Oxidation mechanisms occurring in wines

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A B S T R A C T

The present review aims to show the state of the art on the oxidation mechanisms occurring in wines, as well as the methods to monitor, classify and diagnose wine oxidation. Wine oxidation can be divided in enzymatic oxidation and non-enzymatic oxidation. Enzymatic oxidation almost entirely occurs in grape must and is largely correlated with the content of hydroxycinnamates, such as caffeoyltartaric acid and *para*-coumaroyltartaric acid, and flavan-3-ols. Non-enzymatic oxidation, also called chemical oxidation of wine, prevails in fermented wine and begin by the oxidation of polyphenols containing a catechol or a galloyl group. These phenolic reactions, both enzymatic and non-enzymatic, result in by-products named quinones. However, in non-enzymatic oxidation, oxygen does not react directly with phenolic compounds. The limitation on the reactivity of triplet oxygen is overcome by the stepwise addition of a single electron, which can be provided by reduced transition metal ions, essentially iron(II) and copper(I). The sequential electron transfer leads to the formation of hydroperoxide radical ($HOO^{\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$). The later radical will oxidize almost any organic molecule found in wine and will react with the first species it encounters, depending on their concentration. Sulfur dioxide (SO_2) and ascorbic acid, when added to wine, are able to reduce the quinones. Alternative options have been assessed for the prevention of oxidation during wine storage; nevertheless, these are not fully understood or commonly accepted. During aging, aldehydes are important intermediates in the chemical transformations occurring in wines, leading to color and flavor changes. In the same way, a range of off-flavors can be formed from wine oxidation. At low concentrations these flavors may add to the complexity of a wine, but as these increase they begin to detract from wine quality. In addition to the major chemical browning involving wine phenols, the main oxidation reactions occurring during grape juice heating or storage are caramelization and Maillard reaction, which are temperature dependent. Different methods have been proposed in the literature, addressing the complexity and multi-scale related with the oxidation process, to attempt the quantification of antioxidant activity in wines. These methods can be broadly divided in: i) methods based on chemical reactions and ii) methods based on the chemical-physical properties of antioxidants.

Contents

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1. Oxidation reaction context: ROS definitions

Free radicals species are encountered in various reactions in many biological systems and in processes responsible for the spoiling of foods. Reactive oxygen species (ROS) is a collective term used to describe oxygen radicals, such as superoxide anion ($O_2^{\bullet-}$) and its conjugate acid hydroperoxyl ($HOO^{\bullet}$), hydroxyl ($HO^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$) radicals, and certain other non-radicals that are either potential oxidizing agents or are easily converted into radicals, such as hydrogen peroxide (H_2O_2), ozone (O_3), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), and lipid peroxide (LOOH) (Pourova, Kottova, Voprsalova, & Pour, 2010; Shchepinov, 2007).

In wine, ROS can be produced by reduced transition metals ions [e.g. Fe(II)] in the stepwise addition of a single electron to triplet oxygen (O_2). The initial transfer of an electron leads to the formation of superoxide radical anion ($O_2^{\bullet-}$), which at pH wine exists in the protonated form hydroperoxyl radical ($HOO^{\bullet}$) (Fig. 1). The transfer of a second electron will produce peroxide anion (O_2^{2-}), which at pH wine exists in the protonated form hydrogen peroxide (H_2O_2) (Fig. 1). The next reduction step creates an even more reactive oxidant, the hydroxyl radical ($HO^{\bullet}$) (Fig. 1), which can abstract a hydrogen atom from organic compounds to produce water, the final oxygen reduction product (Danilewicz 2003; Waterhouse & Laurie 2006).

2. Phenolic compounds in wines: Primary substrates for oxidation

Constituents of both red and white wines are capable of reacting with significant amounts of oxygen, polyphenols being among the most readily oxidized wine constituents.

On a sensory perspective, controlled oxidation could be beneficial for red wine by enhancing and stabilizing color and reducing astringency, nevertheless, the quality of white wine is generally damaged by exposure to air (Singleton 1987, Singleton, 2000). Wine polyphenolic substances are usually subdivided into two groups: flavonoids and non-flavonoid compounds. The flavonoids have a common core, the flavane nucleus, consisting of two benzene rings (A and B) linked by an oxygen-containing pyran ring (C) ($C_6C_3C_6$). Differences in the degree of oxidation of the heterocyclic ring (C) and hydroxylation/methoxylation of the three rings results in a large family of structures with essential differences in physicochemical properties and stability. The most common wine flavonoid compounds are flavonols (kaempferol, quercetin, and myricetin), flavan-3-ols (catechin, epicatechin, and tannins), and anthocyanins (cyanidin-3-glucoside, peonidin-3-glucoside, delphinidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside) (Fig. 2a). The concentration of flavonoids in wine are strongly affected by the winemaking practices such as pressing and maceration that affect the degree of extraction from skins and especially from seeds which are rich in flavan-3-ol units. Flavan-3-ols are found in the solid parts of the berry (seed, skin and stem) in monomeric, oligomeric, or polymeric forms. The latter two forms are also known as proanthocyanidins or condensed tannins. While seed tannins are oligomers and polymers composed of the monomeric flavan-3-ols (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate (Prieur, Rigaud, Cheynier, & Moutounet, 1994) (Fig. 2a), skin tannins also contain (−)-epigallocatechin and trace amounts of (+)-gallocatechin and (−)-epigallocatechin gallate (Escribano-Bailón, Guerra, Rivas-Gonzalo, & Santos-Buelga, 1995; Souquet, Cheynier, Brossaud, & Moutounet, 1996) (Fig. 2a). The seeds contain higher concentrations of monomeric, oligomeric, and polymeric flavan-3-ols than the skins (De Freitas, Glories, & Monique, 2000; Sun, Pinto, Leandro, Ricardo da Silva, & Spranger, 1999). Levels of proanthocyanidins or condensed tannins are between 1 g/L and 4 g/L in red wines (Ribéreau-Gayon, Glories, Maugean, & Dubourdieu, 2000), while in white wine, levels are in the range of 100 mg/L and highly dependent on pressing techniques (Ribéreau-Gayon, Glories, Maugean, & Dubourdieu, 2000). Concerning the sensorial properties of these compounds, monomeric catechins are bitter, while polymers are essentially astringents (Peleg, Gacon, Schlich, & Noble, 1999).

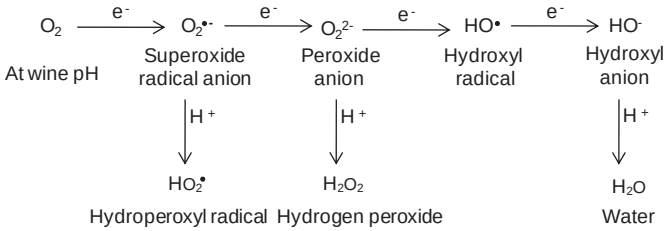


Fig. 1. Oxygen reduction (Waterhouse & Laurie, 2006).

Non-flavonoid compounds are mainly derivatives of benzoic acid and of cinnamic acid (Fig. 2b). Another class of non-flavonoids in grape includes the stilbenes and stilbene glycosides, *trans*-resveratrol being the best known example (Fig. 2b). A different class of non-flavonoids is the hydrolysable tannins. In wine, these compounds are derived from oak and their levels are near 100 mg/L for white wines aged for about 6 months in barrel, while red wines will have levels in the range of 250 mg/L, after aging two or more years (Quinn & Singleton, 1985). Hydrolysable tannins are esters of gallic acid (gallotannins) and ellagic acid (ellagitannins) with glucose or related sugars.

