Universidad de Concepción

Dirección de Postgrado

Facultad de Farmacia - Programa de Doctorado en Ciencias y Tecnología Analítica

Systematic study for the hydroxyl radical production in white wines as a function of the chemical composition

Thesis to qualify for Doctor Degree in Sciences and Analytical Technology

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CONCEPCIÓN-CHILE

2018

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Dedicated to my beloved family.

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Resumen

La exposición del vino blanco al oxígeno puede causar efectos perjudiciales tales como la pérdida de características sensoriales, formación de olores desagradables y alteración del color. Nuevos antecedentes, sobre la oxidación del vino, establecen la importancia de la formación de complejos metálicos con compuestos con hidroxilos adyacentes. Estos complejos podrían reducir Fe(III), promoviendo la formación de radicales a través de la reacción de Fenton. Se ha propuesto un esquema que explica las vías de la oxidación química de los vinos blancos a través de la reacción de Fenton, donde la principal especie oxidante de esta reacción es el radical hidroxilo (•OH). Este radical tiene un alto poder oxidante y podría oxidar los principales componentes del vino.

La formación de •OH, inducida por un corto burbujeo de aire, se determinó en 18 vinos blancos analizados por resonancia paramagnética electrónica (EPR) con *Spin Trapping*. La variación en la producción de •OH se relacionó con la composición fenólica de los vinos. Del análisis de las relaciones lineales, se observó que la concentración de •OH estaba relacionada linealmente con 5 compuestos fenólicos (ácido cafeico, ácido protocatecúico, ácido p-cumárico, ácido gentísico y ácido siríngico). Por lo tanto, en este estudio se estableció la relación entre ciertos compuestos fenólicos y la inducción y amplificación de la producción de •OH.

Aunque la oxidación química de los vinos ha sido ampliamente estudiada, principalmente en vinos modelo. Hasta esta tesis no había un estudio sistemático que relacione la composición de los vinos con la producción de •OH en vinos blancos reales. En esta tesis se generó un modelo de regresión multivariante, utilizando el algoritmo *Partial Least Square* (PLS), y se establecieron correlaciones lineales entre la composición del vino y la producción de •OH. De estos resultados, las variables más importantes se clasificaron como pro-oxidantes (ácido málico, ácido p-cumárico, ácido cafeico, prodelfinidina, procianidinas B1, Mn, Cu y Zn) y antioxidantes (cis-piceido, ácido elágico, ácido gálico, proantocianidinas, glucosa y concentración de protones). Cabe destacar que, a la diferencia de las demás variables antioxidantes, el aumento en la concentración de glucosa y protones, hacen menos eficiente el proceso oxidativo, vale decir que su efecto es indirecto. Finalmente, se discuten las vías en las que los compuestos más importantes del vino participan en la producción de •OH.

Abstract

Exposure of white wine to oxygen can cause detrimental effects such as loss of sensory characteristics, the formation of unpleasant odors and alteration of colour. New antecedents, on the oxidation of wine, establish the importance of the formation of metallic complexes with compounds with adjacent hydroxyls. These complexes could reduce Fe(III), promoting the formation of radicals through the Fenton reaction. A scheme has been proposed that explain the pathways of the chemical oxidation of white wines through the Fenton reaction. Wherein the main oxidant species of this reaction is hydroxyl radical (•OH). This radical has a high oxidizing power and could oxidize the main components of the wine.

The formation of •OH, induced by a short air-bubbling, was studied in 18 white wines analyzed by Electron Paramagnetic Resonance (EPR) with Spin Trapping. The variation in the •OH production was related to the phenolic composition of wines. From the results of linear relationships, was observed that the •OH concentration was linearly related to 5 phenolic compounds (caffeic acid, protocatechuic acid, p-coumaric acid, gentisic acid and syringic acid). Therefore, in this study the relationship between certain phenolic compounds and the induction and amplification of the •OH production was established.

Although the chemical oxidation of wines has been widely studied, mainly in model wines. Until this thesis there was no systematic study that relate the composition of wines with •OH production in real white wines. In this thesis a multivariate regression model was generated, using the Partial Least Square (PLS) algorithm, and linear correlations between wine composition and •OH production were established. From these results, the most important variables were classified as pro-oxidant (malic acid, *p*-coumaric acid, caffeic acid, prodelphinidins, procyanidins B1, Mn, Cu, and Zn), and antioxidant (*cis*-piceid, ellagic acid, gallic acid, proanthocyanidins, glucose, and proton concentration). It should be noted that, unlike the other antioxidant variables, the increase in the concentration of glucose and protons makes the oxidative process less efficient, that is, its effect is indirect. Finally, the pathways in which most important wine compounds participate in the •OH production are discussed.

Preface

Wine is a beverage obtained from the grape by the total or partial alcoholic fermentation of its juice or must. The fermentation is produced by the metabolic action of yeasts that transform the sugars of the fruit into ethanol and gaseous carbon dioxide. In addition, winemaking depends on a set of environmental factors such as climate, during grape production and process conditions.

Some wine components are easily oxidisable by oxygen. To avoid it, certain compounds are added which act as antioxidants (SO_2, ascorbic acid, glutathione, etc.). These additives partially stop the wine oxidation. The wine oxidation is complex processes that determined by several variables that depend in turn on the chemical composition of the wines and the conditions during wine storage and ageing.

In literature, several papers have been published about wine oxidation in function of several factors. In this thesis, it is postulated that the oxidation of white wine depends on the amount and type of pro-oxidant compounds present in this. This is how wines with different chemical composition will generate different $^{\bullet}OH$ concentrations and therefore this will promote or reduce the oxidation of wine by different pathways.

In the first chapter, the study of the state of the art of chemical oxidation of wines is presented. Then, in chapter 2, the research of 18 commercial white wines with respect to the $^{\bullet}OH$ production induced by a short air bubbling is discussed. The concentration of this radical ranged significantly depending on the type of wine used. In this research, linear relationships between five phenolic compounds and $^{\bullet}OH$ production was established and it was postulated as a route of chemical oxidation to the Fenton reaction. In chapter 3 is discussed deeply in a systematic study of the hydroxyl radical production in white wines as a function of their chemical composition. For this, a multivariate analysis and linear correlations with the $^{\bullet}OH$ production were performed. From the obtained results, the variables that contribute most to the oxidative process were obtained. Chapter 5 includes the final conclusions of this thesis. Also, were included chapter 6 with complementary research about Fenton reaction driven by phenolic compounds with adjacent hydroxyls. These investigations complement the knowledge about wine oxidation and are the basis for understanding Fenton reactions in model systems.

CHAPTER 1: State of the art

1.1 The wine

The wine is defined as "exclusively, the drink resulting from the alcoholic fermentation, complete or partial, of fresh grapes, crushed or not, or of grape must" (defined by International Organisation of Vine and Wine, OIV). The wine-growing sector is one of the most important activities of world agriculture, because of the economic value it generates and the role it plays in the conservation of the environment. According to the latest data presented by the OIV (2017), the world wine area would be over 7.6 million hectares. 35% of this area is concentrated between Spain, France, and Italy, which are essentially wine-growing countries. From 2000 to date, this area has been increasing for Chile and is currently in the fourth position as a wines exporting country (in volume). 27.2% of Chilean production correspond to white wine (15.3% Sauvignon Blanc, 9.2% Chardonnay, and 2.7% Muscat of Alexandria).

The winemaking is the set of processes that carry on juice extracted from the grape to become an alcoholic beverage called wine, it begins with destemming and crushing, pressing, maceration, racking, fermentation, decanting/clarification, filtration, and bottling. Each type of wine (red, white, rosé, sweet, sparkling, etc.) has a different elaboration process, which in turn can be changed according to the vineyard or region in which it is produced.

1.2 Chemical composition of the wines

The composition of the wine changes over time thus is considered as a "living" substance. Its chemical composition is very broad and its analysis very complex (Redondo & Aranda, 2013) and its compounds have different origins: some come directly from the grapes while others are developed as a consequence of the biological and chemical processes that are carried out to produce the wine (Bejerano & Zapater, 2013). The wine contains mostly: 86 %water, 0.5-1.0 % organic acids (tartaric, malic, citric, succinic, lactic, acetic, butyric, formic, propionic, and shikimic), 12-14 % alcohol (ethanol 12 %, glycerol 2 %), 0.3% carbohydrates (cellulose, and sugars), 0.2 minerals, 0.004% aldehydes, 0.025% esters, 0.025 % nitrogenous matter (amino acids, proteins), traces of vitamins and 0.4% phenolic compounds (Conradie et al., 2014). Also, wine contains many phenolic compounds that have a number of

important functions in wine. First, affect the tastes of bitterness and astringency. Second, the color of red wine is caused by phenolic compounds (Lago-Vanzela et al., 2014). Third, the phenolic compounds are a key wine preservative and the basis of long aging. Lastly, since certain phenolic compounds oxidize readily, they are a key component in the oxidation (Waterhouse, 2002).

1.2.1 Phenolic compounds in wines

Phenolic compounds in wine are usually classified into two groups: flavonoids and non-flavonoids (Figure 1). Flavonoids are present in the skins, seeds, and stalks, instead, non-flavonoids are present essentially in the pulp (Tsao, 2010). Flavonoids are subdivided, depending on the degree of oxidation of the central ring, into six groups (Spencer et al., 2008).

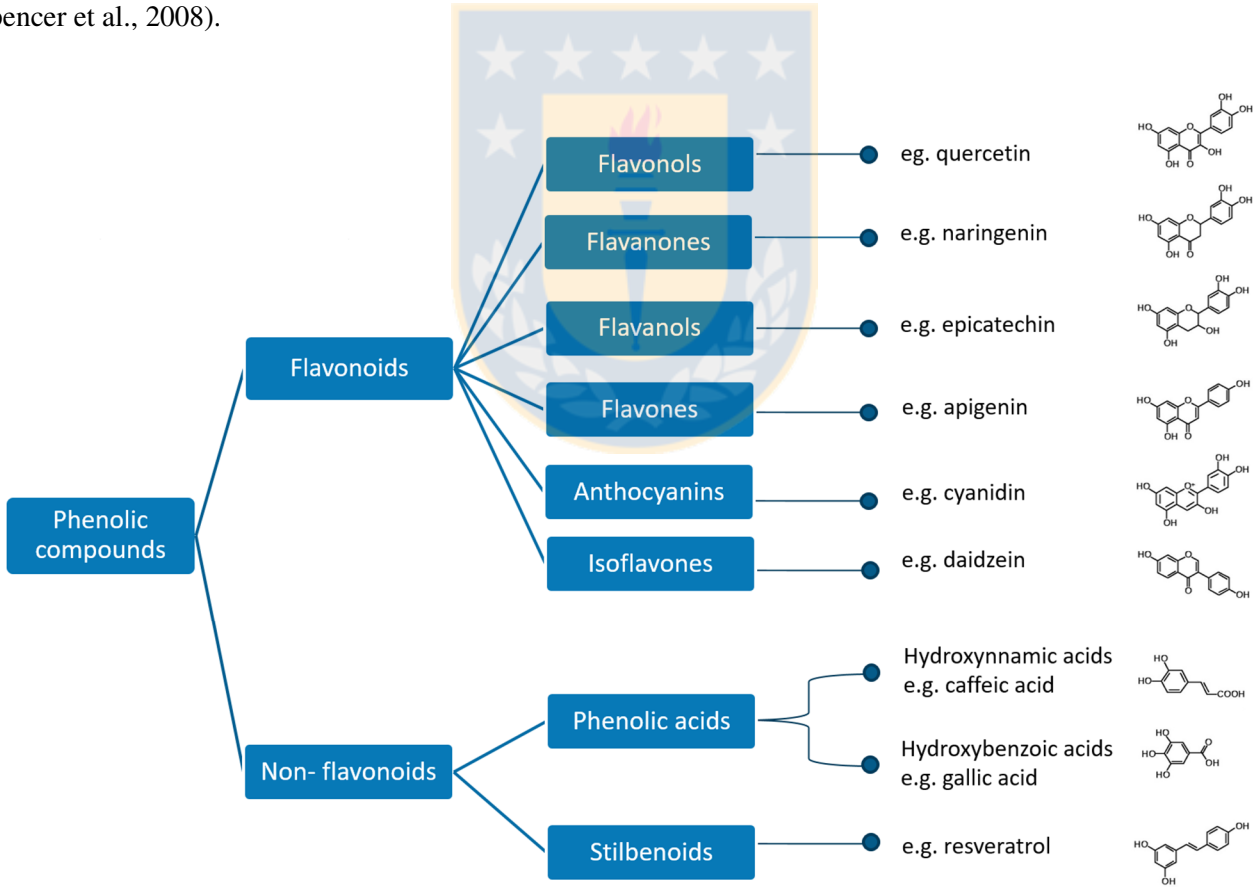

Figure 1: Classification and chemical structure of the main types of phenolic compounds (Source: elaborated by the author).

Flavonols are found in the skin and their color varies from white to yellow. These compounds contribute to the color of white wines and intensify the color of red wines by the formation of complexes with anthocyanins and its content in red wines is 20 more than in white wines (Makris et al., 2006). Flavanones are generally present in grapes and are associated with free radical scavenging (Panche et al., 2016). Flavanols come mainly from seeds and skins and, only a part is transferred to the must during winemaking. These compounds are found in greater amount in red wines than in white wines (Gonzalez-Paramas et al., 2004). Anthocyanins are found in a high concentration in red wines (200 to 500 mg/L), being these compounds responsible for red colour (Kennedy, 2008). Flavanones are present in grapes and have been associated with free radical scavenging. These compounds are in greater concentration in red wines than in white wines (Thompson et al., 2006). Moreover, non-flavonoids are subdivided into phenolic acids and stilbenes. Phenolic acids are which mostly concentrated in the pulp and are classified according to the number of carbon atoms in the aliphatic chain. Then, the compounds of type C6-C1 are hydroxybenzoic acid and compounds of type C6-C3 constitute the hydroxycinnamic acids (Pereira et al., 2009). On the other hand, between the stilbenes, resveratrol stands out. These compounds are found in greater concentration in the skins of the grapes, due to the greater contact of the skin in the maceration stage of red wine (McMurtrey, 1997). In conclusion, the phenolic composition depending on the type of wine (red, white, rosé, etc.) and the most noticeable difference is the color, which depends on grape variety and the winemaking (Girotti et al., 2006).

1.3 The winemaking process

1.3.1 Production of white wine

The fundamentals of winemaking have been maintained over time, although have been improved the environmental impact of the activity, capacity to maintain a sterile environment, improvements in equipment and control of the production process. These improvements contribute to a product of better quality (Conradie et al., 2014). The common stages of the production of white and red wines are shown in Figure 2.

