prevent yeast contaminations
- Use of pure commercial enzymes, if necessary
- Initiatiation of active fermentation with *S. cerevisiae* as soon as possible

These measures are good manufacturing practices of winemaking, irrespective of the risk of phenolic taint. The main preventive measures should be performed during wine storage, aging (mainly in oak barrels) and bottling. As the release of precursors

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is unavoidable, the main preventive measures are directed to reduce the activity of contaminating populations of yeasts and bacteria, especially towards *D. bruxellensis* (see review of Loureiro and Malfeito-Ferreira 2006):

- Use adequate hygienic practices and respective efficiency assessment
- Use adequate levels of sulphur dioxide or DMDC (if legally authorised)
- Minimise residual nutrient contents (sugar or nitrogen)
- Minimise oxygen dissolution
- Handle oak barrel aging properly (disinfection, toppings, rackings, cellar temperature)
- Perform microbiological monitoring especially when wines from external sources are processed and oak aging is used
- Thermal treatment or filter sterilisation is advised when risk of bottle infection is high

11.4 Tetrahydropyridines

Tetrahydropyridines (THP) are secondary metabolites produced by *D. bruxellensis* and lactic acid bacteria in wines and are responsible for a taint described as mousy off-flavour or mousiness. This problem has been known since late nineteenth century (see review of Snowdon et al. 2006) but, in spite of its obnoxious flavours, has been only vaguely studied perhaps due to its low frequency of occurrence.

11.4.1 Chemical Structure and Origin

Tetrahydropyridines (THP) include 2-ethyl-tetrahydropyridine (ETHP), 2-acetyl-tetrahydropyridine (ATHP) and 2-acetylpyrroline (APY) (Fig. 11.6). ETHP is present in tautomeric forms, but the second tautomer is minor. ATHP also occurs in two tautomeric forms, of which the distribution is pH dependent. These molecules are uncommon components of wines and are not currently analysed.

Tucknott (1977) first identified ETHP and other unknown compounds as the molecules imparting mousiness, showing that they were not present in the absence of microorganisms. The origin of THP is related with activity of *D. bruxellensis* and of lactic bacteria, mainly heterofermentative strains, but the possible role of acetic bacteria should not be discarded (Heresztyn 1986b; Snowdon et al. 2006). APY is not produced by *Dekkera* spp. but by lactic acid bacteria, being an indicator of bacterial spoilage. The pathways for production of THP by heterofermentative bacteria or *D. bruxellensis* have been proposed (Costello and Henschke 2002; Snowdon et al. 2006) but require further confirmation. However, it is established that both pathways require L-lysine and ethanol to THP synthesis. L-Lysine and L-ornithine are responsible for ring formation of the heterocycles whereas ethanol and acetaldehyde are responsible for the acetyl side chain (Snowdon et al. 2006;

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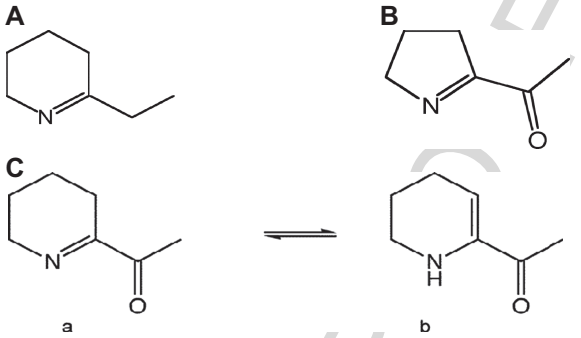

Fig. 11.6 Chemical structure of (A) 2-ethyltetrahydropyridine (ETHP), (B) 2-acetylpyrroline (APY) and tautomers of (Ca) 2-acetyl-3,4,5-tetrahydropyridine (ATHP) and (C) 2-acetyl-1,4,5,6-tetrahydropyridine (adapted from Snowdon et al. 2006, permission to be obtained)

Grbin et al. 2007). ATHP reduction may lead to EHTP. As ethanol is a precursor, mousy off-flavour occurs after alcoholic fermentation, preferably after lactic acid bacteria activity. It seems that the formation of mousiness may be induced by oxidation but it is not clear if the effect is on the microorganisms or in any chemical reaction stimulated by the redox potential. Other agents claimed to affect its production (high pH, low sulphite, residual sugar content) (Lay 2004; Snowdon et al. 2006; Romano et al. 2007) are also stimulators of microbial activity and so the true mechanisms are not yet clarified, but the non-enzymatic chemical synthesis has been ruled out in *D. anomala* (Grbin et al. 2007).