Red wines contain polyphenols at a higher concentration (1 to 5 g/L) than white wines (0.2 to 0.5 g/L), particularly much higher levels of flavan-3-ols and procyanidins. Some of the established effects of O_2 additions to red wine include a decrease in the phenolic compounds such as (+)-catechin, (−)-epicatechin, quercetin, caffeic acid and anthocyanins, and an increase in red polymeric pigments improving the wine color density (Castellari, Matricardi, Arfelli, Galassi, & Amati, 2000). In addition, it is established that oxygenation could improve the evolution of red wines during aging, but also deplete the amount of some monomer and oligomeric phenolic compounds related with health benefits (Castellari et al., 2000). Equally, several recent reports on the effects of micro-oxygenation in red wines have confirmed the loss of monomeric anthocyanins and other polyphenols, along with the formation of polymeric pigments, resistant to sulfur dioxide (SO_2) bleaching, often with an increase in wine color density (De Beer, Joubert, Marais, & Manley, 2008; Tao, Dykes, & Kilmartin, 2007). Additional changes in red wine pigments include the formation of methylmethine-anthocyanin-catechin compounds and pyranoanthocyanin-catechin compounds mediated by acetaldehyde released during wine oxidation processes (ethanol oxidation) (Timberlake & Bridle, 1976; Francia-Aricha, Guerra, Rivas-Gonzalo & Santos-Buelga, 1997; Mateus, Silva, Santos-Buelga, Rivas-Gonzalo, & De Freitas, 2002).

An increase in acetaldehyde has been recorded in the later stages of regular micro-oxygenation (Carlton, Gump, Fugelsang, & Hasson, 2007) and during an electrochemical microoxidation approach (Fell, Dykes, Nicolau, & Kilmartin, 2007). A further influence on the rate of oxidative changes during micro-oxygenation is the level of SO_2 in the wine. In a red wine subjected to micro-oxygenation with additions of 0 to 200 mg/L of SO_2, a large decrease in the amount of monomeric anthocyanins and flavan-3-ols was seen in wines with a lower concentration of SO_2, coupled with an increase in non-bleachable pigments, an increase in tannins, and a greater size and red coloration of a proanthocyanidin extract (Tao et al., 2007). These changes were largely suppressed in wines initially treated with 200 mg/L of SO_2 and occurred more slowly in wines stored in bottles in the absence of O_2. The concentration of SO_2 is shown to regulate the polyphenol chemistry involved in the formation of polymeric pigments and changes in tannin structure affecting wine astringency (Tao et al., 2007).

White wines contain lower levels of polyphenols, mainly hydroxycinnamic acids (Betes-Saura, Andres-Lacueva, & Lamuela-Raventos,

1996), but these remain very important for oxidation related issues in wine browning and losses of varietal aroma. The low concentrations of flavonoids such as catechin and quercetin glycosides remain important particularly for wine browning and are more prevalent in musts exposed to longer skin contact times and harder pressing conditions (Singleton, 1987). Tests on browning rates with different wines have shown variable results with respect to the importance of phenolic content, SO_2 level, pH, and metal ions content (Simpson, 1982).

3. Enzymatic oxidation

Enzymatic browning almost entirely occurs in grape must. A likely mechanism for oxidation of phenolic compounds involves hydroxylation to the *ortho*-position adjacent to an existing hydroxyl group of the phenolic substrate (monophenol oxidase activity), and oxidation of *ortho*-dihydroxybenzenes to *ortho*-benzoquinones (diphenol oxidase activity) (Fig. 3). Several classes of enzymes can catalyze these reactions. According to the *Nomenclature Committee of the International Union of Biochemistry and Molecular Biology* (NC-IUBMB), these enzymes are part of the E.C. 1 class of oxidoreductases. The three main classes of enzymes that catalyze the oxidation of phenolic compounds are the oxidoreductases that use oxygen as electron acceptor (E.C.1.10.3), the monophenol monooxygenase (E.C. 1.14.18.1), and the peroxidases (E.C. 1.11.1).

The E.C. 1.10.3 subclass includes enzymes that use catechols or related compounds as electron donors and oxygen as electron acceptor, leading to the oxidized donor and water. Members include catechol oxidase (E.C. 1.10.3.1), laccase (E.C. 1.10.3.2), and *ortho*-aminophenol oxidase (E.C. 1.10.3.4). Catechol oxidase is also known as diphenoloxidase, phenoloxidase, polyphenoloxidase, *ortho*-diphenolase, phenolase and tyrosinase, whereas, laccase is also known as *para*-diphenoloxidase. Many of these names are also used in reference to a different enzyme, monophenol monooxygenase (E.C. 1.14.18.1). E.C. 1.14 classes

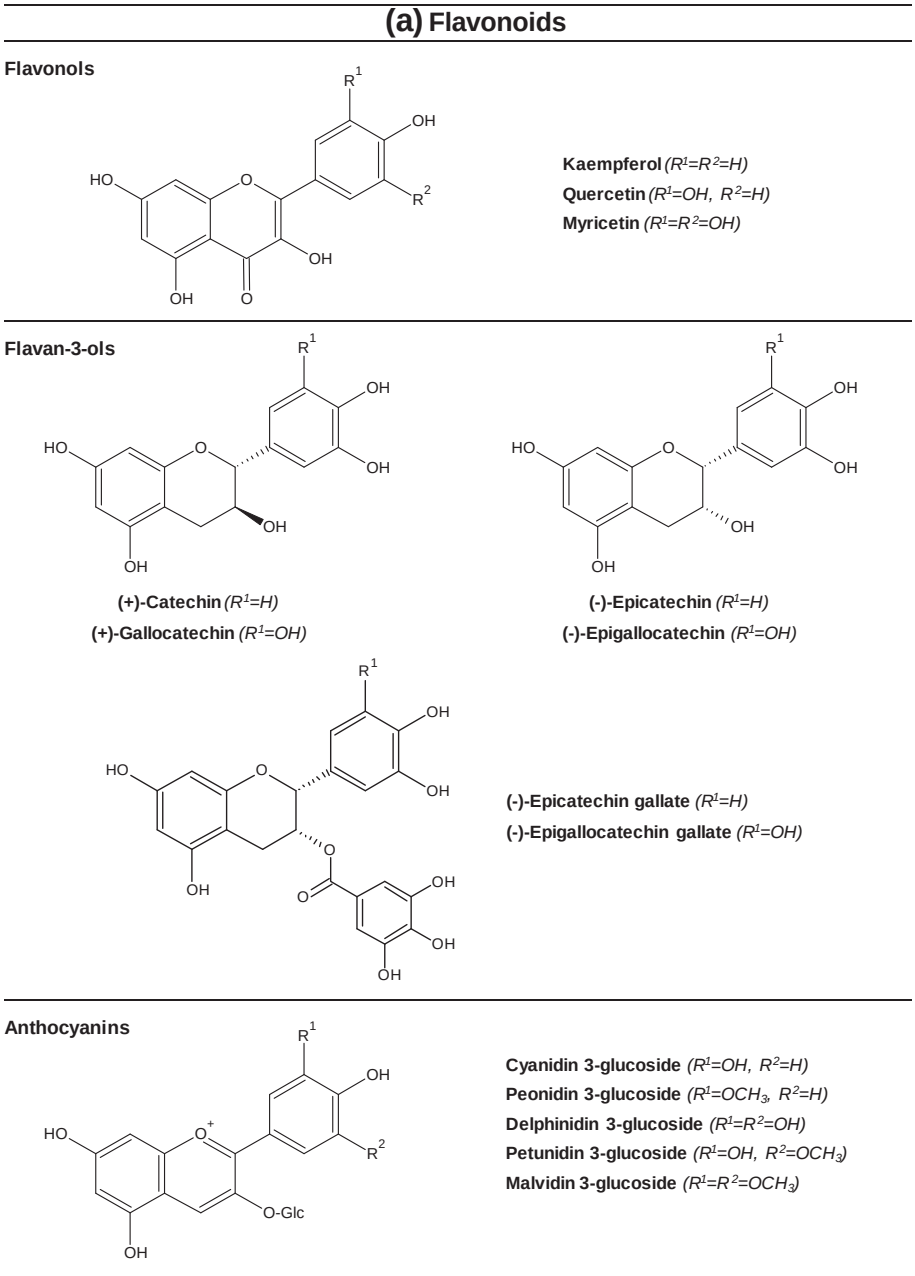
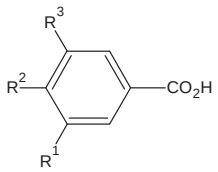
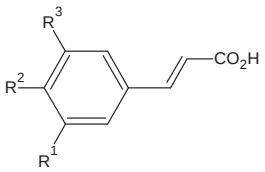


Fig. 2. a. Most common flavonoid compounds in wine. b. Most common non-flavonoid compounds in wine.