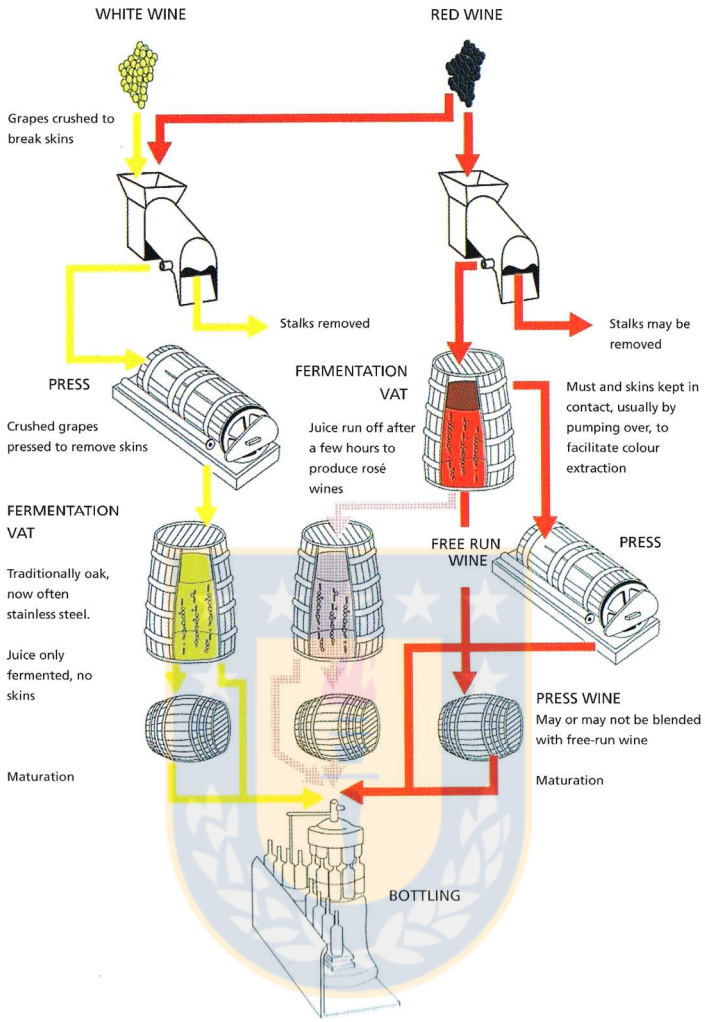

Figure 2: Main stages of the process of winemaking process (Source: elaborated by the author).

As discussed above, phenolic compounds determine the color composition in wine. In the case of red wines, these are subjected to a stage of maceration of the solid parts of the grape to allow the transfer of the phenolic compounds to the wine (Kocabey et al., 2016). Instead in white wines, there is no maceration. In their place, a mechanical pressing of the grapes is carried out. The absence of maceration and incorporation of solid material in the fermentation stage, avoid the transfer of high amounts of phenolic compounds to the white wine.

The antioxidant capacity of several phenolic compounds has been widely reported (Cassino et al., 2016; Dalvi et al., 2017; Gonzalez-Paramas et al., 2004). Due to the differences in the elaboration of white and red wines, white wines contain a lower concentration of antioxidant compounds which, makes them more susceptible to oxidation (Kocabey et al., 2016).

1.4 Oxygen effects in wines

Positive effects can be generated if the oxygen contributions to the wine are controlled, such as lower intensity of vegetable and reductive odors, improvement in varietal fruity characters, decrease in astringency and bitterness (Gómez-Plaza E., Cano-Lopez, 2011). Conversely, excessive amounts of oxygen can cause damaging effects on the wine, such as loss of sensory characteristics, the formation of unpleasant odors and alteration of color (Oliveira et al., 2011; Silva Ferreira et al., 2002). Many red wines benefit to a certain degree from oxidation, although white wines are often damaged by exposure to oxygen (Danilewicz, 2003). The oxidation of white wines is a constant problem for winemakers. Figure 3 summarizes the positive and negative effects of wine exposure to oxygen.

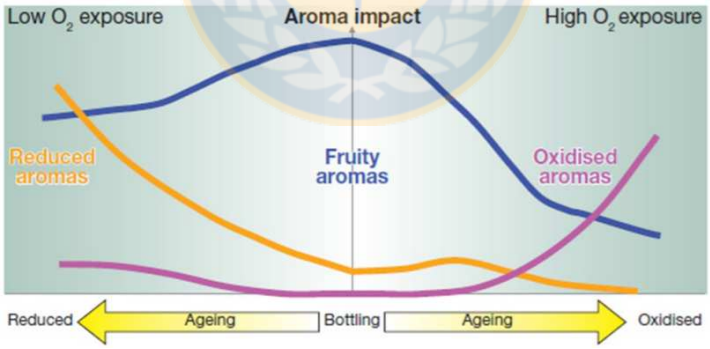

Figure 3: The effect of oxygen exposure on the aroma of bottled wine (cited from (Coetzee & Du Toit, 2015)).

In general, it is accepted that certain grape varieties are especially sensitive to oxidation, suggesting that some of the key chemical components to their sensory attributes are strongly modulated by exposure to oxygen (Bueno et al., 2016; Coetzee & Du Toit, 2015)

1.5 Chemical oxidation of the white wines

Chemical (or non-enzymatic) oxidation of wine requires oxygen and an oxidisable substrate and occurs in the musts such as in the wine. Chemical oxidation is the most frequent, although it occurs at a lower speed than enzymatic oxidation. It is important to consider that oxidative deterioration not only depends on the amount of oxygen, also depending on the chemical composition of the wine (Oliveira et al., 2011). During the process of chemical oxidation, it is favored the oxidation of phenolic compounds that contain units of o-dihydroxybenzene (catechol), such as catechin, epicatechin, gallocatechin, caffeic acid, etc., which are the most easily oxidisable constituents in wine (Danilewicz, 2003; Li et al., 2008). These substrates are sequentially oxidised to their respective, semiquinones and benzoquinones, while oxygen is reduced to hydrogen peroxide. The process is mediated by the redox couple of Fe(II)/Fe(III) (Danilewicz, 2016a; Elias & Waterhouse, 2010).

1.6 Factors involved in the oxidation of wines

1.6.1 Metals

Metal ions have an important place in oenological practice. Some are necessary for the fermentation. Metals also influence organoleptic, toxicological, and nutritional properties of wines (Zoecklein, 2012). Certain metals such as Fe, Cu, and Mn participate in the destabilization of wine and its oxidative evolution (McKinnon & Scollary, 1997). Cu, Fe, Al, Zn, and Ni contribute to the formation of turbidity and undesirable changes of aroma and flavour. All metal ions are naturally present in the wine at a non-toxic level. Some are found in a greater concentration due to agricultural treatments in the vineyards (Pietrzak & McPhail, 2004; Thangaraj et al., 2017).

In recent years, the participation of certain metals in the oxidative process of wines has been reported. These publications postulate the importance of transition metals in the formation of several types of radical species and the consequent aging of the wines (Danilewicz, 2016a; Kreitman et al., 2013).

In chemical oxidation, oxygen does not react directly with phenolic compounds. The limitation of triplet oxygen reactivity is overcome by the addition of an electron, which can be provided by reduced transition metal ions, mainly

Fe(II), Cu(I) and Mn(II) (Cacho et al., 1995; Danilewicz, 2016; Kreitman et al., 2013). Sequential electronic transfer allows the formation of hydroperoxide radical (•OOH), hydrogen peroxide (H_2O_2) and •OH (Figure 4).

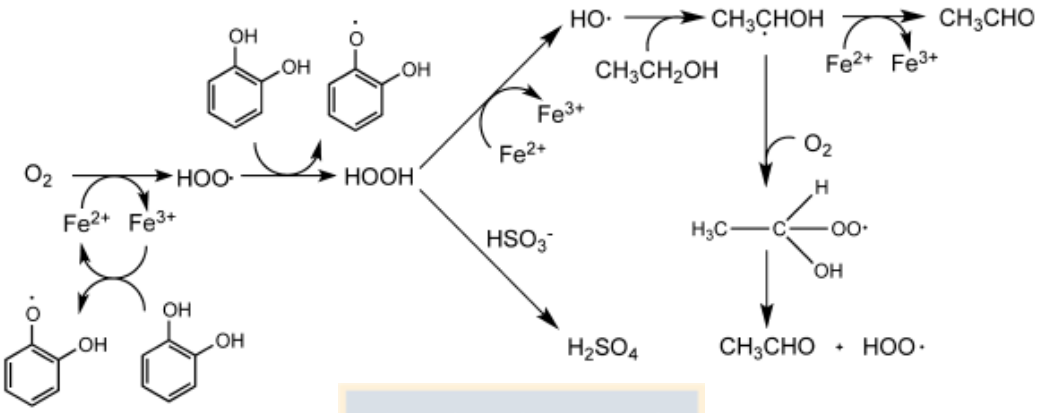

Figure 4: Proposed scheme for the chemical oxidation of wine, catalyzed through a transition metal, reduction of oxygen and the subsequent oxidation of ethanol (cited from (Kreitman et al., 2013)).

The •OH formed is highly oxidant and reacts with the major components of the wine. The oxidation products are aldehydes and quinones. Quinones have a high electrophilic reactivity and lower redox potential than allow their reaction with nucleophiles (phenols, thiols, and amines), resulting in dimers and polymers (Nikolantonaki & Waterhouse, 2012).

1.6.2 Factors of the Fenton reaction

Several authors have been proposed as a source of •OH to the Fenton reaction (Contreras et al., 2006; Melin et al., 2015; Salgado et al., 2013). The most known Fenton reaction is carried out with Fe(II) (Fenton, 1894). This reaction is described in (1) (Chen & Pignatello, 1997).

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\bullet}OH + {}^{-}OH \qquad k = 76\ M^{-1}s^{-1} \qquad (1)$$

In this reaction, the H_2O_2 is reduced to hydroxyl ion (^-OH) and $^•OH$ by a transition metal. The optimal pH for this reaction is close to 3.0. This due to that at high pH values Fe(III) precipitates as $Fe(OH)_3$ (pKps = 38.8), displacing the equilibrium of the redox couple Fe(II)/Fe(III). At lower pH (less than 3), the formation of complex $Fe(H_2O)_6^{3+}$ is favoured. In this complex, the exchange of one molecule of H_2O by one molecule of H_2O_2 is not favored to form the respective peroxo-complex. The most reactive species for the exchange of H_2O by H_2O_2 is $[Fe\ (H_2O)_5(OH)^{2+}$. The optimum pH for the formation of this complex is 3.0 (Salgado et al., 2013). When the decomposition of H_2O_2 is catalyzed by a transition metal other than Fe(II), this reaction is called Fenton-like. Thus, when the metal is Fe(III) the reaction is described (2) (Chen & Pignatello, 1997):

$$Fe(III) + H_2O_2 \rightarrow Fe(II) + {}^•OOH + H^+ \quad k = 0.01\ M^{-1}s^{-1} \qquad (2)$$

The $^•OOH$ generated has lower oxidant power than other reactive oxygen species (1.44-1.65 V/SHE) (Brillas et al., 2009). The Fenton-like reaction (2) is two orders of magnitude slower than the Fenton reaction (1). However, the Fenton-like can induce the Fenton reaction, since the $^•OOH$ generated can react with Fe(III) reducing it to Fe(II) and leaving it available to react with H_2O_2 (3) (Rothschild & Allen, 1958).

$$Fe(III) + {}^•OOH \rightarrow Fe(II) + O_2 + H^+ \qquad (3)$$

The Fenton-like reaction can be carried out with Fe, Cu, Co, Mn, and Zn. All these metals can be present in wines (Cacho et al., 1995; Kreitman et al., 2013; Pham et al., 2013). Moreover, the reactivity of an oxidant system based on the Fenton reaction is strongly dependent on metal speciation, which in turn depends on the pH (Melin, et al., 2015; Salgado et al., 2018).

1.6.3 Organic compounds with adjacent hydroxyls

Organic acids

Organic acids with adjacent hydroxyls (tartaric, malic, citric and succinic acids) can form complexes with Fe(III), being able to modify the redox properties of these systems (Gomathl, 2000; Vukosav et al., 2010). The formation

of iron complexes with tartaric, malic, and citric acids has been widely reported. The formation of these complexes will depend on the dissociation constant and stability constant of the complexes formed.

Phenolic compounds

Certain phenolic compounds act as a ligand, forming a complex with Fe. Some ligands act as an antioxidant by sequestering the Fe necessary for the Fenton reaction (Zaman et al., 1999; Zheng et al., 2005). Other ligands increase the reactivity of the Fenton reaction by solubilisation of iron (Keenan & Sedlak, 2008). Also, there are ligands that chelate Fe(III) and reduce to Fe(II), by driving and amplifying the Fenton reaction. Among these, the 1,2-dihydroxybenzenes (1,2-DHBs) are highlighted (Contreras et al., 2011; Salgado et al., 2013, 2018).

Therefore, the speciation of Fe is very important to define the reactivity of the Fenton reaction. In consequence, some authors have postulated that the coordination number of organic ligands are mainly dependent on pH and its concentration (Hider et al., 2001; Zhou & Elias, 2013). Sun and Pignatello proposed that ligands can modify the reactivity of Fenton systems in three ways: (i) modification of the redox properties of the metal, (ii) creating a labile coordination site that can be occupied by H_2O_2 and (iii) competition with the substrate for the oxidizing compounds (Yunfu Sun & Pignatello, 1992).

According to pathway (i), it has been reported that for pH values between 6 and 12, certain flavonols can completely chelate iron, acting as antioxidants (Gülçin, 2008; Kumamoto et al., 2001). At lower pH (between 5.5 and 7.4), the chelating properties of certain flavonoids with Fe and Cu have been reported. All flavonoids studied have great capacity to reduce Cu over Fe (Mira et al., 2002).

According to pathway (ii), Fe(III) complexes can have several labile positions for H_2O_2 to enter and if the complex formed is redox-active it would reduce Fe(III) (Graf et al., 1984; Salgado et al., 2018, 2013). Other researchers reported the reaction of Fenton promoted by catecholamines. Catecholamines can form mono, bis and tris-complexes with iron depending on the pH (Figure 5) (Melin et al., 2015).

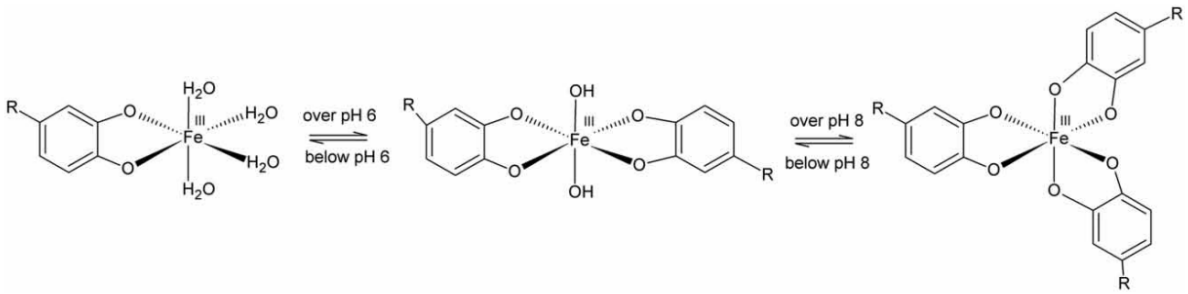

Figure 5: Catecholamine-Fe(III) complexes at different pH values (cited from (Melin et al., 2015)).

At pH less than 5.5, the formation of mono-complexes with Fe(III) increases the oxidative capacity of Fenton systems. On the contrary, at pH greater than 8.0, the formation of tris-complexes show an antioxidant behavior, due to a complete chelation of Fe(III) (Figure 4) (Melin et al., 2015). Several phenolic compounds with pro-oxidant activity have been reported (Bossmann et al., 2004; Georgi et al., 2007; Šnyrychová et al., 2006; Kang et al., 2012; Kerem et al., 1999). The pro-oxidant capacity has been associated with the reduction of Fe(III) and making the Fenton process more efficient.

On the other hand, the antioxidant activity of iron ligands has been associated with free radical scavengers and inhibition of Fenton reaction by metal chelation (Andrade et al., 2005; Nenadis et al., 2003; Polyakov et al., 2001; Qian et al., 2002; Terashima et al., 2012). Notably, several of these ligands known for their antioxidant activity have shown a pro-oxidant activity and vice versa (De la Lastra & Villegas, 2007; Rietjens et al., 2002; Strlič, Radovič, Kolar, & Pihlar, 2002). This dual behavior has been attributed to its concentration and pH (Mahmoud et al., 2013; Zhou & Elias, 2013).