11.4.2 Effect on Wine Quality and Occurrence

The "mousy off-flavour" is described as resembling the smell of mice urine and once tasted becomes unforgettable. The taint is mainly perceived by the after-mouth sensation and has a long persistence (may exceed 10 min). The compounds responsible are not volatile at wine pH and so are only perceived by the increase in pH due to saliva. These features justify the use of practical sensorial detection methods without the need to swallow the wine, like rubbing the wine in the palm of the hand and sniffing the skin, or dipping an alkaline paper strip in the wine and smelling. ATHP is the main molecule responsible for the fault (Strauss and Heresztyn 1984), being present in levels up to 108 μg/L (Snowdon et al. 2006) and having a detection threshold of 1.6 μg/L in water (Colagrande et al. 1988). The imino tautomeric form provides the mousy perception and its prevalence at high pH explains the detection after increasing sample pH. Its off-flavour also resembles cracker biscuit, and this molecule is also present in some cereal based products (Snowdon et al. 2006). EHTP has been detected in wines only recently and has a detection threshold of 150 μg/L in wines (Snowdon et al. 2006). APY is also a major contributor to mousy off-flavour, with detection threshold of 0.1 μg/L in water and being detected in

trace levels up to 7.8 μg/L (Snowdon et al. 2006). In other food products it has been described as "roasty" and "popcorn-like". Acetamide, although occasionally linked to mousiness, is not the cause of mousy off-flavour because it is odourless (Snowdon et al. 2006).

The incidence of mousiness in wines is not known. In our experience we have only tasted it a handful of times. Earlier reports and classical enology treatises already mention this problem (Grbin and Henschke 2000 and references cited therein). The activity of *Dekkera/Brettanomyces* spp. has been linked to this fault since early studies leading to some confusion between mousiness and phenolic taint. The development of each other is independent (Romano et al. 2007) and we are not aware of the factors stimulating one instead of the other. Although it is not a common taint, it is known to affect wines all over the wine countries (Grbin and Henschke 2000). As it depends on *Dekkera*/*Brettanomyces* activity it is likely that red wines are more affected. Also, white wines are usually not subjected to malolactic fermentation and so the activity of lactic bacteria may preferentially affect red wines. These yeasts also produce THP in grape juices (Grbin and Henschke 2000) but the real incidence in this product is not known.

11.4.3 Control Measures

There is no available method to remove this taint effectively (Lay 2004). The removal of precursors (L-lysine and ethanol) is not feasible. As it depends on microbial activity, the preventive measures are similar to those suggested for volatile phenols when there is the risk of *D. bruxellensis* infection. The prevention of spoilage by heterofermentative lactic bacteria usually advised, like decreasing wine pH values and rapid inactivation by sulphur dioxide, once malolactic conversion is finished, should also be effective against bacterial mousiness.

11.5 Final Remarks

The wine spoilage effects described in this chapter illustrate two different approaches undertaken to solve the problems. One, OTA production, is associated with food safety and so profits from the allocation of relatively large funds under specific EU research frameworks. The other problems, associated mainly with VP, are of technological nature and are not specifically supported by research frameworks and so the respective scientific outputs are much less relevant. The present awareness of OTA incidence and knowledge of preventive measures make this a relatively minor problem to the wine industry, which can easily be kept under control by using an adequate HACCP system from vineyard to the bottle.In contrast, concerning phenolic taint, this continues to be a major problem of wine microbial spoilage which involves significant economical losses. The primary sources and routes of contamination of *D. bruxellensis*, the role of esterified VP precursors, the role of lactic

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acid bacteria, the white wine enigmatic resistance to VP production, the reasons explaining unexpected blooms, the choice of effective prevention measures, and the different wine susceptibility to spoilage are only some of the issues not fully understood by scientists and wine technologists. From a practical point of view, the slow response of the production sector to the problem is surprising, particularly concerning *D. bruxellensis* monitoring in wines and equipment surfaces. The utilisation of microbiological control and the adoption of guidelines by attributes for bulk and bottled wines would certainly avoid most problems, given that the essential of the knowledge on *D. bruxellensis* spoiling activity is already available.

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Chapter-11

Query No.	Page No.	Line No.	Query
AQ1	615	07	Should this not be "implying that any pathogenic bacteria DID NOT HAVE the capacity to grow or survive in wine"??? Please check sense of statement as it is!!!
AQ2	620	17	I do not understand what you mean by 'busted' – have substituted 'greatly increased'. Please check and change as required.
AQ3	622	05	"Bellí et al., 2005" is not listed in the reference list. Please provide.
AQ4	626	45	a or b? There are two such references reference list.
AQ5	627	TABLE 11.3	a or b? There are two such references reference list
AQ6	631	38	"Heresztyn, 1986" whether it is "1986a" or "1986b"?
AQ7	633	17	a or b? There are two such references reference list.
AQ8	634	13	a or b? There are two such references reference list.
AQ9	642	38	Please update.
AQ10	644	06	Please update.

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