(b) Non - Flavonoids

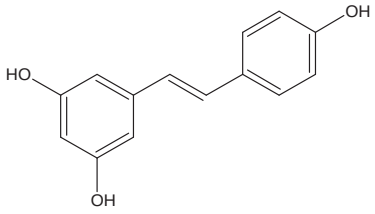
Derivates of benzoic and cinnamic acid





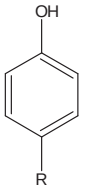
	R^1	R^2	R^3	
Benzoic acid	*H*	*H*	*H*	**Cinnamic acid**
***p*-Hydroxybenzoicacid**	*H*	*OH*	*H*	***p*-Coumaric acid**
Protocatechuic acid	*OH*	*OH*	*H*	**Caffeic acid**
Vanillic acid	OCH_3	*OH*	*H*	**Ferulic acid**
Gallic acid	*OH*	*OH*	*OH*	
Syringic acid	OCH_3	*OH*	OCH_3	**Sinapic acid**

Stilbenes

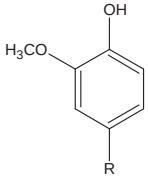


***trans*-Resveratrol**

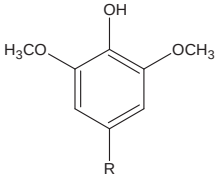
Volatile Phenols



Ethylphenol *(R=CH_2CH_3)*
Vinylphenol *(R=$CHCH_2$)*



Guaiacol *(R=H)*
Methylguaiacol *(R=CH_3)*
Ethylguaiacol *(R=CH_2CH_3)*
Vinylguaiacol *(R=$CHCH_2$)*



Syringol *(R=H)*
Methylsyringol *(R=CH_3)*

Fig. 2 (*continued*).

of monooxygenases contain enzymes acting on paired donors, with the incorporation or reduction of molecular oxygen. Monophenol monooxygenase (E.C. 1.14.18.1) catalyzes the same reactions as catechol oxidase (E.C. 1.10.3.1) if only catechols are available as substrate.

The phenol oxidizing enzyme tyrosinase has two types of activity: (i) phenol *ortho*-hydroxylase (cresolase) activity, whereby a monophenol is converted into a catechol via the incorporation of oxygen, and (ii) catecholase activity, whereby the catechol is oxidized to the brown pigment melanin (Sánchez-Ferrer, Rodríguez-López, García-Cánovas, & García-Carmona, 1995). Laccase catalyzes the oxidation of *para*-hydroquinones to *para*-benzoquinones.

The E.C. 1.11.1 subclass contains the peroxidases, which use hydrogen peroxide (H_2O_2) as electron acceptor to oxidize the donor, thereby forming the oxidized donor and water. Members include horseradish peroxidase (E.C. 1.11.1.7) also known as guaiacol peroxidase and scopoletin peroxidase, manganese peroxidase (E.C. 1.11.1.13) and diarylpropane peroxidase (E.C. 1.11.1.14). All three classes are hemoproteins. Mechanistically, hydrogen peroxide oxidizes the active

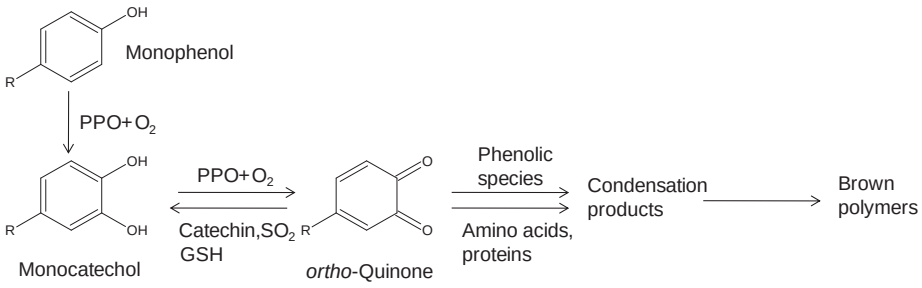


Fig. 3. Enzymatic browning process in grape must (Li et al., 2008).

site of the peroxidase enzyme, and upon substrate binding in the active site, the substrate becomes oxidized and the enzyme returns to its reduced state. Peroxidases are encoded by a large multi-gene family, which has complicated the study of individual peroxidase enzymes (Wilfred & Ralph, 2006).

The most important oxidoreductases responsible for browning during grape processing are polyphenoloxidases (PPO), namely, catechol oxidase or tyrosinase (E.C. 1.10.3.1) and laccase (E.C. 1.10.3.2), and peroxidases (POD), namely, horseradish peroxidase (E.C. 1.11.1.7) (Li, Guo, & Wang, 2008; Whitaker, 1995). PPO is a Cu-containing enzyme, while POD is a Fe-containing enzyme. Tyrosinase, is naturally produced in grape berry and can catalyze the oxidation of monophenols and catechols (Li et al., 2008; Singleton, 1987). Laccase is produced by molds and are able to oxidize a great number of substrates, especially 1,2- and 1,4-dihydroxybenzenes. Moreover, the browning caused by POD seems insignificant in fruits although some researchers found that it did increase the degradation of phenols when coexisting with PPO (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999).

In grape must, enzymatic browning (Fig. 3) is largely correlated with the content of hydroxycinnamates such as caffeoyltartaric acid (caftaric acid) and *para*-coumaroyltartaric acid (coutaric acid) (Cheynier, Trousdale, Singleton, Salgues, & Wylde, 1986), and is promoted by flavan-3-ols (Cheynier, Fulcrand, Guyot, Oszmianski, & Moutounet, 1995). When grapes are crushed, polyphenoloxidases (PPO) are released, and rapidly oxidize the hydroxycinnamates to benzoquinones. Meanwhile, the benzoquinones produced by enzymatic oxidation will undergo further reactions, according to their redox properties and electronic affinities (Robards et al., 1999). Being oxidants, quinones can oxidize substances which have a lower potential such as polyphenols and ascorbic acid as well SO_2. The quinone is then reduced back to its original catechol. As electrophiles, they can also react with nucleophiles like amino derivatives (Kutyrev & Moskva, 1991).

In must oxidation, the initial oxygen uptake by *ortho*-dihydroxybenzenes is slowed by addition of thiols like cysteine (Cys) or glutathione (GSH) (Cheynier & Van Hulst, 1988). When caftaric acid is oxidized to its corresponding quinone by tyrosinase, GSH will quickly react with the quinone forming a colorless product called grape reaction product (GRP; 2-S-glutathionyl caftaric acid), which is no longer a substrate for further oxidation by tyrosinase (Salgues, Cheynier, Gunata, & Wylde, 1986; Singleton & Cilliers, 1995). Therefore, the formation of GRP is believed to limit the must browning and depends on the relative amounts of GSH. Analysis of aged bottled wines shows that GRP is slowly hydrolyzed to the GSH-caffeic acid derivative (the tartrate ester is hydrolyzed) (Cheynier et al., 1986). The specific brown products are not well characterized, but it appears that the hydroxycinnamate quinones react with flavan-3-ols to form colored products (Cheynier et al., 1995, Rigaud, Cheynier, Souquet, & Moutounet, 1991).