1.6.4 pH

The pH of the musts and wines have a great impact on the oenological practices and quality of wine. The acidity sensation is influenced by the nature and concentration of acids, and the pH (Sowalsky & Noble, 1998). Tartaric acid is more ionized than malic and citric acids. Therefore it liberates more protons and has a great influence on pH

changes of wine. Generally, the pH of the wines is adjusted between pH 3.0 and 3.4. Variations to these values do not necessarily represent a problem in wine. However, it is always sought to avoid pH values over 4.0. The optimum pH for the growth of bacteria in wine is between 4.2 and 4.5 (Sowalsky & Noble, 1998). Therefore, wines with a pH higher than 4.0 have a greater potential to suffer microbiological problems than wines with pH close to 3.5.

As discussed in the previous item, pH variations significantly affect the formation of complexes with transition metals (Melin et al., 2015). Because the pH of the wines affects the dissociation of the species. Hence, it is important to know the fraction of ligand that is under the ionized form to form the complex at that pH. Similarly, if another ligand has the possibility of forming complexes with the metal is present in the medium, it will compete with the first ligand, and so the competition for the formation of one complex will depend on stability constant and dissociation constant.

1.6.5 Sulphur dioxide

Sulphur dioxide (SO_2) is found naturally in wines at low concentrations and is added to prevent bacterial growth, oxidation and to control enzymatic reactions during process and storage. Without the use of this additive, it is difficult to secure the quality of the wines. SO_2 may be present in wine under two forms: free (HSO_3^- or SO_2) or bound (carbonyl, unsaturated compounds, and phenols) (Barbe et al., 2000). Free SO_2 has reducing and antiseptic properties and its level must be adjusted before bottling. The SO_2 at pH of wines is found as HSO_3^-. This ion can react with H_2O_2 according to (4), and this reaction is more kinetically favored than the reaction between H_2O_2 and Fe(II). For this reason, HSO_3^- had been used extensively for quenching the Fenton reaction (McArdle & Hoffmann, 1983; Velásquez et al., 2014).

$$2e^- + 3\ H_2O_2 + 2\ HSO_3^- \rightarrow 4\ H_2O + 2\ SO_4^{2-} \quad (4)$$

The HSO_3^- could act as an antioxidant by two pathways, decomposing the H_2O_2 (Boulton et al., 1999) and/or reacting with the quinones (Figure 6). So, considering the presence of H_2O_2 and HSO_3^- in wines (Elias &

Waterhouse, 2010; Danilewicz, 2016b), the fate of H_2O_2 is determined by the competition between HSO_3^-/H_2O_2 and Fe(II)/H_2O_2 reactions.

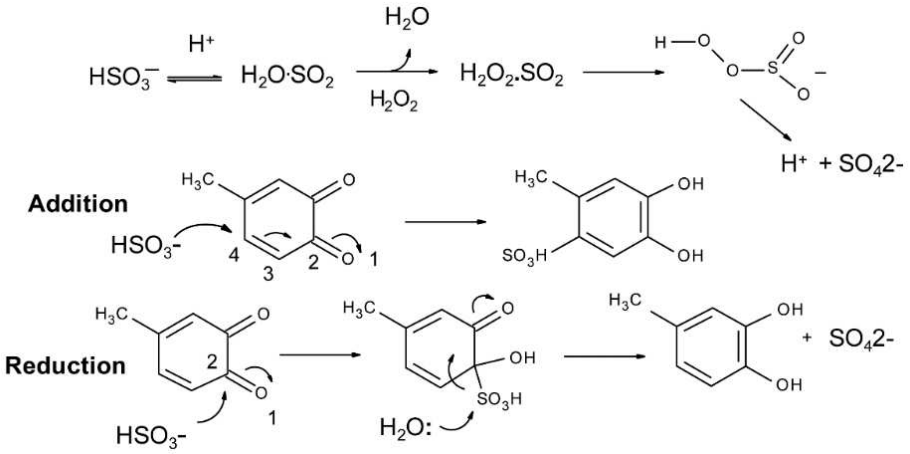

Figure 6: Reactions proposed by Danilewicz to explain to fate of HSO_3^- in wines (Danilewicz & Standing, 2018).

However, the kinetics of these reactions are not simple in a wine due to several reasons. The competitions of these reactions depend on: concentration and phenolic composition (Voelker & Sulzberger, 1996), pH (McArdle & Hoffmann, 1983), oxygen (Danilewicz & Standing, 2018), and concentration of HSO_3^- (Danilewicz et al., 2008). Evidently, the oxidation chemistry of wine in the presence of SO_2 is complex. To date, there are reports of reaction rate for SO_2/H_2O_2 and Fe(II)/H_2O_2 but only in model systems.

Based on the literature reports, these indicate a 1:2 ratio of O_2:SO_2 in model wines. This means that 1 equivalent mol of SO_2 reacts with H_2O_2 and other with quinone (Danilewicz et al., 2008). Although a recent study in real wine indicates that these proportions can be much lower, suggesting that SO_2 may be an antioxidant much less effective in practice (Danilewicz & Standing, 2018).

Therefore, to increase the knowledge of the oxidative process of wines, it is necessary to deepen the mechanism of the reaction between SO_2 and H_2O_2. For this, the formation of free radicals is relevant. Finally, based on the multiple

reports of the literature, it is postulated that all the factors involved (mentioned in section 1.6) could have some kind of relationship with the production of radicals.

1.7 Reactive oxygen species

It has been proposed that free radicals are key intermediaries in the oxidation of wine, but its nature and proportions have not been fully established. Electronic Paramagnetic Resonance spectroscopy (EPR) is a widely used technique that allows the direct detection of paramagnetic species, such as free radicals and ions of transition metals. The radicals with a very short half-life, (superoxide radicals, ˙OH, alkoxy radicals, etc.), can be detected by the use of spin trapping reagents. The spin trap is capable of forming an adduct that is more stable than the original free radical and can be analyzed by EPR (Buettner, 1987). EPR-Spin Trapping has been used successfully to elucidate problems in the oxidation of beer and other complex matrices (Jenkins et al., 2018; Kuresepi et al., 2018; Pietrzyk et al., 2018). However, this technique has not been widely used in the study of wines, only a limited number of studies have been carried out to understand the chemistry of wine free radicals (Elias et al., 2009a; Elias & Waterhouse, 2010; Zhang et al., 2015). EPR-Spin trapping has been used to identify some free radical species in wine under oxidative conditions. To date, species such as 1-hydroxyethyl (1-HER) radical using R-(4-pyridyl-1-oxide-N-tert-butylnitrone) (POBN) as spin trapping have been identified in wines. Spin trapping 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide (BMPO) has been used to identify sulfite radicals and hydroperoxyl radicals in wine (Singleton & Cilliers, 1995). In addition, the reaction between the 1-HER radical, phenolic compounds and thiols present in wine has been studied, using N-tert-butyl-α-phenylnitrone (PBN) as a spin trapping (Kreitman et al., 2013). DMPO has also been used to detect the ˙OH in model wines Thus, the first direct evidence of the Fenton reaction in the wine was provided (Elias et al., 2009a). Subsequent reports confirm the formation of ˙OH in wines (Elias & Waterhouse, 2010; Kreitman et al., 2013; Zhang et al., 2015; Márquez et al., 2018).

1.8 Chemometric analysis

Instrumental methods of chemical analysis utilize the relationships between the signal obtained and a property of the system studied (generally a concentration). Advances in electronics and computing have made possible rapid progress in data acquisition, transmission, and processing. Then, it is increasingly common the use of signals which are acquired quickly and efficiently by electronic or electro-optical instruments. Where the property of interest must be related in some way to the intensity of the signal obtained. The study of these relationships is known as chemometrics and is an area with powerful activity, due to its wide applications in the chemical, food, environmental, and process studies (Miller & Miller, 2005; Roussel et al., 2014).

1.8.1 Multivariate regression analysis

Multivariate regression analysis is known as the statistical method that allows establishing a mathematical relationship between a set of variables X_1, X_2,..., X_k (covariant or factors) and a dependent variable Y. It is used mainly in studies in which it has a dataset where there are many correlated variables and significant amounts of random noise (Wold et al., 2001).

Data pre-treatment

The pre-processing of the data is used to eliminate the variation of the data that does not belong to the analytical information. Preprocessing is a column-oriented operation, so preprocessing is applied to the samples. Among the typical preprocessing we have the autoscale, variance scale, range scale, mean-center, among others. In addition, it is possible to incorporate treatments to variables such as Log10, normalize, baseline correction, smooth, among others (Chau, 2004; Miller & Miller, 2005).

Exploratory analysis

The first step in a chemometric analysis is based on an exploratory analysis of the data. This type of analysis is carried out without prior knowledge of the nature or grouping of the samples. The exploratory algorithms reduce a

set of large and complex data to a data set with a better graphics visualization of the association patterns in the independent variables (**X** Matrix).

One of the best-known exploratory techniques is the Principal Components Analysis (PCA). The PCA analysis provides information on the correlations between samples and/or variables, and also finds linear combinations of the original independent variables (Chau, 2004; Miller & Miller, 2005).

Regression methods

Principal Components Regression (PCR) is a method of analyzing multivariate with a data multicollinear. In this analysis, the **X** Matrix is decomposed employs the Singular Value Decomposition (SVD) (Press et al. , 1986). PCR is explaining in the next equation (5) (user Guide, Pirouette).:

$$X = USV^T \tag{5}$$

The **U** matrix corresponding to eigenvectors of the row space, the **V** matrix holds eigenvectors of the column space, and **S** is a diagonal matrix whose diagonal elements are the singular values.

Partial Least Squares (PLS) is a robust method that combines two techniques of multivariate analysis: principal component analysis and multiple linear regression (Alciaturi et al., 2003; Miller & Miller, 2005). The PLS relate two matrices of data, **X** and **Y**, by means of a multivariate linear model. Unlike the traditional regression in which the structure of **X** and **Y** is also related, PLS has the capacity to analyze data with many, noisy and collinear variables. PLS also has the advantage that the accuracy of model parameters improves with the increasing number of variables (Kourti & MacGregor, 1996) (6).

$$X = \bar{U}R\bar{V}^T \tag{6}$$

$\bar{U}$ and $\bar{V}$ are not identical to U and V, which means that PLS the results of graphics "scores" and "loadings" are different from PCR. R it is a bi-diagonal matrix, other than the **S** matrix in PCR analysis. Then, the diagonal elements of the **R** matrix are not equivalent to the SVD singular values (Infometrix, 2011). The main objectives of a regression model can be two:

I. Obtain an equation that allows you to predict the value of Y. These regression models are known as "predictive models".

II. Quantify the relationship between X_1, X_2,..., X_k and the variable Y in order to know or better explain the mechanisms of that relationship. These are "explanatory models", widely used for searching the variables that affect the values of Y.

The PCR or PLS regression could be applied, in order to establish a valid systematic relationship between the several independent variables (**X** matrix) and the reactivity of the system (**Y** Matrix).

In summary, this chapter presents the state of the art of the chemical oxidation of white wines and the multiple factors that can affect it. The oxidation of wines has been studied for many years, although the mechanistic basis of the reactions involved and that promote the oxidative process is not completely known. To the date, there are several proposed schemes (Elias et al., 2009a; Kreitman et al., 2013; Danilewicz & Standing, 2018; Márquez et al., 2018). These schemes support that the chemical oxidation of the wines not only depends on the amount of dissolved O_2 but also varies according to the chemical composition of the wine (Oliveira et al., 2011).

The oxidation of white wines depends on multiple factors that influence individually and/or synergistically, among the most relevant factors are the concentration of the main organic acids, phenolic compounds, metals, SO_2, and protons. These factors have been studied mainly in model wines. In this thesis, the oxidative process has been studied in function of radical intermediaries, being $^{\bullet}OH$ a key oxidant. To date, there is no a systematic study that relates the white wine oxidation by $^{\bullet}OH$ with the chemical composition of the wines. Therefore, this thesis established the effect of the chemical composition of the wine on the amplification or abating $^{\bullet}OH$ production.

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CHAPTER 2: Hypothesis, objectives and analytical strategy

HYPOTHESIS

The oxidation of white wines depends on concentration and type of variables that influence individually or through interactions to oxidation. The oxidative process can be partially explained through a systematic study that relates the chemical composition with •OH production of white wines.

OBJECTIVES

2.1 General objetive

To generate a multivariate regression model through a systematic study that relate the chemical composition of white wines with •OH production.

2.2 Specific objetives

1) To characterize different types of white wines.
2) To determine •OH in different types of white wines by EPR with Spin Trapping.
3) Evaluate the relationship between the chemical composition of white wine and the •OH production.
4) To propose a pathways for explaining the white wine oxidation due to •OH.

ANALYTICAL STRATEGY

Analytical chemistry, the fundamental basis of this research, can be defined as the science that develops and improves methods and instrumentation with the aim of acquiring information on the composition and chemical nature of the samples. In practice, the instrumental chemical analysis applies these methods of analysis to solve problems. Therefore, in order to carry out this research, it is fundamental to define the following components of instrumental chemical analysis:

2.3 Samples

All the samples correspond to commercial white wines produced in Chile and were chosen in order to obtain differences in their chemical composition, mainly in the concentration of organic acids, and phenolic compounds. In addition, late harvest wines were selected to broaden sugar levels. Another point taken into consideration was to include wines of the same variety, but different geographical origin to see the effect of different metals and relationship with the oxidative process of wines.

The samples they have to be representative of the study system and this should not be modified during the research process, or their alteration must be minimized. For this, the sampling strategy is to acquire enough sample volume for all the analysis (750 mL per sample). The content of commercial wine bottles was mixed and immediately subdivided into 15 centrifuge tubes of 50 mL (under argon atmosphere). Also, before sealing the tubes, we will make sure to displace the oxygen with argon gas. After this, the tubes were closed with film and refrigerated at 4 °C.

So, for a total of 18 type of wines, 270 independent samples are available (15 for each type of wine). These samples were labeled with a number, varietal or assemblage, year and vineyard. In addition, one letter was assigned to each wine as a label, which will facilitate the subsequent taking of data.

2.4 Analytes

As is well known, wine is a complex matrix and its chemical composition is very variable. Therefore, this research focuses on the determination of compounds that influence the oxidation process. These variables have been widely reported in model wines and are the following: SO_2, pH, metals, phenolic compounds (flavonoids and non-flavonoids), main sugars and organic acids.

2.5 Methods

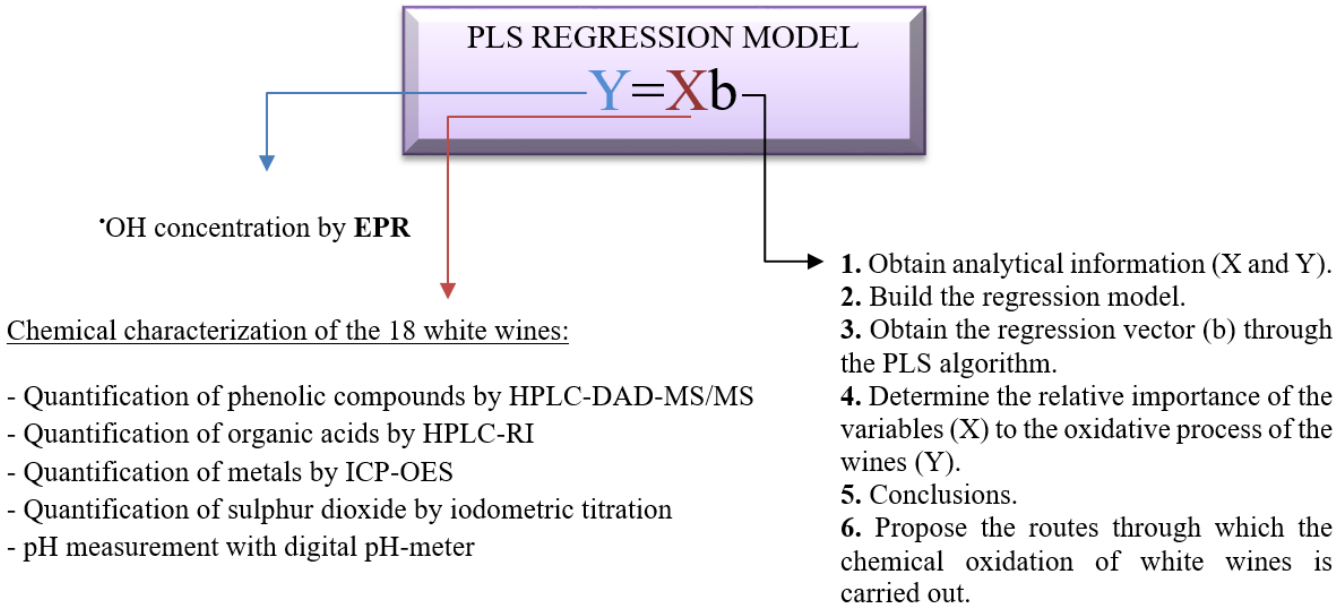
The selected analytical techniques (HPLC-DAD, HPLC-MS, HPLC-RI, ICP-OES and EPR-Spin Trapping) were chosen based on previously studied and validated methods. For all analysis, an alternative method was considered, in case the first method chosen was not suitable for the samples under study. In addition, the analytical validation of each of the methods used in this investigation was carried out and is reported in each chapter.

In the case of EPR spectroscopy, only the identification of radicals in wines had been reported in literature. Hence, the instrumental parameters were optimized for these samples. For the EPR quantification, a small experimental design was carried out, that considered the concentration of the "spin trap" reagent, amount of oxygen and time. Once these parameters were optimized, the method was validated using the TEMPOL standard (for more details go to Chapter 3, Section 3.2).

2.6 Analysis

The analysis have an established order to be carried out, but this can be modified depending on the availability of the instruments. The modification in the order of the analysis will not alter the results in any way, since the samples were stored in separate tubes for the different types of analysis. The only analysis that must be carried out first is the determination of radicals by EPR spectroscopy. Because this analysis is critical because it supports the basis of the hypothesis of this thesis.

Also, was considered sufficient tubes with independent samples (15 samples per wine) to test the optimization of the methods and in the case that it is necessary, to evaluate more than one method for one type of analysis. The following diagram (Scheme 1) shows how the research was carried out to meet the objectives established here:



Scheme 1: General scheme of the analytical strategy of this thesis and analytics techniques used (Source: elaborated by the author).

Scheme 1 shows the required items to carry out this thesis, where the main objective is to establish a multivariate regression model that allows to relate the chemical composition of a wide range of white wines with their chemical oxidation of them. Once the analytical information has been registered by all the methods described here, the multivariate model can be constructed (explanatory model). Finally, through the regression vector, it is possible to know the contribution of each component of wines in the chemical oxidation. In addition, it is feasible to propose the pathways by which oxidation is promoted.

CHAPTER 3: Production of hydroxyl radicals and their relationship with phenolic compounds in white wine

Manuscript accepted and published in Food Chemistry, volume 271, 15 January 2019, pages 80-86.

Production of hydroxyl radicals and their relationship with phenolic compounds in white wine.

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Available in: https://doi.org/10.1016/j.foodchem.2018.07.165

INTRODUCTION

Controlled exposure of wine to oxygen (O_2) can generate several positive effects, such as lower intensity of vegetal and reductive odours, improvement in fruity varietal traits, and decreased astringency and bitterness (Cano-López et al., 2008; Lesica & Kosmerl, 2009). However, excessive amounts of O_2 can cause detrimental effects to the wine, such as loss of sensory characteristics, the formation of unpleasant odours and alteration of the colour (Berradre et al., 2007; Silva Ferreira et al., 2002). In certain red wines, oxidation is necessary, especially in the case of wines with maderized tones where it is essential for the characteristic flavour and aroma of the products. On the other hand, white wines are often damaged by exposure to O_2 (Bueno, et al, 2016; Danilewicz, 2014; Kreitman et al., 2013).

Oxidation and ageing of white wines are a constant problem. In general, it is accepted that certain grape varieties are especially sensitive to oxidation, which suggests that certain key chemical components are strongly modulated by O_2 exposure. Within the study of the oxidative process, researchers should take into account the preservation of aromatic compounds while avoiding the formation of unpleasant odours and colour alteration over a long period.

Chemical oxidation of wine requires O_2 beside an oxidisable substrate and occurs both in the grape must and the wine. This oxidation is the most frequent, although it occurs at a slower rate than enzymatic oxidation. It should be taken into account that the oxidative deterioration of wine not only depends on the absolute amount of dissolved O_2 but also varies depending on the chemical composition of the wine (Oliveira et al., 2011). The chemical oxidation process is favoured by the oxidation of polyphenols that contain 1,2-dihydroxybenzene units (catechol) such as catechin, epicatechin, gallocatechin, and caffeic acid, which are the constituents that are most easily oxidized in wine (Kilmartin et al., 2001; Li et al., 2008). These substrates are oxidized sequentially to semiquinone and quinone (Figure 1, steps 1 and 2).

New antecedents were added to the traditional model of wine oxidation, which establishes the importance of transition metals in the formation of several types of radical species (Danilewicz, 2016a; Kreitman et al., 2013), as well as a series of secondary reactions that produce the changes observed during the oxidation and ageing of the

wines. In chemical oxidation, O_2 does not react directly with phenolic compounds due to the kinetic limitations of its spin state. The low reactivity of triplet oxygen, it could be increased by the monoelectronic reduction of Fe(II) (Fig. 1, step 2a) (Elias et al., 2009b; Danilewicz, 2014). However, we propose a most likely way to step 2b, since this step is kinetically favoured over step 2a (Sun et al., 2016). Moreover, the wines have several types of phenolic compounds that could act as ligands with redox activity (non-innocent ligands), forming complexes with Fe(III). In this way, Fe(III) is reduced to forming semiquinones ($SQ^{\bullet}$) (Fig. 1, step 1). Therefore, the $SQ^{\bullet}$ is generated by the oxidation of dihydroxybenzene (DHB) by Fe(III) (Salgado et al., 2013; Melin et al.,2013). Next, $SQ^{\bullet}$ is oxidized to quinone by O_2, while the O_2 is reduced to hydroperoxyl radical ($HOO^{\bullet}$), which subsequently dismutates to hydrogen peroxide (H_2O_2) (Figure 1, step 2b). The H_2O_2 reacts with Fe(II) producing hydroxyl radical ($^{\bullet}OH$) by a Fenton reaction (Fig. 1, step 3). The $^{\bullet}OH$ formed is highly oxidizing and reactive and reacts with the majority of the components in the wine. The main oxidation products are aldehydes (Fig. 1, step 4) and quinones (Fig. 1, step 2b).

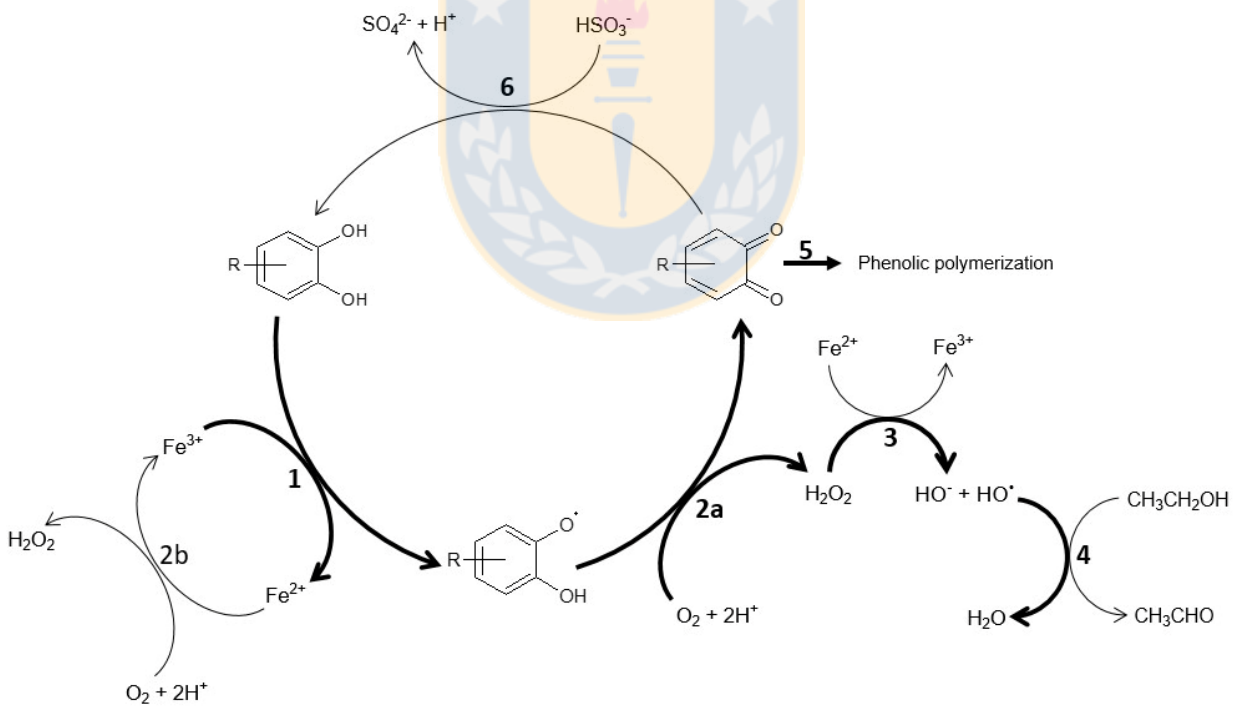

Figure 1: Proposed scheme for the chemical oxidation of white wine, catalysed through iron, that in turn activates triplet O_2 by action of $SQ^{\bullet}$ and subsequent oxidation to quinone. Additionally, the $^{\bullet}OH$ react with the major components of the wine (Source: elaborated by the author).

The quinones have high electrophilic reactivity that allows their reaction with nucleophiles, resulting in dimers or polymers (Fig. 1, step 5). Conversely, through the oxidation of hydrogen sulphite (HSO_3^-) to sulphate ion (SO_4^{2-}), quinones are reduced to their respective DHB (Fig. 1, step 6) (Nikolantonaki & Waterhouse, 2012).

It should be noted that the content of metals in wines can be broad. The transition metals would act as catalysts involved in the reduction of H_2O_2 to $^{\bullet}OH$ by Fenton reaction. The most known Fenton reaction is the one that occurs with Fe(II) (Fig. 1, step 3), the rate constant of which is 76 $M^{-1}s^{-1}$ (Chen & Pignatello, 1997). This reaction is performed at pH values close to 3.0. When the transition metal is different from Fe(II), it is called a Fenton-type reaction. The Fenton-type reaction can occur with Fe(III), Cu(I), and Mn(II). All of these metals can be present in wines (Laurie, et al., 2010).

Another point to consider is that the reactivity of an oxidant system based on a Fenton reaction is strongly dependent on metal speciation (Melin et al., 2015; Salgado et al., 2017), which is how some iron chelators act as antioxidants by capturing the iron needed to carry out the reaction (Zheng et al., 2005). Together with the above findings, there are ligands that increase the reactivity of the Fenton reaction by solubilization of Fe(II) and Fe(III) (Keenan & Sedlak, 2008). Other ligands chelate Fe(III) and reduce Fe(II), driving and amplifying the Fenton reaction. Among these ligands, the 1,2-DHBs is highlighted (Melin et al., 2015; Salgado et al., 2013).

Sun et al.(Sun & Pignatello, 1992) proposed that phenolic ligands can modify the reactivity of the Fenton systems in three ways: (i) modifying the redox properties of metal, (ii) creating a labile coordination site that can be occupied by H_2O_2 and (iii) generating competition with the substrate by oxidizing compounds. Considering the abovementioned literature, ligands have been reported with known antioxidant properties that have shown pro-oxidant activity and vice versa (Rietjens et al., 2002; Strlič et al. , 2002). This dual behaviour has been attributed to reaction variables, such as metal speciation and pH (Mahmoud et al., 2013; Rietjens et al., 2002). In addition, several authors have postulated that the coordination number of 1,2-DHB is pH-dependent. At pH values lower than 5.5 would form mono complexes with metals, allowing the entry of H_2O_2, which promotes the oxidative process (Melin et al., 2016; Melin et al., 2015).

Therefore, in a matrix of real wine, it is completely feasible that the Fenton and Fenton-type reactions by phenolic compounds are developed because they are favoured by the acidic pH values (2.8 – 3.6) of wine. At these pH values, the mono complexes are formed with labile positions that allow the exchange of H_2O by H_2O_2, necessary for the Fenton reaction. In addition, the wine contains intrinsic traces of transition metals that catalyse these reactions. However, the wines contain an important amount of phenolic compounds, and these compounds could chelate the transition metals by modifying the reactivity of these systems, either by increasing the reactivity or acting as antioxidants in the system.

The process that has been proposed as one of the main mechanisms for the oxidation of white wines is the Fenton reaction (Elias et al.2009a; Waterhouse, 2012) induced by hydroxybenzenes (Figure 1, arrows highlighted in bold). In the proposed system, the Fe(III) present in the wine could be chelated and reduced by phenolic compounds in addition to generating H_2O_2 by O_2 reduction. As a product of the oxidation mediated by O_2 in wines, researchers (Elias et al., 2009a; Kreitman et al., 2013) have identified the $^{\bullet}OH$ and 1-hydroxyethyl radical in model and real wines using electronic paramagnetic resonance (EPR) spectroscopy with 5,5-dimethyl-1-pyrroline-N-oxido (DMPO) and phenyl-N-tert-butyl nitrone (PBN) as the spin trap. However, to the best of our knowledge, the amount of radicals generated has not been quantified or compared in different types of white wines to date. This paper will determine, by means of quantitative EPR spectroscopy, the $^{\bullet}OH$ production induced by air in 18 commercial white wines that differ in grape varietal, geographical origin, and production process. The production of radicals will be correlated with the phenolic composition of the wines.

EXPERIMENTAL

3.1 Sample collection

This methodology was applied to 18 different types of white wines: Chardonnay (4), Sauvignon Blanc (5), Muscat of Alexandria (2), Riesling (1), Pedro Jimenez (1), Semillon (1), Viognier (1) and a wine blends (3). The wines tested are detailed below in Table 1.

Table 1: Information about the wines in this study (Source: elaborated by the author).

Labels	Grape wine varieties	Geographic origin (Chile)	Other characteristics
A	Chardonnay	Central Valley	Varietal
B	Sauvignon Blanc	Rapel Valley	Varietal
C	Riesling	Central Valley	Late Harvest
D	Sauvignon Blanc	Central Valley	Varietal
E	Sauvignon Blanc	Central Valley	Varietal
F	Pedro Jimenez	Limarí Valley	Varietal
G	Chardonnay	Leyda Valley	Varietal
J	Muscat of Alexandria	Limarí Valley	Late Harvest
K	Semillon	Central Valley	Varietal
L	Sauvignon Blanc and Riesling	Central Valley	Assemblage
M	Gewürztraminer and Semillon	Colchagua Valley	Late Harvest
N	Sauvignon Blanc	Casablanca Valley	Varietal
O	Muscat A., Gewürztraminer and Semillon	Central Valley	Assemblage
P	Viognier	Cachapoal Valley	Late Harvest
Q	Chardonnay	Limarí Valley	Varietal
R	Muscat of Alexandria	Loncomilla Valley	Late Harvest
S	Sauvignon Blanc	Rapel Valley	Late Harvest
T	Chardonnay	Maule Valley	Late Harvest

All commercial wines (2016 harvest) were from different vineyards throughout Chile (north, central and south) with different production processes and varied types of soil.