SO_2 inhibits tyrosinase (Dubernet & Ribéreau-Gayon, 1973) and prevent the production of GRP, which will maintain a high level of free hydroxycinnamates with high browning potential. Moreover, unlike tyrosinase, laccase will readily oxidize GRP. GRP was shown to be oxidized by laccase from *Botrytis cinerea* to the corresponding *ortho*-quinone with substitution of the latter by glutathione. When no glutathione is available, polymerization of the quinones led to browning of the juice (Salgues et al., 1986). It is accepted that tyrosinase is more sensitive to SO_2, while laccase is more resistant to SO_2 and has a wider substrate oxidation spectrum (Du Toit, Marais, Pretorius, & Du Toit, 2006). In general, not all the enzymatic oxidation occurring in white grape must is detrimental. White must hyperoxygenation decreases the browning potential of wine in two ways: by the tyrosinase disappearance and by the depletion of oxidizable polyphenols during the oxidation reactions. This results in wines with low polyphenol concentrations and high GRP content, which are more stable than those made from non-oxidized juice, in which high polyphenols contents are maintained with a high browning potential (Li et al., 2008). However, during red wine processing the impact of enzymatic oxidation is limited (Cheynier, Fulcrand, & Moutounet, 2000).

4. Non-enzymatic oxidation

During the non-enzymatic oxidation process, also called chemical oxidation of wine, the oxidative processes is favored by the oxidation of polyphenols containing an *ortho*-dihydroxybenzene moiety (a catechol ring) or a 1,2,3-trihydroxybenzene moiety (a galloyl group), such as (+)-catechin/(−)-epicatechin, gallocatechin, gallic acid and its esters, and caffeic acid, which are the most readily oxidized wine constituents (Singleton, 1987; Singleton, 2000; Kilmartin, Zou, & Waterhouse, 2001; Danilewicz, 2003; Li et al., 2008). These substrates are sequentially oxidized to semiquinone radicals and benzoquinones while oxygen is reduced to hydrogen peroxide, and the whole process is mediated by the redox cycle of Fe^{3+}/Fe^{2+} and Cu^{2+}/Cu^{+} (Fig. 4) (Danilewicz, Seccombe, & Whelan, 2008). Further compounds with more isolated phenolic groups such as malvidin, the major colored anthocyanin in red wines, *para*-coumaric acid and resveratrol are oxidized at higher potentials (Kilmartin et al., 2001).

Danilewicz (2003) and then Waterhouse and Laurie (2006) have examined the proposed mechanisms by which oxygen and its intermediate reducing products react with wine constituents, as well the participation of transition metal ions in these reactions. The authors have concluded that oxygen does not react directly with phenolic compounds without the presence of transition metal ions. Moreover, an intervention of iron, copper, and manganese ions in wine oxidation had already been observed (Cacho, Castells, Esteban, Laguna, & Sagristá, 1995).

The quinones formed from the oxidation of polyphenols, as the primary products, are unstable and may undergo further reactions. Quinones can spontaneously combine with nucleophilic compounds (including some phenols, thiols and amines) due to their high electrophilic character. Furthermore, the produced dimers or polymers may rearrange their structure through an enol-like conversion reaction to form new dihydroxybenzene moieties (Li et al., 2008). Moreover, these dimers or polymers in coupled oxidation reactions have lower redox potentials than their initial phenols and are much more easily oxidized (Li et al., 2008; Singleton, 1987). Consequently, it is proposed that oxidation of these products results in an acceleration

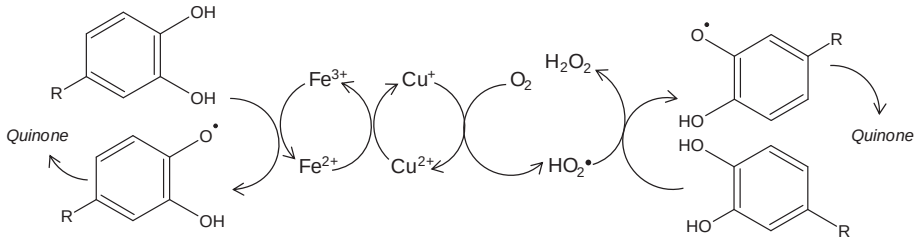


Fig. 4. Proposed catalytic action of iron and copper ions in the oxidation of catechols to produce quinones and hydrogen peroxide (Danilewicz et al., 2008).

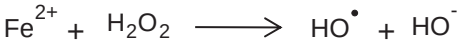


Fig. 5. Fenton reaction.

of the polymerization process (Boulton, Singleton, Bisson, & Kunkee, 2001; Zhai, Du, Guan, Qiao, & Pan, 2001).

Hydrogen peroxide in association with ferrous ions generates hydroxyl radicals ($HO^{\bullet}$), which is known as the Fenton reaction (Fig. 5). Hydroxyl radical is a reduced product of oxygen and it is recognized to oxidize almost any organic molecule found in wine (Waterhouse & Laurie 2006). Moreover, because of its non-selectivity it will react with the first species it encounters, depending on their concentration (Danilewicz, 2003; Danilewicz 2007; Li et al., 2008), such as ethanol, tartaric acid, glycerol, sugars and organic acids (Danilewicz, 2003, Waterhouse & Laurie 2006). Fenton oxidation of ethanol and tartaric acid generates, respectively, acetaldehyde and glyoxylic acid (Danilewicz, 2003; Es-Safi, Fulcrand, Cheynier, & Moutounet, 1999; Li et al., 2008; Singleton, 2000), however, the oxidation of tartaric acid by hydroxyl radicals was described to produce dihydroxyfumaric acid (Danilewicz, 2003). This oxidative degradation pathway was confirmed by Clark (2008), where dihydroxyfumaric acid reacted itself with (+)-catechin in a wine-like solution and the formed yellow pigments were identified as xanthylium cations. Furthermore, the concentration of these pigments increased if the wine-like system also contained 0.6 mg/L of Cu^{2+}. The results clearly demonstrate a link between the production of the yellow xanthylium cation pigments from (+)-catechin and the degradation of tartaric acid by hydroxyl radicals (Clark, 2008). In addition, butane-2,3-diol is oxidized to butan-2-one, 3-hydroxybutan-2-one, and butane-2,3-dione under Fenton conditions (Danilewicz, 2003). The wine α-hydroxyacids are also substrates, L(−)-lactic and L(−)-malic acids being oxidized to pyruvic and 2-oxobutanedioic acid, respectively.

Ortho-dihydroxybenzene rings are oxidized to quinones in a sequential transference of two hydrogen atoms (Danilewicz, 2003). Under normal wine conditions there is a slow rate of phenolic oxidation reactions. On the other hand, electrons are rapidly transfer from phenols to semiquinones at pH 7 (Danilewicz, 2003), and in alkaline conditions wine reacts much more quickly with oxygen (Singleton, Trousdale, & Zaya, 1979).

The reaction rate of phenolic compounds with ROS depends on its ability to form a stable product radical. As seen before, polyphenols containing 1,2-dihydroxybenzene or 1,2,3-trihydroxybenzene moieties are easily oxidized because the resulting phenoxyl semiquinone radical can be stabilized by a second oxygen atom. Moreover, nearly all wine phenolic compounds are very reactive toward the hydroperoxyl radical. Monophenols and their equivalent *meta*-dihydroxybenzene rings and substituted phenols (especially methoxy derivatives) are not as readily oxidized because they do not produce stabilized semiquinone radicals. In the same way, malvidin-3-glucoside, the main anthocyanin present in red wine, is not readily oxidized. Oligomeric and polymeric phenolic compounds (procyanidins and condensed tannins) react similarly with ROS as the monomeric catechol derivatives (Waterhouse & Laurie, 2006).

The presence of phenol radicals in red wine has been supported by electron spin resonance (ESR) spectroscopy studies (Troup, Hutton, Hewitt, & Hunter, 1994). The authors detected stable free radicals in red and white wines (Troup & Hunter, 2002). Recent studies with spin traps detected 1-hydroxylethyl radical that appears to arise from ethanol oxidation via the hydroxyl radical, thus providing the first direct evidence of the Fenton reaction in wine (Elias, Andersen, Skibsted, & Waterhouse, 2009a). Moreover, the addition of iron, copper, or iron and copper in combination to a red wine resulted in a marked increase in spin adducts formation. The addition of catechin to a white wine containing an excess of sulfur dioxide had no effect on the initial rate of radical formation, but was prooxidant in the latter stages of the experiment. Finally, sulfur dioxide was shown to inhibit radical formation in a concentration-dependent manner (Elias, Andersen, Skibsted, & Waterhouse, 2009b). More recently, the metal-catalyzed reduction of H_2O_2, by Fenton reaction, was confirmed to be a key step in non-enzymatic wine oxidation yielding to hydroxyl radicals capable of oxidizing ethanol to acetaldehyde in a model wine system (Elias, Andersen, & Waterhouse, 2010).

5. Other antioxidants in wines: Sulfur dioxide and ascorbic acid

Sulfur dioxide (SO_2) is widely used from pressing to bottling, especially in white wines, in order to protect musts and wines. Antimicrobial and antioxidant activities are the two most important properties of SO_2. It protects wine against browning and regulates the growth of harmful yeast and bacterial in wine. SO_2 have also the ability to participate in addition reactions with carbonyl compounds to form non volatile bisulfite adducts and thus preventing unpleasant sensory properties. Concentrations of added SO_2 to wine generally range from 50 to 200 mg/L. In wine, there is an equilibrium between molecular and ionic forms of sulfur dioxide. At wine pH, 94 to 99% exists in the ionic form as the bisulfite ion HSO_3^- and so only a small proportion is present as free SO_2. Once in wine solution, sulfur dioxide can bind with several molecules such as acetaldehyde, anthocyanins, pyruvic acid, glutaric acid, glucose or phenolic compounds, particularly, caffeic acid and *para*-coumaric acid. There are commonly two fractions of SO_2 in the wine, the "free SO_2", referred to HSO_3^- and SO_2, and the "bound SO_2", indicating sulfur dioxide that is mainly bound to unsaturated compounds. Sulfur dioxide does not react directly with oxygen but with the oxygen reduced form, hydrogen peroxide (Fig. 6). In this way, SO_2 can inhibit aldehydes formation by competing for hydrogen peroxide (Elias et al.,

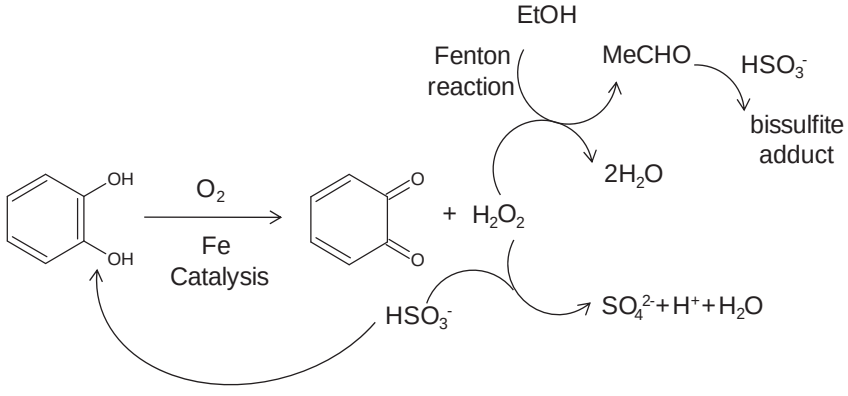


Fig. 6. The interaction of SO_2 with hydrogen peroxide and quinones following catechol oxidation, so preventing oxidation of ethanol by the Fenton reaction.

2010). SO_2 also plays an important role in reducing quinones formed during oxidation process back to their phenol form (Fig. 6) (Danilewicz, 2007; Danilewicz et al., 2008).

In a wine-model system having 4-methylcatechol (4-MeC) in a concentration that simulate the reducing capacity of red wine, no significant SO_2 oxidation is observed without addition of iron and copper ions. Had the catechol been oxidized, hydrogen peroxide would have been generated and reacted with the SO_2. In the presence of both metal ions the rate of SO_2 oxidation is markedly increased and is dependent on the concentration of the catechol. These results demonstrate the crucial importance of metal ions in allowing polyphenol oxidation and that the rate of SO_2 consumption is dependent on the rate of catechol oxidation. Adding iron and copper ions separately, only a modest increase in the rate of catechol oxidation is observed. However, when combined, marked synergism is observed and the rate then became very sensitive to copper concentration. It is proposed that copper, by interacting with oxygen, facilitates redox cycling of iron (Fig. 4). Exposure of red wines to the above conditions produced similar results regarding SO_2 oxidation (Danilewicz, 2007). The oxygen and SO_2 molar reaction ratio is 1:2, which is consistent with one mole equivalent of SO_2 reacting with hydrogen peroxide and a second with the quinone. Moreover, the oxygen/SO_2 molar reaction ratio found in red wines was 1:~1.7, suggesting that some nucleophilic substances may be competing with bisulfite for quinones. The rate of the reaction of oxygen with wine constituents is also accelerated by SO_2 in red wine (Danilewicz et al., 2008).

Ascorbic acid is naturally present in grapes but is usually rapidly consumed after crushing, typically due to either scavenging oxygen or reducing *ortho*-quinone derivatives formed from the enzymatic oxidation of phenolic compounds. The ascorbic acid present in white wine is mostly due to exogenous additions, often just before the bottling, although it may be used at various stages in the wine production process. The levels of ascorbic acid added may vary considerably, but it is typically added at rates ranging from 50 to 150 mg/L (Barril, Clark, Prenzler, Karuso, & Scollary, 2009). Ascorbic acid is added to white wine due to its ability to scavenge molecular oxygen efficiently, but in the process it is initially converted to dehydroascorbic acid and hydrogen peroxide. The dehydroascorbic acid then undergoes rapid degradation into a variety of species including numerous carboxylic acids, ketones, and aldehydes. In fact, ascorbic acid is highly reducing, its reduction potential being estimated as ~210 mV (Ag/AgCl) at pH 3.6 by cyclic voltammetry, much lower than the common polyphenols (Kilmartin et al., 2001). For this reason, and when ascorbic acid is added, it is important to have adequate SO_2 present, to remove hydrogen peroxide and to react with the various carbonyl compounds that result from ascorbic acid oxidation.

Additions of ascorbic acid to *Riesling* and *Chardonnay* wines at bottling proved their effect on wine color, since assessment between two weeks and two years after bottling suggested that wines without addition were browner and had more overall color intensity (Skouroumounis et al., 2005). On the contrary, recent studies have shown the increased production of phenolic pigments in model wine systems from ascorbic acid and (+)-catechin and demonstrated that a degradation product emanating from ascorbic acid was able to react with (+)-catechin and to form colored xanthylium cations (Barril et al., 2009; Barril, Clark, & Scollary, 2008; Bradshaw, Prenzler, & Scollary, 2001; Bradshaw, Prenzler, Scollary, & Cheynier, 2003). Furthermore, the stereochemical influence of ascorbic acid and erythorbic acid on the oxidation processes in a model wine system was studied and xanthylium cation pigments were identified as the major contributor to color development. Also, the production of pigment precursors, previously identified as furanone-substituted flavanols, was confirmed and their corresponding xanthylium cation pigments were lower in the presence of erythorbic acid than ascorbic acid (Clark et al., 2010).

In recent studies alternative options for SO_2 and ascorbic acid have been assessed for the protection of wine aromatic volatiles, during wine storage, including the addition of caffeic acid, gallic acid and glutathione (Lambropoulosa & Roussis, 2007; Lavigne, Pons, & Dubourdieu, 2007; Roussis, Lambropoulos, & Tzimas, 2007; Roussis & Sergianitis, 2008). Results suggested that mixtures of glutathione with caffeic acid or gallic acid protect several aromatic volatiles (esters and terpenes) of white wine with reduced amount of SO_2. Furthermore, cyclic voltammetric studies have demonstrated that glutathione can readily protect wine polyphenols against oxidation by reacting with quinones and reducing them back to polyphenols (Makhotkina & Kilmartin, 2009).