3.2 Standards and reagents

For the EPR spectroscopy, 99% 5,5-dimethyl-1-pyrroline-N-oxido (DMPO) was used as the spin trap, 97% 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL) was used as the standard and was supplied by Sigma Aldrich Germany.

For high-performance liquid chromatography (HPLC), the following standards were used and supplied by Sigma Aldrich Germany: gallic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid, (+)-catechin hydrate, (-)-epicatechin, (-)-epigallocatechin, quercetin, vanillin, syringic acid, and rutin. Sinapic acid, gentisic acid, ellagic acid, tartaric acid and malic acid were supplied by Acros Organics Belgium. Cinnamic acid, -*trans*-resveratrol, myricetin, kaempferol and protocatechuic acid were supplied by Merck (USA). The purity of all polyphenolic standards was greater than 95%. Polyphenol stock solutions of 1 g/L were prepared by dissolving the appropriate amount of each compound in ethanol. These solutions were stored at 4 °C and diluted before use with Milli-Q water to prepare the working standard solutions.

HPLC grade acetonitrile was obtained from Sigma-Aldrich, and ultra-pure water was obtained from a Milli-Q system (Millipore, Milford, MA, USA). 99% disodium hydrogen phosphate 85% dihydrate, orthophosphoric acid and 0.05 mol/L sulphuric acid were supplied by Merck Millipore, USA. The eluents were previously filtered with membrane filters obtained from Merck Millipore membrane (0.22 µm GV, USA).

For the UV-Vis spectroscopy and iodometric titration, the following reagents were used and supplied by Merck Millipore USA: 37% formaldehyde, mercury(II) chloride, p-rosaniline chloride, 37% hydrochloric acid, sodium chloride, sodium hydroxide, 96% sulphuric acid, 40% sodium hydrogen sulphite solution, starch from potato, 99.8% iodine, 99.5% potassium iodide, ethylenediaminetetraacetic acid di-sodium salt (EDTA), sodium thiosulfate 0.01N Titripur, ferrozine (monosodium salt hydrate of 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulphonic acid), ferric nitrate nonahydrate ($Fe(NO_3)_3 \cdot 9H_2O$) and potassium fluoride.

3.3 EPR spin trapping assay

EPR spectra were measured for the white wine/30 mM DMPO systems using the Bruker model EMX micro spectrometer, and the cavity corresponds to model ER 4119HS. The reagent and standard solutions were prepared in Milli-Q water. The reactor consists of a 25 mL glass that contained an aliquot of 2.5 mL of wine together with 50 µL of 1.5 mol/L DMPO. The chemical oxidation process is induced by air bubbling for 3 minutes with an air pump (2.5 mL/min). This process is timed and occurs in the sealed reactor. The quartz cell (AquaX-bore cell) is immediately filled, and all readings are started a minute later.

The EPR microwave power was set to 2.000 mW, the modulation frequency was 100 kHz, and a sweep time of 30 s was used. Each sample was scanned a total of 10 times. A sweep width of 100 G was used for experiments with DMPO as the spin trap. The receiver gain was set to 30 dB. EPR calibration was performed using TEMPOL. Simulation and fitting of the EPR spectra were performed using the Xenon software.

3.4 Sulphur dioxide determination

The content total and free SO_2 was determined by acid titration by the Ripper method. Free SO_2 is defined as the SO_2 present in wines in the following forms: molecular SO_2 and bisulphite ion (HSO_3^-) and was measured directly in acid medium. Total SO_2 was determined with a previous alkaline hydrolysis. The procedures for preparing the samples and reagents are specified in the Method OIV-MA-AS323-04B described in the "Compendium of international methods of analysis", published in 2009 by the International Organisation of Vine and Wine (OIV).

3.5 Organic acid determination

The quantification of tartaric acid and malic acid (main acids in wines) was determined based on a modified method from the literature (Schneider et al., 1987). An isocratic HPLC system was set up with a column block heater and

RI detector. The mobile phase was 8 mmol/L H_2SO_4, 0.6 mL/min, and 75 °C on an Aminex HPX-87H 300 x 7.8 mm column. The wines were diluted by a factor of five with water. Next, the wines were directly injected into the column at an injection volume of 20 µL.

In addition, in order to assign chemical forms to the iron in aqueous media, equilibrium speciation calculations were performed with the CHEAQS software programme. The software requires values for acid constants (K_a) and formation constants (K_f). An initial concentration of Fe(III) was also included (we considered 5 µmol/L based on the content of an average white wine), the pH remained constant at 3.0, and the concentration of the organic acids was varied in relation to their content in the wines studied.

3.6 Polyphenol determination

The identification and quantification of phenolic compounds in wines was determined by a method previously described in the literature (Pereira et al., 2010). Chromatographic analysis of polyphenols was carried out using a Perkin Elmer HPLC PDA Flexar equipped with photodiode array detector (Perkin Elmer Flexar Series 200). To separate polyphenols, a Purospher STAR RP-18 endcapped column (250 mm x 4.6 mm id; 5 µm; Milford, Merck, Germany) was selected as the analytical column using the following mobile phases: A was 10 mM phosphate solution buffered at pH 2.70 with concentrated phosphoric acid, and B was 100% acetonitrile. For the separation of polyphenols, it is necessary to use gradient elution applied as follows: 0–30 min at 0–20% B, linear, 30–50 min at 20–50% B, linear, and 50–60 min washing and re-equilibration of the column. The mobile phase was set to a flow rate of 1.0 mL/min, and the column was thermostated at 30 °C. The injection volume was set to 10 µL, and all standards and wine samples were injected in triplicate after being filtered through Rephile Quik PES membrane filters from Rephile (0.22 µm). Some phenolic compounds were detected at 254 nm (flavan-3-ols, benzoic acids, ellagic acid and cinnamic acid) and other at 350 nm (hydroxycinnamic acids, resveratrol and flavonols).

3.7 Iron reduction determination

The iron reduction in wines was determined by the spectrophotometric method modified by Salgado et al 2017 (Salgado et al., 2017). In this method, the ferrozine reacts with Fe(II) to form a stable, magenta complex. The maximum absorbance was recorded at 565 nm and yields, between pH 4 and 9, a molar absorption coefficient close to 30,000 $Lmol^{-1}cm^{-1}$. When Fe(III) is also present in the solution, it can react with ferrozine (FZ), thereby interfering with the colouration of the ferrous complex (Jeitner, 2014). To avoid this interference, KF was used, which formed a stable complex in solution with Fe(III), $[FeF_6]^{3-}$ (Salgado et al., 2017). The formation kinetics of the Fe(II)-FZ_3 complex were measured for 90 minutes with an Agilent 8453 spectrophotometer. Reagent solutions and standards were prepared in Milli-Q water. The reaction was performed in the cuvette, which contained 0.4 mL of FZ, 1.2 mL of KF, 0.2 mL of buffer MES adjusted to a pH of 5.5 with NaOH, and 40 µL of phenolic compound. The reaction was commenced with aliquots of 160 µL of Fe(III). The final concentrations were 5.0 mmol/L, 12 mmol/L, 10 mmol/L, 0.1 mmol/L and 0.5 mmol/L, respectively. The kinetics were followed at 563 nm for 90 minutes.

RESULTS AND DISCUSSION

3.8 EPR signal identification

The EPR spectra of 18 white wines with a DMPO probe were scanned centring at 3510 G with a range of 100 G. These spectra were compared with simulated EPR spectra of the DMPO/•OH adduct. The hyperfine coupling constants of the experimental spectra were in agreement in all the samples (aH = 14.70 and aN = 15.20). The characteristic DMPO/•OOH adduct signals (or organic radicals) were not detected in any sample.

3.9 Hydroxyl radical quantification

The ˙OH concentration over 90 minutes after air bubbling for all of the wine samples was quantified using DMPO/˙OH quantification. For this process, a direct spin quantification method was implemented according to previous reports in the literature (Weber, 2011). For all the assayed samples, the only adduct identified was DMPO/˙OH.

The implementation of this method was performed using a method for spin quantitation modified from previous reports (Elias et al., 2009a; Zhang et al., 2015) based on the stable radical standard, TEMPOL. For this propose, a calibration curve for TEMPOL was used in the range from 0.125 µmol/L to 64 µmol/L. In addition, we used another method called "Spin Count". Spin Count is a tool in Xenon software that uses the calculation of the double integral of the signal and an algorithm to obtain the absolute spin number. Afterwards, both methods were compared (R = 0.998, slope = 1.06). Non-significant differences were observed between both methods. The coefficient of variation (% CV) of the direct spin quantification method was of 3.7% (by 11 replicates of 9.50 µmol/L) and the limit of quantitation (LOD) was 0.125 µmol/L. More details on this procedure are shown in Table 1S.

Both methods are compared versus TEMPOL standard. There was a lineal correlation between the expected value and predicted values by both methods. The method 1 corresponds to the absolute number of spins ($Y = 1.06\ X$, $R^2 = 0.998$). Besides, the method 2 corresponds to the use of a calibration curve ($Y = 1.00X$, $R^2 = 0.998$). The slope of both methods is close to 1, therefore the quantification by both methods is proportional to the true concentration of the analyte. Moreover, it was analyzed the error associated with each point with the relative error percentage (% Er), to define which method was more suitable for quantifying radicals in wine.

From table 2S, we can note that by quantifying with the method 1, the % Er is less than the method 2. Therefore, the method 1 was used for quantification of ˙OH in white wines.

3.10 Kinetics of DMPO/•OH adduct formation

The formation of the DMPO/•OH adduct in the 18 wines was recorded for 90 minutes, sampling every 5 minutes in triplicate. The samples showed three different kinetic profiles. Examples of these profiles are shown in Figure 2 (wines B, Q and T).

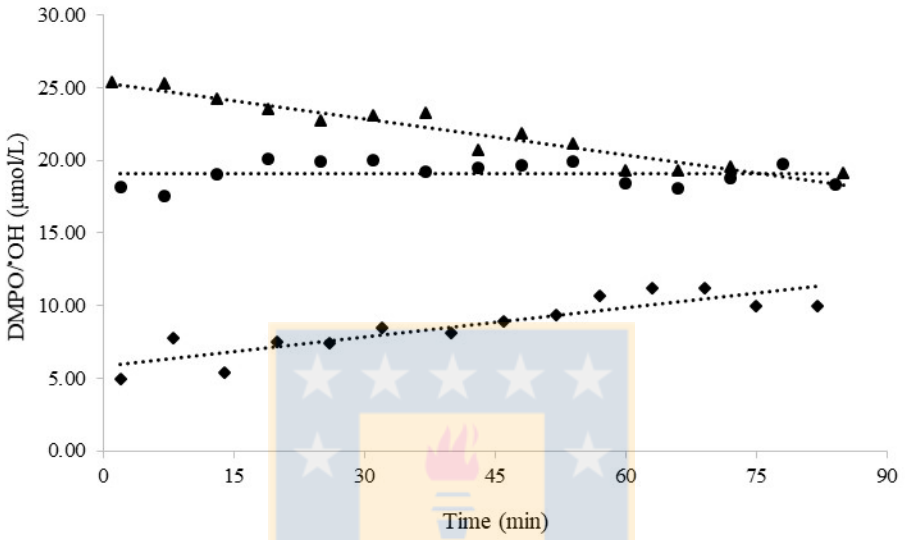

Figure 2: DMPO/•OH adduct formation kinetics for 90 minutes (▲: Wine B, ●: Wine Q and ♦: Wine T; source: elaborated by the author).

The wines A, B, D, G and M showed a decay profile for DMPO/•OH adduct concentration. This behaviour has been associated with the presence of an oxidant compound in the solution that reacts with the DMPO/•OH adduct (Contreras et al., 2007). The wines E, F, L, N, O, P, and Q produced a DMPO/•OH adduct with a concentration that was constant along the reaction time. The wines C, J, K, R, S, and T produced an increase in DMPO/•OH adduct over the assayed reaction period.

3.11 Comparative determination of •OH produced by the wines

To establish comparative parameters for •OH production relative to the different assayed wines, the following factors were considered: the initial amount of radical produced by the system (Figure 3, black bars), the maximum

amount of radical produced (Figure 3, dashed bars) and the reaction time to reach the maximum radical amount (value over dashed bars).

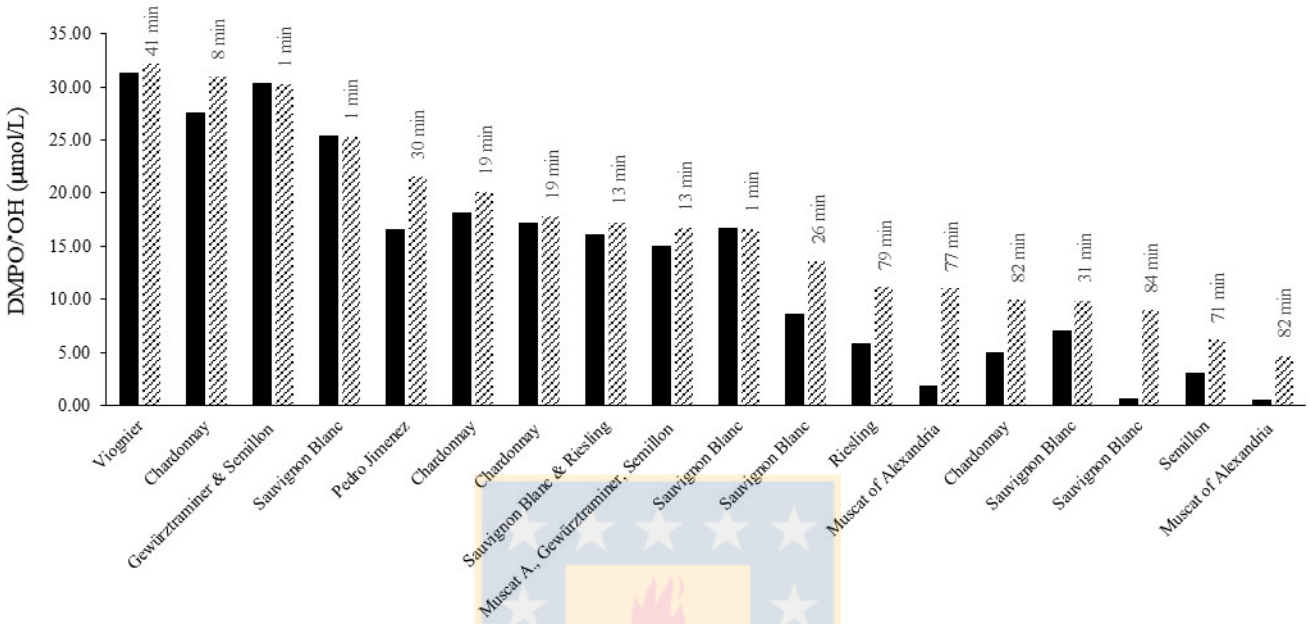

Figure 3: Initial and maximum concentrations of DMPO/•OH adducts for each of the 18 white wines. The dashed bars correspond to the initial concentration of formed radicals; black bars correspond to the maximum concentration of formed radicals by the wine. The legend above corresponds to the time in which the maximum concentration of radicals was obtained (Source: elaborated by the author).