6. Mechanism of color and flavor generation in wines

During aging, aldehydes and mostly acetaldehyde, resulting essentially from ethanol oxidation, are important intermediates in the chemical transformations occurring in red wine, leading to color and flavor changes. One of the first reactions described in red wines was the polymerization reaction between anthocyanins and flavanols (catechins and condensed tannins) mediated by acetaldehyde (Lee, Swinny, & Jones, 2004; Rivas-Gonzalo, Bravo-Haro, & Santos-Buelga, 1995; Timberlake & Bridle, 1976). Acetaldehyde may also cross-link flavanols yielding methylmethine-linked flavanol adducts (Cheynier, 2005; Danilewicz, 2003; Fulcrand, Dueñas, Salas, & Cheynier, 2006). The reaction of acetaldehyde with flavanoids is believed to start with the protonation of acetaldehyde to a carbocation under acidic conditions, followed by a nucleophillic addition of the nucleophilic position C-8 and less likely C-6 of the phloroglucinol ring of the flavanol to the formed carbocation (Fig. 7a, b). After losing a water molecule, the ethanol adduct forms a new carbocation intermediate that is attacked by another flavanol unit (Fig. 7a) or a anthocyanin (Fig. 7b) to yield a methylmethine-linked flavanol adduct (Fig. 7a) or a methylmethine-linked anthocyanin-flavanol adduct (Fig. 7b). The reaction may begin again from the newly formed dimers leading to polymers. Acetaldehyde may also mediate the self-condensation of anthocyanins leading to the formation of oligomeric methylmethine-linked anthocyanins (Atanasova, Fulcrand, Le Guernevé, Cheynier, & Moutounet, 2002; Salas et al., 2005), and also reacts directly with malvidin-3-*O*-glucoside to produce vitisin-B (Bakker & Timberlake, 1997).

The methylmethine-linked flavanol adducts generated with acetaldehyde are not stable and cleave into vinylflavanol oligomers (Fig. 7a (1)) (Es-Safi et al., 1999; Francia-Aricha et al., 1997; Francia-Aricha, Rivas-Gonzalo, & Santos-Buelga, 1998; Fulcrand et al., 2006; Fulcrand, Cheynier, Oszmianski, & Moutounet, 1997). These vinylflavanols can further react with malvidin-3-*O*-glucoside and carboxypyrano-malvidin-3-*O*-glucoside (vitisin A) to produce pyranoanthocyanins-flavanols (orange color) (Cruz et al., 2008) and vinylpyranoanthocyanin-catechins (portisins, blue color) pigments, respectively (Mateus, Silva, Rivas-Gonzalo, Santos-Buelga, & De Freitas, 2003).

Some others aldehydes present is wines such as glyoxilic acid, resulting from the tartaric acid oxidation, furfural and 5-(hydroxymethyl)furfural (5-HMF), which are both sugar degradation products (by caramelization or Maillard reaction) formed during the processing and storage of wine, react directly with flavanols leading to the formation of yellow-orange xanthylium compounds (Es-Safi, Le Guernevé, Cheynier, & Moutounet, 2000). The reaction mechanism resembles an acetaldehyde-induced condensation. However, the colorless dimer would form yellow pigments by a dehydration follow by an oxidation process (Fig. 7a (2)). Furthermore, furfural and methylfurfural have also shown to react directly with malvidin-3-glucoside leading to the formation of anthocyanin-furanic aldehyde adducts (Sousa et al., 2010). The acetaldehyde-mediated condensation was found to occur more generally than with glyoxylic acid, furfural, and 5-HMF (Es-Safi, Cheynier, & Moutounet, 2002).

A range of off-flavors can be formed from wine oxidation. At low concentrations these flavors may add to the complexity of a wine, but as these increase they begin to detract from wine quality. It has been reported that aromatic degradation occurs before chromatic degradation (Escudero, Asensio, Cacho, & Ferreira, 2002; Silva Ferreira, Oliveira, Hogg, & Guedes de Pinho, 2003; Singleton et al., 1979; Singleton & Kramling, 1976), resulting manly from the oxidation of phenolic compounds (Singleton, 1987), which occurs at high redox potentials (E > + 400 mV) (Kilmartin et al., 2001).

Various researchers have tried to reproduce in laboratory the "aroma degradation" associated with oxidative spoilage (Escudero et al., 2002; Escudero, Cacho, & Ferreira, 2000; Silva Ferreira, Guedes de Pinho, Rodrigues, & Hogg, 2002; Silva Ferreira, Oliveira, et al., 2003). The identification of the most important descriptors related to the typical aroma of "oxidative spoiled white wines" was reported as being "honey-like", "farm-feed", "hay", and "woody-like" (Silva Ferreira et al., 2002). Furthermore, it was observed that wines stored at high temperatures and supplemented with high levels of dissolved oxygen suffered a rapid and pronounced oxidative spoilage aroma, which were related with the presence of 3-(methylthio)propionaldehyde (methional), responsible for "boiled-potato" odor notes, phenylacetaldehyde, with "honey-like" odor notes, 3-hydroxy-4,5-dimethyl-2(5 *H*)-furanone (sotolon), with "nutty" and "spicy" odor notes (Silva Ferreira et al., 2002; Silva Ferreira, Hogg, & Guedes de Pinho, 2003), and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), responsible for the kerosene odor in aged *Riesling* (Escudero, Cacho, & Ferreira, 2000; Pisarnitskiĭ, Bezzuboy, & Egorov, 1988; Silva Ferreira, Hogg, & Guedes de Pinho, 2003; Simpson 1980).

In addition to the major chemical browning involving wine phenols, the main oxidation reactions occurring during grape juice heating or storage are caramelization and Maillard reaction (Maillard, 1912), which are temperature dependent.

Caramelization can occur with carbohydrates but requires higher temperatures. In fact, wine caramelization occurs during production of baked sherry and during excessive pasteurization of sweet wines. In the same way, browning overtones derived from caramelization may be present in red and white wines vinified from raisined berries (Zoecklein, Fugelsang, Gump, & Nury, 1995).

Maillard reaction, a reaction involving condensation of reducing sugars with amino acids and proteins, occurs in foods during processing and cooking, even during storage, and proceeds well at 50 °C at a favored pH of 4 to 7. Although the Maillard reaction has been found in beer and other foods, there is little supportive evidence for its occurrence in wine browning (Main, 1992; Zoecklein et al., 1995). However, because wine contains the necessary Maillard reaction substrates such as amino acids, proteins, and reducing sugars, the possibility of its occurrence should not be overlooked (Zoecklein et al., 1995). Recent studies indicate that a great number of