All of the assayed wines produce •OH by air bubbling induction. The assayed wines showed a wide variation in •OH production. Additionally, there is a wide variation between the initial amount of •OH and the maximum amount reached. This variation is a consequence of the kinetic profile of the •OH discussed above. The difference in the kinetic profiles have to be related to the composition of the samples. However, there are a number of wines with systems that slowly produce •OH, and others that produce •OH more quickly. The DMPO/•OH generated by this latter type of wine is degraded by other oxidant compounds present in the samples (such as H_2O_2). A similar phenomenon was reported with the •OH production by the Fenton reaction driven by DHB (Contreras et al., 2007). In this paper, the rate of •OH production was related to complex stability and Fe(III) reduction rate. This effect could be present in the wine samples.

In a related investigation, we searched for patterns related the geographical origin, type of wine and variety of grape with the •OH production. However, in this study, it was not possible to establish any relationship between •OH production and these three variables (Figure 1S). As a consequence, it could be established that the •OH production in the wines did not only depend on the productive process and variety of grape but also depended on a complex mixture of chemical composition.

In previous reports, it was established that the antioxidant and pro-oxidant properties of phenolic compounds were achieved through the Fenton reaction driven by DHB (Melin et al., 2016; Salgado et al., 2017). The main reactives of these systems are present in wines (Fe, DHB, •OH).

Hence, phenolic compounds seem to be an important factor involved in the oxidative stability of white wines, but their role has not yet been completely characterized. Then, in order to determine relationships between •OH production and the wine composition, correlations among the amount of sulphur dioxide, main organic acids and phenolic compounds with DMPO/•OH were calculated.

3.11.1 Sulphur dioxide

The concentration of total and free SO_2 in wines was determined by iodometric titration. The %CV is 1.3% for the total SO_2 and 1.2% for free SO_2. From the results obtained, we found a wide range of concentrations of free and total SO_2 in white wines was 5.1 to 13.2 mg/L for free SO_2 and 34 to 217 mg/L for total SO_2. The concentrations of total and free SO_2 were related to the concentrations of •OH in each wine.

From previous studies, it is known that the oxidation of wine depends on the concentration of free SO_2 (Coetzee & Du Toit, 2015; Danilewicz, 2016b), although in the range of concentrations of the wines studied, no significant difference was observed in the impact it could generate in the •OH production (Figure 2S).

In several studies, SO_2 was attributed antioxidant properties in wines (Nardini & Garaguso, 2018), although the mechanism by which SO_2 participates in the process has not been fully established. Previous studies proposed that HSO_3^- reacts with H_2O_2 (Danilewicz, 2016b) stopping the Fenton reaction and thus the formation of •OH. However,

in this study and other studies (Elias et al., 2009a, 2009b; Kreitman et al., 2013), the •OH was identified. On the other hand, it was proposed that HSO_3^- react with the quinones formed by the oxidative process (Danilewicz & Standing, 2018). This process is how HSO_3^- can be oxidized to SO_4^{2-}, while the quinones are reduced to their respective phenolic compounds (Figure 1, step 6). Therefore, it could be that the reason there is no inverse linear relationship (antioxidant) between the concentration of SO_2 and the •OH production is that SO_2 acts by more than one mechanism in the redox process in the wines.

3.11.2 Organic acids

The concentration of organic acids that are more important in wines (tartaric and malic acids) was determined by a modified HPLC/IR method (Schneider et al., 1987). From the results obtained, we have found concentrations ranging from 1.12 to 3.14 g/L for tartaric acid and 0.63 to 2.61 g/L of malic acid. More details about validation parameters are shown in Table 2S. Next, the concentration of these organic acids was related to the concentration of •OH in wines.

It is known that tartaric and malic acids form complexes with Fe(III), thus influencing the redox process Fe(III)/(FeII) and therefore the oxidation of the wine (Danilewicz, 2014). However, in the range of concentrations (1.12 to 3.14 g/L for tartaric acid and 0.63 to 2.61 g/L for malic acid) of the wines studied, there was no relationship with the •OH production (Figure 3S).

Additionally, the speciation of Fe(III) in model wines was studied, and the speciation of Fe(III) was calculated according to the range of concentrations of these organic acids in the wines studied. The speciation was calculated with CHEAQS software. Then, the concentrations of all the possible species formed from Fe(III), the concentration of free organic acid and the formation of the Fe(III)-Tartrate and Fe(III)-Malate complexes were calculated with the pH set at 3.0 (average pH of the wines under study) and the initial concentration of Fe(III) set at 5 µmol/L.

Therefore, the effect of these acids in the speciation of Fe(III) in wines was calculated, although in the range of concentrations and pH values of wines in this study, there were no significant differences because iron was chelated in all wines (Figure 4S).

Of the results obtained, in both cases, the iron formed complexes with tartrate and malate over the entire range of concentrations of the wines studied. Therefore, all the wines under study were affected in the same way by tartaric and malic acid because the Fe(III) was always with these ligands.

3.11.3 Phenolic compounds

A chromatographic methodology to determine 21 phenolic compounds was implemented. To validate the developed methodology, several parameters, such as linearity, analytical determination limits, precision and accuracy, were considered. The obtained parameters are listed in supplementary material (table 3S). Among these compounds, 13 were found in the 19 assayed wines (gallic acid, protocatechuic acid, epigallocatechin, gentisic acid, p-hydroxybenzoic acid, catechin, vanillic acid, caffeic acid, syringic acid, vanillin, p-coumaric acid, rutin and ellagic acid).

The total phenolic concentration and the individual compounds were related to $^{\bullet}OH$. There were only 5 linear relationships between the phenolic compounds and $^{\bullet}OH$ concentrations (figure 4). Protocatechuic acid (PA), caffeic acid (CA), and p-coumaric acid (pCA) had a direct linear relationship between the concentrations in the wines and with $^{\bullet}OH$ production (figure 4a). The linear relationships showed lower or upper limits for linearity. This limit value was called the "critical concentration". For PA, the pro-oxidant effect was observed only for a concentration over 2.8 mg/L. For CA, the direct linear relationship was observed up to 17 mg/L. For pCA, the pro-oxidant effect was shown through 3.0 mg/L. Conversely, an inverse linear relationship between the phenolic concentration and $^{\bullet}OH$ production was observed for gentisic acid (GA) and syringic acid (SA) (figure 4b). In these systems, an inverse linear relationship with GA was observed over 48 mg/L, and the effect was observed below 2.3 mg/L for SA.

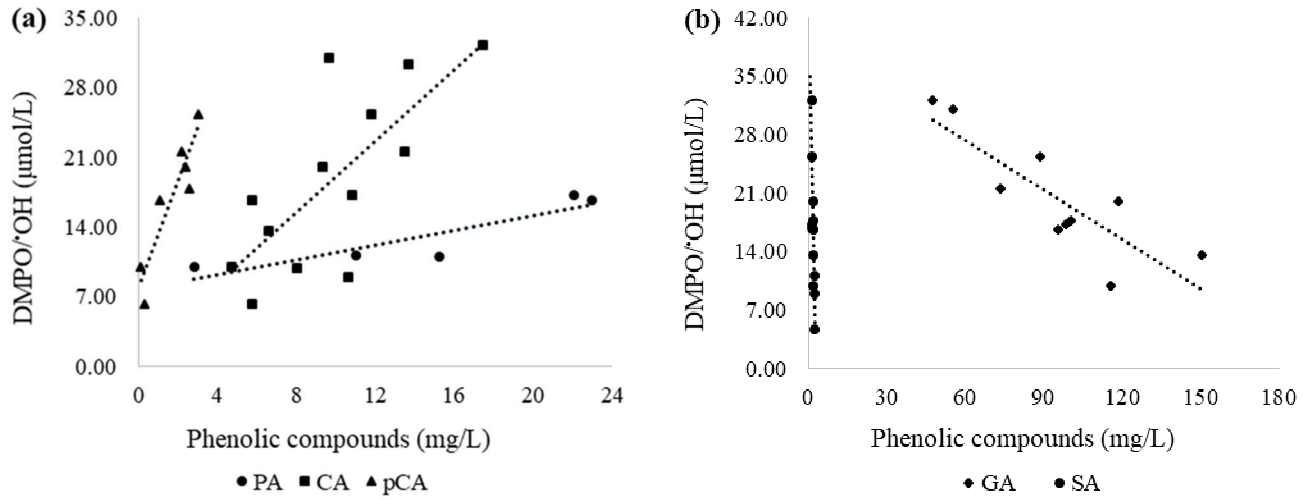

Figure 4: Relationship of •OH and phenolic compounds in white wines. (a) The protocatechuic acid, caffeic acid and p-coumaric acid show pro-oxidant effects (▲: pCA, ●: PA and ■: CA) (b) Gentisic acid and syringic acid show antioxidant effects (●: SA and ♦: GA) (Source: elaborated by the author).

The concentrations of these phenolic compounds in the wine samples were linearly correlated (Table 2). The amplification or abating of the •OH production in wines was mainly due to the contribution of these 5 phenolic compounds.

According to the proposed scheme in Figure 1, the pro-oxidant activity of DHB was explained by their ability to reduce Fe(III) and promote a Fenton reaction (Melin et al., 2016; Salgado et al., 2017). The driven Fenton reactions have to be the result of the synergistic and antagonistic effects of the components in the wine (especially DHB). To determine the individual effect of the wine's phenolic compounds on the Fe(III) reduction, a kinetic determination of Fe(III) reduction by the 5 phenolic compounds was related to •OH production.

3.11.4 Iron reduction

The Fe(III) reduction kinetics were determined for standards of the five phenolic compounds that were related to •OH production. All of these compounds reduced Fe(III) to Fe(II). Different initial rates (until 1500 seconds) were observed for these compounds, ranging from $2.4x10^{-3}$ to $2.5x10^{-5}$ mol $L^{-1}s^{-1}$ (Table 2, sixth column).

Table 2: Linear relationships found between phenolic compounds and •OH and comparison of Fe^{3+} reduction kinetics through the formation of Fe(II)-FZ_3 complexes (Source: elaborated by the author).

	PA	**GA**	**CA**	**SA**	**pCA**	**Initial rate (mol $L^{-1}s^{-1}$)**	**Maximum concentration (mol L^{-1})**
PA	-	0.8186	0.8548	0.9344	0.9544	$1.6x10^{-4}$	$3.27x10^{-5}$
GA	0.8186	-	0.9825	0.9348	0.8099	$1.1x10^{-3}$	$9.00x10^{-5}$
CA	0.8548	0.9825	-	0.9660	0.8528	$1.8x10^{-3}$	$1.10x10^{-4}$
SA	0.9344	0.9348	0.9660	-	0.9259	$3.4x10^{-4}$	$6.04x10^{-5}$
pCA	0.9544	0.8099	0.8528	0.9259	-	$3.1x10^{-6}$	$9.29x10^{-7}$

The reduction rates of each phenolic compound were evaluated separately, demonstrating that all of these compounds reduce Fe(III). The pCA shows that the lower the initial rate (Ri), the lower the amount of Fe(II).

It is highlighted that all the assayed phenolic compounds reduced Fe(III) to Fe(II), but this behaviour was not correlated with •OH production. This result means that •OH production is a complex process in which other wine components were also involved. There was a critical concentration (for all the studied phenolic compounds) observed that limited the concentration range and showed pro-oxidant effects.

In addition, it is remarkable that the phenolic compounds interacted in different ways with Fe(III) depending on their chemical structure. Thus, there were different pathways to induce and amplify a Fenton reaction in these systems (Perron & Brumaghim, 2009; Salgado et al., 2017).

CONCLUSIONS

All of the 18 commercial white wines were assayed and generated •OH during a short air bubbling period. The amount of these radicals ranged significantly depending on the type of wine used. The effect of tartaric acid, malic acid and SO_2 in wines was studied. In all of these investigations, no relationship was found with •OH production. At all of the concentration ranges of organic acids in the wines, the iron was completely chelated. Additionally, the SO_2 concentration was not significantly different in its antioxidant effects on the wines. This could be explained due to SO_2 can react with H_2O_2 and quinones, reducing the oxidation of wines. Therefore, the antioxidant effect of SO_2 has several pathways and is not necessarily linear.

It was previously established that phenolic compounds have antioxidant capacity and have health benefits. However, some of these compounds demonstrate pro-oxidant effects in a real wine matrix. The concentration of these compounds in wines was proportionally related to the increase in •OH production. Therefore, in this study, a relationship between certain phenolic compounds (caffeic acid, protocatechuic acid, p-coumaric acid, gentisic acid and syringic acid) and induction and amplification of the •OH production was established and was postulated to be a chemical oxidation path of the Fenton reaction.

SUPPLEMANTARY MATERIAL

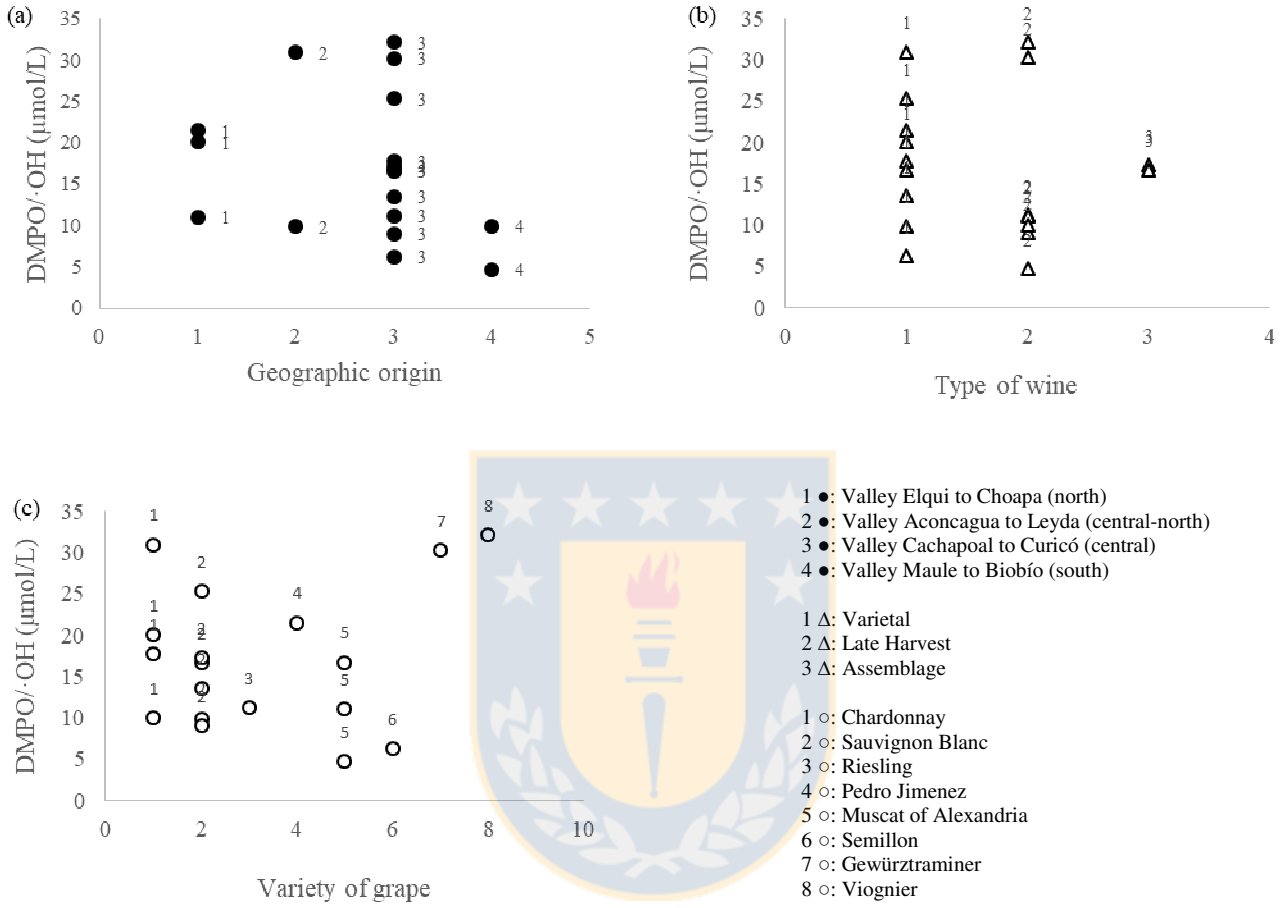

Figure 1S: The relationship between ·OH production and geographic origin, type of wine or variety of grape (Source: elaborated by the author).

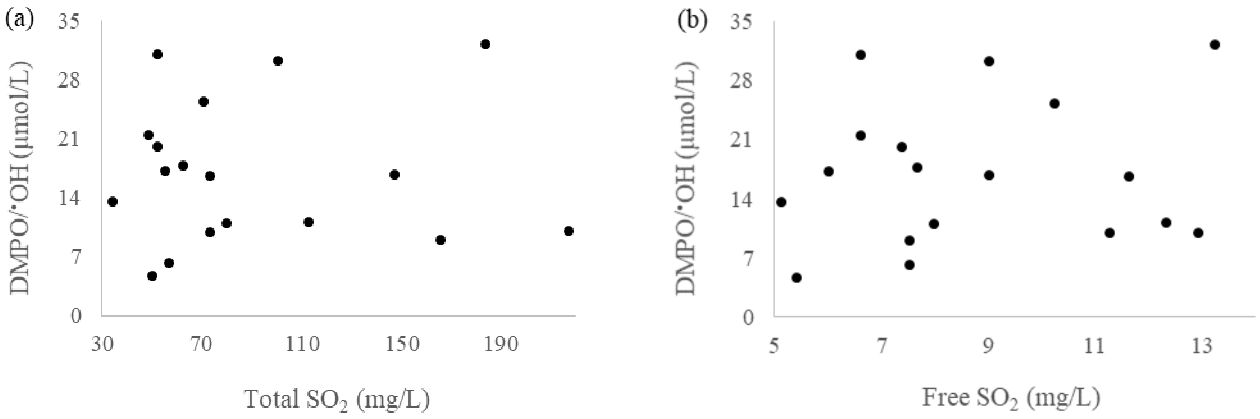

Figure 2S: Relationship between the concentration of •OH and (a) total SO_2 in total range of concentrations; (b) free SO_2 in total range of concentrations (Source: elaborated by the author).

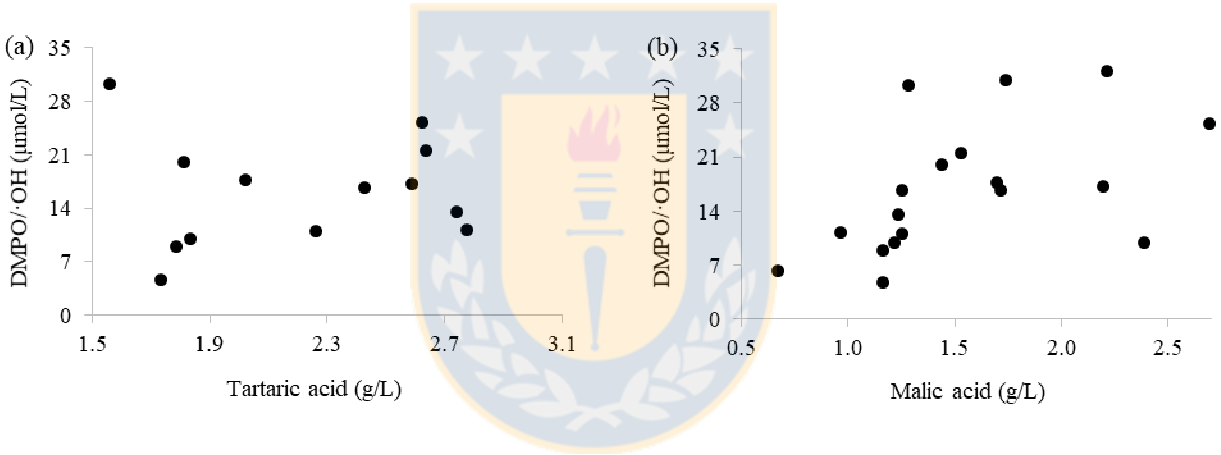

Figure 3S: Relationship between the concentration of •OH and organic acids. (a) Relationship between tartaric acid and •OH production. (b) Relationship between malic acid and •OH production (Source: elaborated by the author).

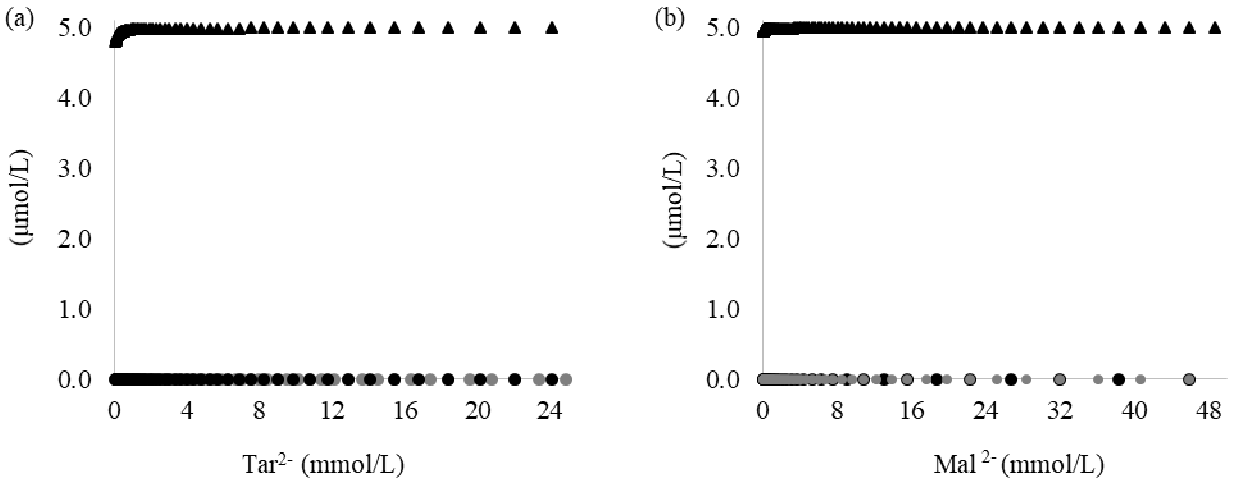

Figure 4S: Relationship between organic acids and Fe(III) species. (a) Tartrate free (Tar^{2-}) in function of concentration H_2Tar (●), $Fe(III)(OH)_3$ (●) and Fe(III)-Tar complex (▲). (b) Malate free (Mal^{2-}) in function of concentration H_2Mal (●), $Fe(III)(OH)_3$ (●) and Fe(III)-Mal complex (▲) (Source: elaborated by the author).

Table 1S: Comparison of quantification methods. Method 1 corresponds to the quantification of the absolute number of spins; Method 2 corresponds to the quantification by a calibration curve (Source: elaborated by the author).

Standard (µmol/L)	**Method 1 (µmol/L)**	**% Er (Method 1)**	**Method 2 (µmol/L)**	**% Er (Method 2)**
0.125	0.018	85.8	0.594	-374.8
0.250	0.208	16.9	0.773	-209.4
0.500	0.417	16.6	0.971	-94.2
1.000	0.842	15.8	1.400	-39.7
2.000	1.730	13.3	2.270	-13.4
4.000	3.630	9.2	4.010	-0.3
8.000	7.290	8.9	7.470	6.6
16.00	15.20	5.3	14.90	6.8
32.00	31.40	1.8	30.30	5.3
64.00	68.60	-7.2	65.20	-1.8

Table 2S: Validation parameters for the tartaric and malic acid quantitation method in wines (Source: elaborated by the author).

Organic acid	**λ detection (nm)**	**Linearity (R^2)**	**Precision (%CV)**	**LOD (g/L)**	**LOQ (g/L)**
Tartaric acid	210	0.970	3.50	0.308	1.030
Malic acid	210	0.994	2.80	0.134	0.445

Table 3S: Validation parameters for the identification and quantification of phenolic compounds in wines (Source: elaborated by the author).

tr (min)	**λ detection (nm)**	**Compound**	**Linearity (R^2)**	**LOD (mg/L)**	**LOQ (mg/L)**
10.0	254	Gallic acid	0.978	0.32	0.97
15.9	254	Protocatechuic acid	0.992	0.30	1.00
20.6	254	Epigallocatechin	0.985	1.07	3.57
21.0	254	Gentisic acid	0.980	0.51	1.68
21.6	254	p-Hydroxybenzoic acid	0.979	0.38	1.28
22.3	254	Catechin	0.972	0.54	1.81
24.6	254	Vanillic acid	0.995	0.59	1.97
25.2	254	Caffeic acid	0.989	0.34	1.14
25.7	254	Syringic acid	0.987	0.22	0.72
29.1	254	Vanillin	0.975	1.08	3.61
30.1	254	p-Coumaric acid	0.990	0.52	1.73
32.9	254	Rutin	0.970	0.49	1.64
38.6	254	Ellagic acid	0.988	0.30	0.99

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CHAPTER 4: Systematic study of hydroxyl radical production in white wines as a function of chemical composition

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Systematic study of hydroxyl radical production in white wines as a function of chemical composition

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INTRODUCTION

Although the chemistry of the oxidation of white wines has been studied for many years, the mechanism behind the reactions that promote the oxidative process has not been thoroughly elucidated to date. Until now, several schemes have been proposed (Danilewicz, 2014; Elias et al., 2009a; Kreitman et al., 2013; Márquez et al., 2018). The chemical oxidation of wines depends not only on the amount of dissolved O_2, but also on the chemical composition of the wine (Oliveira et al., 2011). Chemical oxidation is favoured by the oxidation of polyphenols containing units of 1,2-dihydroxybenzene (1,2-DHB) and 1,2,3-trihydroxybenzene (1,2,3-THB), which are the components in wine that are most easily oxidised (Oliveira et al., 2011).

These dihydroxybenzenes (DHBs) are sequentially oxidised to semiquinone (SQ$^{\bullet}$), catalysed by transition metals, such as iron (Kreitman et al., 2013; Márquez et al., 2018). The oxygen reacts with SQ$^{\bullet}$ to form quinones, which possess high electrophilic reactivity and can form dimers and polymers. While SQ$^{\bullet}$ is oxidised to quinone, the oxygen is reduced to hydrogen peroxide (H_2O_2). In the presence of Fe(II), H_2O_2 produces hydroxyl radical ($^{\bullet}OH$) a reaction known as the Fenton reaction (1) (Elias & Waterhouse, 2010).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH \quad (1)$$

The $^{\bullet}OH$ radical that is formed is highly oxidising and reactive, and reacts with the majority of the components in the wine. In this way, the primary products of oxidation are aldehydes, and quinones. These products change the colour and flavour of the wines, degrading its organoleptic properties.

Thus, this research is based on the fact that the chemical composition of the wine will notably affect $^{\bullet}OH$ production. The $^{\bullet}OH$ radicals can be quantified directly by electronic paramagnetic resonance (EPR) spectroscopy using the spin trap DMPO. The adduct DMPO/$^{\bullet}OH$ has been reported in model wines (Elias et al., 2009; Zhang et al., 2015) and real wines (Márquez et al., 2018).

Several authors have proposed schemes to explain the chemical oxidation of wines (Danilewicz, 2014; Elias et al., 2009; Kreitman et al., 2013; Márquez et al., 2018). For example, the participation of organic acids with iron

chelating properties has been described (Danilewicz, 2014). These organic acids increase the availability of iron and modify its redox potential. Phenolic compounds that can chelate and reduce Fe(III), promoting the Fenton reaction, have also been described (Melin et al., 2015; Salgado et al., 2018). Such phenolic compounds can react with •OH, stopping the reaction of these radicals (Cassino et al., 2016; Dalvi et al., 2017). In addition, several metals have been associated with wine oxidation through Fenton-like reactions. This phenomenon has been described in wines and other natural matrices (Kreitman et al., 2013; Salgado et al., 2018; Tony et al., 2009).

In conjunction with the variables described above, SO_2 (found in aqueous solution as HSO_3^-), plays the main role in preventing the oxidation of wines. The HSO_3^- decomposes H_2O_2 (McArdle & Hoffmann, 1983), avoiding the Fenton reaction as well as the reduction of quinones formed by chemical oxidation (Danilewicz et al., 2008; Danilewicz & Standing, 2018).

Based on the aforementioned discussion, the oxidation of white wines depends on multiple factors, the influences of which—both individual and synergistic—have been reported widely in the literature. The chemical oxidation in wines has been studied through a variety of oxidation products and radical intermediates, with the •OH radical being one of the main oxidants. To the best of our knowledge, there exists no systematic study that relates the oxidation of white wine by •OH to the chemical composition of the wine. Therefore, this paper will establish the effect of the chemical composition of the wine on •OH production induced by air bubbling.

EXPERIMENTAL

4.1 Samples

Fifteen varietal and three assembly wines were chosen for this study. The wines were selected to maximise the variety in their chemical compositions, mainly in the concentrations of organic acids and phenolic compounds. In addition, late harvest wines were selected to broaden sugar levels. Wines with varied pH (between 2.8 to 3.6) were also chosen. Finally, wines of the same variety but of different geographical origin were selected to determine the effect of different metals on oxidation of the wines.

4.2 Standards and reagents

5,5-dimethyl-1-pyrroline-N-oxido (DMPO), 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL), vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid, (+)-catechin hydrate, (-)-epicatechin, (-)-epigallocatechin, quercetin, vanillin, syringic acid, rutin, fructose, glucose, and HPLC-grade acetonitrile were supplied by Sigma Aldrich Germany. Sinapic acid, gentisic acid, ellagic acid, tartaric acid, malic acid, citric acid, succinic acid, acetic acid, lactic acid and shikimic acid were supplied by Acros Organics Belgium. Cinnamic acid, *trans*-resveratrol, myricetin, kaempferol and protocatechuic acid, sodium hydroxide, nitric acid 65%, sulphuric acid 96%, starch from potato, iodine, potassium iodine potassium dihydrogen phosphate solution, ammonium sulfate, phosphoric acid, and multi-element standard solution for ICP were supplied by Merck Millipore, USA. The *trans*-caftaric acid, *trans*-piceid were supplied by Phytolab (Vestenbergsgreuth, Germany). Procyanidins B1 and B2, kaempferol, quercetin, isorhamnetin, and the 3-glucosides of kaempferol, quercetin and isorhamnetin were supplied by Extrasynthese (Genay, France). Gallic acid, *trans*-resveratrol, (+)-catechin, (-)-epicatechin, (-)-epicatechin 3-gallate, and (-)-gallocatechin 3-gallate were supplied by Sigma (Tres Cantos, Madrid, Spain). Quercetin 3-glucuronide were previously isolated from Petit Verdot grape skins (Castillo-Muñoz, Gómez-Alonso, et al., 2009). Procyanidin B4 was kindly supplied by Prof. Fernando Zamora (Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Spain). The *trans* isomers of resveratrol and its 3-glucoside (piceid) were transformed into their respective *cis* isomers by UV-irradiation (366 nm light during 5 min in quartz vials) of 25% methanol solutions of the *trans* isomers. The purity of all standards was greater than 95% and ultra-pure water was obtained from a Milli-Q system (Millipore, Milford, MA, USA). The eluents were previously filtered with membrane filters obtained from Merck Millipore membrane (0.22 µm GV, USA).