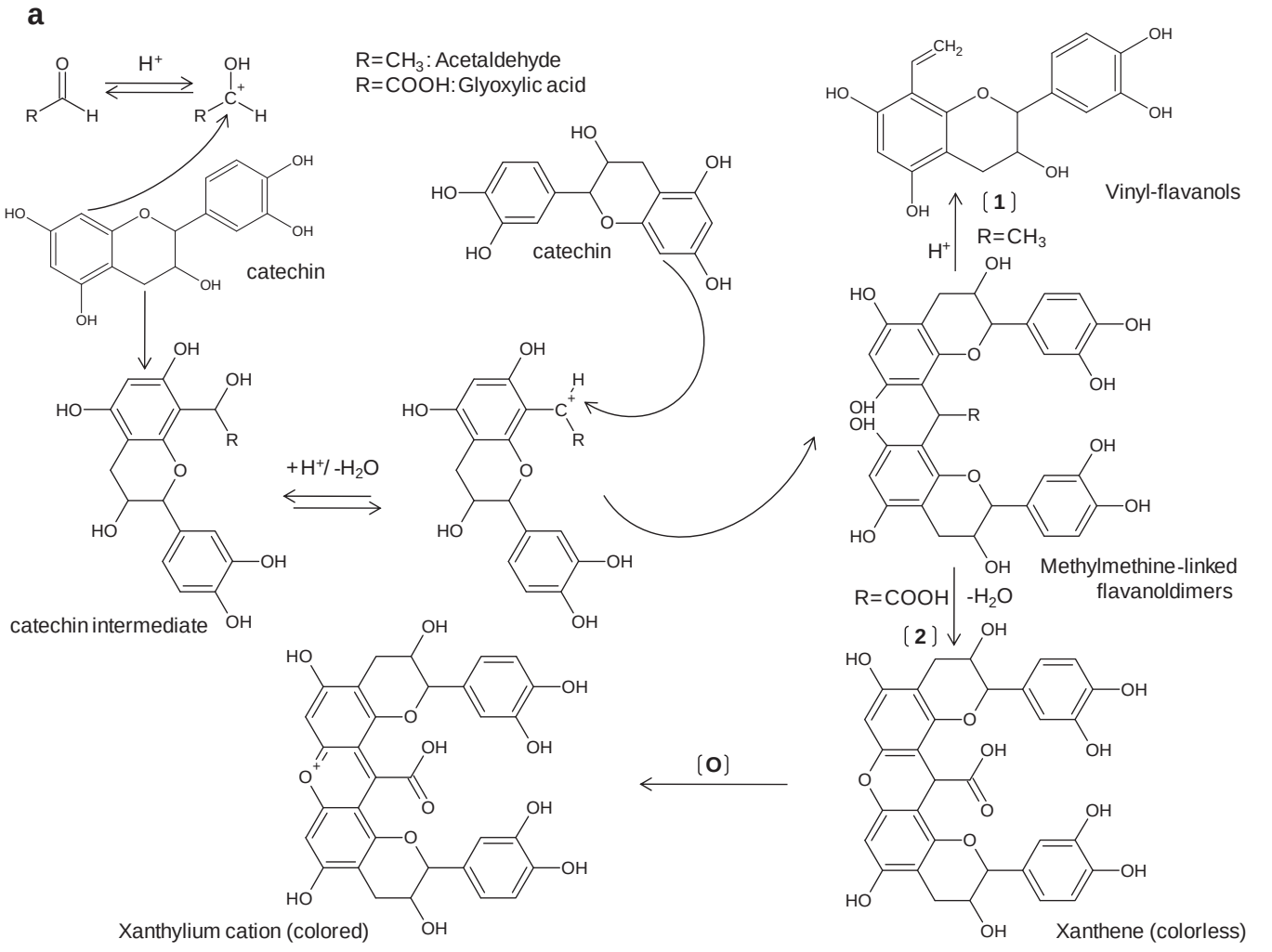


Fig. 7. a. Mechanisms of acetaldehyde- and glyoxylic acid-mediated polymerization of flavanols (Fulcrand et al., 2006). b. Mechanisms of acetaldehyde- and glyoxylic acid-mediated polymerization of flavanols and anthocyanins (Sousa et al., 2010).

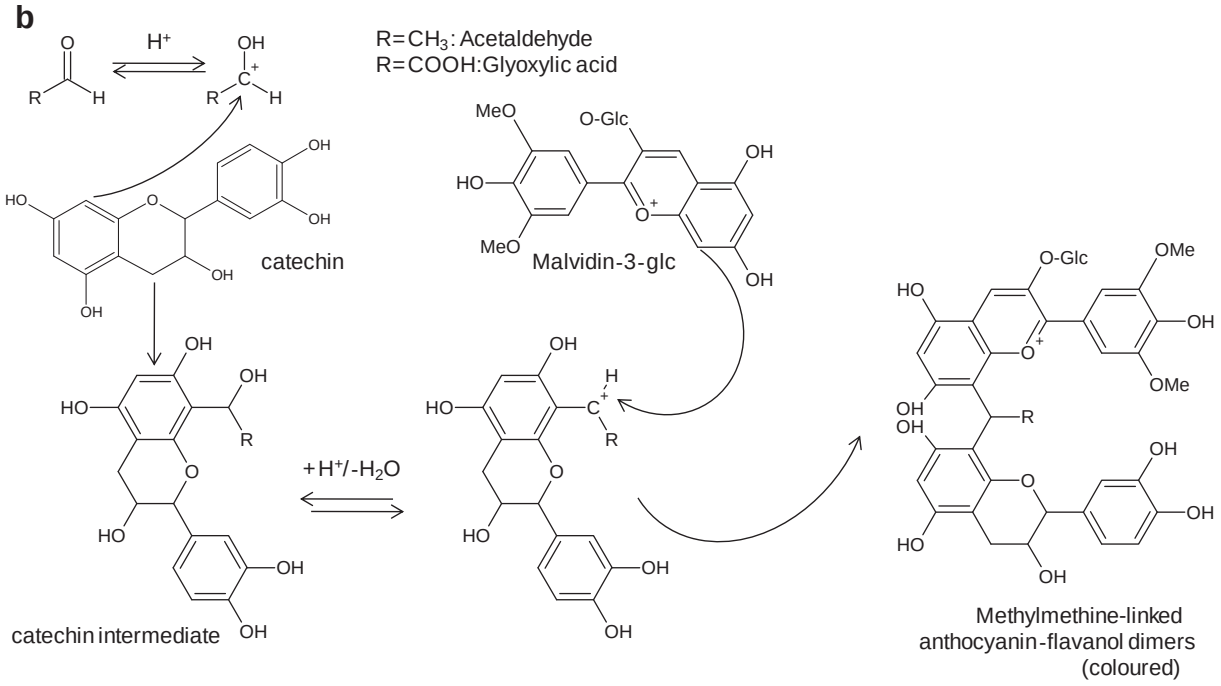


Fig. 7 (*continued*).

volatile compounds responsible for typical aroma or aging aromas of some sweet natural wines seem to be linked with chemical like Maillard reactions between sugars and amino acids (Marchand, De Revel, & Bertrand, 2002; Potman & Van Wijk, 1989; Vernin, 1982).

The Strecker degradation of amino acids is described as a result of the Maillard reaction and involves the interaction of sugar-derived α-dicarbonyl compounds with free amino acids. The amino acid in the presence of α-dicarbonyl compounds is decarboxylated and deaminated, forming an aldehyde with one carbon atom less than the amino acid known as "Strecker aldehyde" (Keim, Revel, Marchand, & Bertrand, 2002; Marchand, De Revel, & Bertrand, 2000; Pripis-Nicolau, Revel, Bertrand, & Maujean, 2000; Shonberg & Moubacher, 1952). Typically, α-dicarbonyls with $n=0$, such as glyoxal, pyruvaldehyde, butane-2,3-dione, and others, are reported as Strecker degradation reagents but, in principle, any dicarbonyl compound with extended conjugation ($n>0$) can be used (Rizzi, 2006). The latter structural category can be extended to include *ortho*-quinones, formed during the oxidation process. In fact, by using ferricyanide ion as oxidant, caffeic acid, chlorogenic acid, (+)-catechin, and (−)-epicatechin will react with methionine and phenylalanine to produce the Strecker aldehydes methional and phenylacetaldehyde, in pH 7.17 phosphate buffer at 20 °C (Rizzi, 2006).

Along with the Strecker degradation of the respective amino acid (Escudero, Cacho, & Ferreira, 2000; Escudero, Hernandez-Orte, Cacho, & Ferreira, 2000; Silva Ferreira et al., 2002), methional and phenylacetaldehyde can also be formed by the direct oxidation of the respective alcohol, but the main pathway for their formation is not clearly established. Some researchers suggest that the Strecker mechanism is the main pathway for the formation of methional (Silva Ferreira et al., 2002). Nevertheless, due to the temperature, pH, and dissolved oxygen regimes related to the storage conditions of wines, the question remains unanswered. Between the amino acid and the Strecker aldehyde some authors suggest that an intermediary ketoacid is involved (Silva Ferreira, 1998). Other mechanisms, like esterification, can also contribute to amino acid consumption in wine, and the respective products could also participate in wine aroma (Herraiz, Huang, & Ough, 1993; Herraiz & Ough, 1992; Herraiz & Ough, 1993).

Oxidative reactions occurring during the aging process of Port wine stored in barrels increased the contents of aldehydes and methyl ketones (Silva Ferreira & Bertrand, 1996). Among these substances, acetaldehyde was the major aliphatic aldehyde in wine and the one that presented the most significant increasing trend with time of barrel storage.

7. Methods to monitor, classify and diagnose wine oxidation

Antioxidant activity in biological systems and foods can be assessed by methods based on chemical reactions or on the chemical-physical properties of antioxidants.

Methods involving potentiometric titrations were used to measure the resistance to oxidation of white wines under normal and forced aging conditions. In this method an oxidation titration was preceded by a pre-reduction step in order to assure the visualization of the end-point indicator of the oxidation titration in all wines. The titrants chosen were trichlorotitanium (TCT) as reductant and dichlorophenolindophenol (PIP) as oxidant. The pH of analyzed wines decreased during reduction titration (due to the presence of HCl in the TCT solution) reaching final values close to $pH=1.5$. Redox species, present in white wine, will be oxidized by the dichlorophenolindophenol in the potential range from −400 to +400 mV. Considering the formal potential of PIP (close to 400 mV) it will selectively pick out the most powerful reducing agents of the wine, which includes ascorbic acid, polyphenols with a trihydroxybenzene moiety on the flavonoid B-ring, and to some extent SO_2 and catechol-containing polyphenols (Oliveira, Silva Ferreira, Pinho, & Hogg, 2002; Silva Ferreira, Oliveira, et al., 2003a).

Recently, studies using cyclic voltammetric measurements enables the grouping of both quantitative and qualitative information concerning antioxidants (Kilmartin et al., 2001; Roginsky et al., 2006; Rodrigues, Silva Ferreira, Guedes de Pinho, Bento, & Geraldo, 2007; Martins et al., 2008; Makhotkina & Kilmartin, 2009). Wines originated from a forced aging degradation along with wines of different ages were monitored by cyclic voltammetry and the consumption rates of oxidizable species were estimated (Rodrigues et al., 2007). Moreover, the influence of sulfur dioxide, glutathione

and ascorbic acid on the cyclic voltammograms of four representative wine polyphenols (catechin, caffeic acid, rutin and quercetin), in a model wine solution, along with five different wines was investigated using a glassy carbon electrode (Makhotkina & Kilmartin, 2009). Results have showed that sulfur dioxide increased the anodic current and decreased the cathodic current for all four polyphenols and all wines, pointing to a rapid interaction of SO_2 with the oxidized polyphenol quinones. A similar trend was seen for glutathione, except in the case of quercetin, where the addition of glutathione led to the formation of a second set of voltammetric peaks, corresponding to the redox activity of a glutathione derivative (Makhotkina & Kilmartin, 2009). In addition, voltammogram fingerprinting was already used for monitoring oxidation management (Martins et al., 2008). In this study, a supervised multivariate control chart was developed using a control sample as reference, and when white wines are plotted onto the chart, it is possible to monitor the oxidation status and to diagnose the effects of oxygen regimes and antioxidant activity (Martins et al., 2008).

A simple method based on a single-line FIA (Flow Injection Analysis) system with amperometric detection, at a potential of +0.4 V (Ag/AgCl reference electrode) and wine pH, has been developed to evaluate the antioxidant capacity of red and white wines (Mannino, Brenna, Buratti, & Cosio, 1998). In this method, the antioxidant capacity of white wines was well correlated with total phenols content obtained by the traditional Folin–Ciocalteu assay (FCR). The FCR based assay gained popularity and is commonly known as the total phenols (or phenolic) assay. However, no such good correlation exists for red wines due to the fact that some red wines showed a high antioxidant capacity even though they have low concentrations of total phenols.

Another single-channel FIA system with amperometric detection using a glassy carbon disk electrode has been introduced and used in wines (Blasco, Rogerio, Gonzalez, & Escarpa, 2005). Basically, the electrochemical protocol consisted in the measurement of the amperometric current at neutral pH (7.5) and at different oxidation potentials. Since the selectivity increases after a decrease of the oxidation potential, different degrees in the total polyphenolic fractions could be attained. In this way, the total polyphenolics measured under no selective oxidation conditions (+0.8 V versus Ag/AgCl reference electrode) represent the "electrochemical index (EI)".

The most common methods used to determine the antioxidant capacities of wines, based on chemical reactions, are the electron transfer (ET) based assays. These methods involve one redox reaction with the oxidant as an indicator of the reaction endpoint (Fernández-Pachón, Villaño, García-Parrilla, & Troncoso, 2004; Lachman, Šulc, & Schilla, 2007; Oliveira, Silva Ferreira, Pinho, & Silva, 2008). Due to its operational simplicity, the *Trolox Equivalence Antioxidant Capacity* (TEAC) assay has been used in many research laboratories for studying wine antioxidant capacity. In the same way, DPPH (diphenyl-1-picrylhydrazyl) assay involves a stable and commercially available organic nitrogen radical and provides a technically simple assay for evaluation the wine antioxidant capacity (Fernández-Pachón et al., 2004; Lachman et al., 2007; Oliveira et al., 2008). Other methods are also used, namely, *Ferric Ion Reducing Antioxidant Power* (FRAP), "*Total Antioxidant Potential*" assay, using a Cu^{2+} complex as an oxidant, and Folin Ciocalteu Reagent (FCR).

The total antioxidant capacity (TAC) of five different wines (four red and one white) was determined in five different steps of winemaking carried out in a commercial wine cellar by a chemiluminescence (CL) assay. The CL method is suitable to determine the antioxidant capacity of beverages, and preliminary trials showed that the TAC immediately after the bottle was opened was greater than the day after (about 25% decrease). The wines were characterized by different levels of total phenolic compounds and TAC, and the differences were related to grape composition and winemaking technologies (Girotti et al., 2006).

Finally, the level of wine oxidation can be followed by measuring the formation of primary or secondary oxidation products. With ESR it is possible to study the radicals that are involved in the oxidative reactions directly, which makes it possible to determine the level of oxidation more directly during forced aging experiments (Elias et al., 2009a; Elias et al., 2009b; Elias et al., 2010).

8. Conclusions

Although, phenolics are recognized to be related with health benefits by limiting lipid oxidation, in wine they are the primary substrates for oxidation participating in both enzymatic and non-enzymatic browning resulting in the by-products quinones. In this way, oxygen in must and wine is progressively depleted, and it becomes unavailable to oxidize other wine compounds. Conversely, phenolics oxidation generates hydrogen peroxide, a potent oxidant that undergoes further oxidation reactions. In addition, phenolics have a number of important functions in wine, affecting the tastes of bitterness and astringency.

Sulfur dioxide and ascorbic acid, when added to wine, are able to reduce the quinones, while metal ions will catalyze the oxidation reactions. Nevertheless, when ascorbic acid is added, it is important to have adequate SO2 present, to remove hydrogen peroxide result from ascorbic acid oxidation as well the various carbonyl compounds formed.

During aging, aldehydes are important intermediates in the chemical transformations occurring in wines, leading to color and flavor changes. In the same way, a range of off-flavors can be formed from wine oxidation. At low concentrations these flavors may add to the complexity of a wine, but as these increase they begin to detract from wine quality.

In addition to the major chemical browning involving wine phenols, the main oxidation reactions occurring during grape juice heating or storage are caramelization and Maillard reaction, which are temperature dependent, nevertheless, due to the temperature, pH, and dissolved oxygen regimes related to the storage conditions of wines, the question remains unanswered.

In conclusion, the complexity of the mechanisms implicated in wine oxidation is not fully understood and the identification of all mediators' reactions and its characterization needs to be done.

Acknowledgments

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