4.3 EPR assays

EPR spectroscopy was utilised to identify and quantify $^{\bullet}$OH radicals. Additionally, DMPO as a spin trap and TEMPOL as a standard for the calibration curve were used. The instrumentation and conditions were as follows: the reactor contained 2.5 mL of white wine and 50 µL of DMPO 1.5 mol/L, and the spectra were measured in a

Bruker model EMX-micro spectrometer. The chemical oxidation process was accelerated by air bubbling for 3 minutes. The measurements were made in a quartz cell (AquaX-bore cell) and the readings were started a minute later. The instrumentation conditions were described in the literature (Márquez et al., 2018).

4.4 Chemical characterisation

4.4.1 Determination of flavonols and hydroxycinnamic acid derivatives by HPLC-ESI-MS/MS

The determination of flavonols and hydroxycinnamic acid derivatives (HCAD) was performed by high-performance liquid chromatography (HPLC) with an Agilent 1100 Series chromatograph equipped with a diode-array detector (DAD) and electrospray ionization mass spectrometry (ESI-MS^n) detectors. An Agilent Chem Station data-processing station was also used. For pre-treatment of samples, a rotary evaporator was utilised at a temperature of 35 ºC, in which the samples were concentrated to double the volume with methanol 20%, of which 40 µL was injected on a reversed-phase column (ZORBAX Eclipse XDB-C18 2.1 x 150 mm; 3.5 µm particle, Agilent) and set to a temperature of 40 ºC. The analytical conditions and instrumentation parameters are described in the literature (Castillo-Muñoz et al., 2007). Identification and quantification of flavonols and HCAD were performed at 360 nm and 320 nm, respectively, and based on the spectroscopic data (UV-Vis and MS/MS) obtained from the authentic standards or using previously reported data (Rebello et al., 2013).

4.4.2 Determination of flavan-3-oles, proanthocyanidins and stilbenes by HPLC-MS-MRM

The determination of flavan-3-ols, proanthocyanidins, and stilbenes was performed by HPLC with an Agilent 1200 series chromatograph equipped with DAD and coupled to an AB Sciex 3200 TRAP with a triple quadrupole, turbo spray ionization (electrospray assisted by a thermos-nebulization) mass spectroscopy system mass spectrometry system (ESI–MS/MS). Two MS scan types were used: enhanced MS (EMS) for compound identification; and multiple reaction monitoring (MRM) for quantification, based on a method previously described in the literature

(Rebello et al., 2013). The chromatographic system and mass spectra data were managed and processed using Analyst MSD software (Applied Biosystems, version 1.5). For the samples used a pre-treatment with solid phase extraction (SPE) system with C18 cartridges (Sep-Pak Plus C18, Waters Corp.) was performed, based on a procedure previously described in the literature (Rebello et al., 2013). Furthermore, a pyrogallol-induced acid-catalysed depolymerization method was employing to obtain structural information on proanthocyanidins. The samples, before and after the acid-catalyzed depolymerization reaction, were injected (20 µL) into an Ascentis C18 reversed-phase column (150 mm × 4.6 mm with 2.7 µm of particle size) (Supelco, Bellefonte, USA), with the temperature controlled at 16°C. The solvents and gradients used for this analysis and the multiple reaction monitoring settings as well as all the mass transitions (m/z) for identification and quantitation were according to the methodology reported by Lago-Vanzela.(Lago-Vanzela et al., 2011).

4.4.3 Determination of other phenolic compounds by HPLC-DAD

The determination of phenolic compounds was performed by HPLC with a Perkin Elmer HPLC PDA Flexar chromatograph equipped with a photodiode array detector (Perkin Elmer Flexar Series 200). To separate polyphenols, a Purospher STAR RP-18 endcapped column (250 mm x 4.6 mm id; 5 µm; Milford, Merck, USA) was utilised. The chromatographic conditions are described in the literature (Pereira et al., 2010). The injection volume was 10 µL, and all standards and wine samples were filtered through (Rephile Quik PES 0.22 µm) membrane filters. Certain phenolic compounds were detected at 254 nm (flavan-3-ols, benzoic acids, ellagic acid), and others were detected at 320 nm (hydroxycinnamic acids, and flavonols).

4.4.4 Determination of sugars and organic acids by HPLC-IR and HPLC-DAD

Quantification of the main sugars and organic acids (citric acid, succinic acid, acetic acid, lactic acid, fructose, and glucose) in wine was determined by an isocratic HPLC system was set up with a column block heater and refractive index (RI) detector. The mobile phase was 8 mmol/L H_2SO_4, 0.6 mL/min, and set at 75 °C on an Aminex HPX-

87H 300 x 7.8 mm column. The wines were filtered and 10 µL the sample was injected into the column. Also, tartaric acid, malic acid, and shikimic acid by an HPLC system with a DAD detector to 210 nm were determined. The mobile phase was potassium dihydrogen phosphate solution, 70 g/L, ammonium sulfate, 14 g/L, and adjusted to pH 2.1 by adding phosphoric acid, 0.8 mL/min, and 20 °C on three C18 250 x 4.6 mm column in series. The wines were filtered and 10 µL the sample was injected into the column. Both methods are based on a method described in the literature (OIV-MA-AS313-04).

4.4.5 Determination of sulphur dioxide by iodometric titration

The free and total SO_2 content was determined by titration with triiodide (I_3^-) in acid medium—a method known as the Ripper method. For the measurement of free SO_2, the sample was placed in an acid medium and titrated with I_3^- following standardisation with sodium thiosulphate ($Na_2S_2O_3$). For the measurement of bound SO_2, pre-treatment in an alkaline medium is necessary. Further detail on the procedures for sample preparation and reagents may be found by reviewing method OIV-MA-AS323-04B described in the "Compendium of International Methods of Analysis", published in 2009 by the International Organisation of Vine and Wine (OIV).

4.4.6 Determination of metals by ICP-OES

The metals content on the wines was determined by inductive coupled plasma atomic emission optical spectroscopy (ICP-OES) with a Perkin Elmer Optima 5300 DV spectrometer. This method allows for the simultaneous determination of Al, B, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, Pb, and Zn. The operating conditions for the analysis of each element were as follows: 1300 W, 15 L/min by plasma gas, 0.85 L/min by gas nebulizer, 0.2 L/min auxiliary gas and 15-mm observation height. The determination was simultaneous, and a simple pre-treatment of the samples was required. The pre-treatment consisted of an acid digestion with 9 mL of HNO_3 and 1 mL H_2O_2, and 5 mL of wine inside a microwave digester.

4.5 Chemometric analysis

4.5.1 Data processing

The data are pre-processed to minimise the contributions of variables that incorporate irrelevant information into the data matrix to construct simpler and more robust models. The **X** matrix was pre-processed by auto-scaling (AS) and variance scaling (VS) (Lavine & Workman, 2013) as follows.

The AS consists of centered on the mean ($\bar{x}_m$) followed by normalization with the standard deviation (S_m):

$$X'_{i,m} = \frac{X_{i,m} - \overline{X_m}}{S_m} \quad (2)$$

Equation (2) shows the mathematical transformation performed, where $X'_{i,m}$ are the AS data, $X_{i,m}$ are the data before AS, $\bar{x}_m$ is the mean of column m and S_m is the standard deviation of column m. Thus, the mean and variance of the new AS variables are 0 and 1, respectively (Lavine & Workman, 2013).

VS is used when the data corresponding to some variables possess very different ranges in magnitude. In such a case, the largest variable dominates in any variance computations. In this study, measurements of pH, molar concentration, and ppm concentration were used. A change of one unit in the pH, for example, could be masked by a change of 10 units in the concentration. This masking can be removed by VS. First, the variance is calculated for each variable, after which each independent variable is divided by its standard deviation (Lavine & Workman, 2013).

4.5.2 Multivariate analysis

The partial least-squares (PLS) algorithm was used for this study. This technique constructs new predictor variables, known as principal components, as linear combinations of the original predictor variables. The algorithm constructs these components while considering the observed response values, comparing observed values vs prediction values, after which a model with reliable predictive power is generated (Wold et al., 2001).

The most influential variables in the multivariate model were correlated by PLS regression with the experimentally obtained reactivity parameters.

The **X** matrix corresponds to the chemical characterisation, the Y column corresponds to the reactivity data for each wine (•OH production) and the **B** matrix corresponds to the regression vector. This vector, which possesses bi components, characterises the influence of each variable in the calibration model. To validate the explanatory PLS model, it is important to keep a sufficient number of factors. Otherwise important information will be missing from the model and it will not be reliable. To that end, we must choose an optimal value between the number of factors, the % variance of the samples (> 80%) and the standard calibration error (SEC).

$$SEC = \sqrt{\frac{\sum_{i=1}^{n}(y_i - Y_i)^2}{n-p}} \quad (3)$$

In equation (3), n corresponds to the number of samples, p is the number of factors used, Y_i corresponds to the value predicted by the model and y_i is the value measured or considered true. This model is explanatory for the Y variable, and unlike a predictive model, external validation is not necessary, since it will not be used for future predictions but rather to understand how the Y variable behaves as a function of the independent variables.

4.6 Speciation analysis

The speciation calculations for Fe(III) in aqueous solution were made with the CHEAQS software programme. The speciation was calculated for a model wine that contained the main organic acids of white wine (tartaric and malic acid), caffeic acid (as a pro-oxidant model compound) and gallic acid (as an antioxidant model compound). To perform the speciation calculations, the software requires the values of the acid constants and the formation constants for the experimental Fe(III)-complexes, which were obtained from the literature (Gomathl, 2000 et al., 2010). The concentrations of all the compounds present in the model wine were constant and corresponded to the average concentrations found in real white wines. These concentrations were as follows: 24.0 µmol/L of Fe(III), 17 mmol/L of tartaric acid, 28 mmol/L of malic acid, 55 µmol/L of caffeic acid, 30 µmol/L of gallic acid. Finally, the pH was varied between 2 and 4 to study the formation of complexes with Fe(III).

RESULTS AND DISCUSSION

4.7 **Chemical characterisation**

4.7.1 HPLC measurements

Phenolic compounds

The determination of several families of phenolic compounds and their derivatives was performed by three chromatographic methods with DAD and MS detectors. The families of compounds were analysed at different wavelengths. Flavonols (e.g., quercetin, myricetin, rutin, and kaempferol) were analysed at 360 nm, the family of flavan-3-ols (e.g., (+)-catechin, (-)-epicatechin, and (-)-epigallocatechin) was analysed at 280 nm, the benzoic acid derivatives at 254 nm and the hydroxycinnamic acid derivatives at 320 nm. Based on the results, each parameter was considered to be a discrete variable. These variables were the following: *cis*- and *trans*-Grape Reaction Product (*cis*-GRP and *trans*-GRP), caftaric acid, *cis*- and *trans*-cutaric acid, *p*-coumaroyl-glucose (*p*-coumaroyl-glc), *cis*- and *trans*-fertaric acid, quercetin-3-glucoside (Q-3-glc), quercetin-3-glucuronide (Q-glcU), medium degree of polymerization (mDP), % galloylation, % prodelphinidin, proanthocyanidins, total flavan-3-ols monomers, total flavan-3-ols dimers, stilbenes, (-)-epicatechin, (-)-gallocatechin, (-)-epigallocatechin, flavan-3-ols monoglycosides, procyanidin B1, B2 and B4, other flavan-3-ols dimers, galloylated flavan-3-ols dimers, *cis*- and *trans*-piceid and *cis*-resveratrol, gallic acid, 3,4-dihydroxybenzoic acid (3,4-DHB acid), gentisic acid, *p*-hydroxybenzoic acid, (+)-catechin, vanillic acid, caffeic acid, syringic acid, vanillin, *p*-coumaric acid, ferulic acid, rutin and ellagic acid. These 42 variables are included in the chemometric analysis and their correlative numbers are described in Table 1 (numbers 17 to 58) and its concentrations were included in supplementary material (tables 1S to 3S).

Sugars and organic acids

The determination of major organic acids and their main sugars was performed by two chromatographic methods (HPLC-RI and HPLC-DAD). From the results obtained, the concentrations of these compounds were considered to be discrete variables. These variables are the following: tartaric acid, malic acid, citric acid, succinic acid, lactic

acid, acetic acid, shikimic acid, glucose and fructose. These nine variables are included in the chemometric analysis and their correlative numbers are described in Table 1 (numbers 3 to 9 and 59 to 60). The concentrations of these variables and the validation of method were included in supplementary material (tables 4S and 5S).

4.7.2 Spectrophotometric measurements

The concentrations of metals in the wines were determined by ICP-OES. The determination was simultaneous and a simple pre-treatment of the samples was required. From the results, the concentrations of these metals were treated as independent variables. These variables are following: Al, Cu, Fe, Mn, Zn, and S. These six variables, are included in the chemometric analysis and their correlative numbers are described in Table 1 (numbers 11 to 16). The concentrations of these variables and the validation of method were included in supplementary material (tables 6S and 7S).

4.7.3 Other measurement

The concentrations of free and total SO_2 was determined by titration with I_3^- in acid and alkaline media, respectively. The concentrations of free and total SO_2, together with the concentration of protons (mol/L), are included in the chemometric analysis and their correlative numbers are described in Table 1 (numbers 1, 2 and 10). The concentrations of these variables were included in supplementary material (tables 6S) and the validation of the SO_2 quantification method in white wines has been described in the literature (Márquez et al., 2018).

Table 1: The correlative number assigned to the 60 variables in the multivariate regression model (Source: elaborated by the author).

1	Free SO_2	**16**	S	**31**	Catechin	**46**	Stilbenes
2	Total SO_2	**17**	*trans*-GRP	**32**	Vanillic acid	**47**	Epicatechin
3	Tartaric acid	**18**	caftaric acid	**33**	Caffeic acid	**48**	Gallocatechin
4	Malic acid	**19**	*cis*-GRP	**34**	Syringic acid	**49**	Epigallocatechin
5	Citric acid	**20**	cis-cutaric acid	**35**	Vanillin	**50**	Flavan-3-ol
6	Succinic	**21**	trans-cutaric acid	**36**	*p*-coumaric acid	**51**	Procyanidin B1
7	Lactic acid	**22**	p-coumaroyl-glc	**37**	Ferulic acid	**52**	Procyanidin B2
8	Acetic acid	**23**	trans-fertaric acid	**38**	Rutin	**53**	Procyanidin B4
9	Shikimic	**24**	cis-fertaric acid	**39**	Ellagic acid	**54**	Other flavan-3-ol
10	$[H^+]$	**25**	Q-3-glcU	**40**	mDP	**55**	Galloylated flavan-3-
11	Al	**26**	Q-3-glc	**41**	% Galloylation	**56**	*trans*-piceid
12	Cu	**27**	Gallic acid	**42**	% Prodelphinidin	**57**	*cis*-piceid
13	Fe	**28**	3,4-DHB acid	**43**	Proanthocyanidin	**58**	*cis*-resveratrol
14	Mn	**29**	Gentisic acid	**44**	Flavan-3-ol	**59**	Glucose
15	Zn	**30**	*p*-hydroxybenzoic	**45**	Flavan-3-ol dimers	**60**	Fructose

Finally, all the results of the analysis of the chemical characterization of the wines was included in **X** Matrix of the multivariate regression model.

4.8 Hydroxyl radical production

The ˙OH production for all the wine samples was quantified by EPR spectroscopy using DMPO for spin trapping. For all the assayed samples, the only adduct identified was DMPO/˙OH (Figure 1S in the supplementary material). These results are used as the dependent variable (**Y** matrix) in the PLS regression model. The validation of the DMPO/˙OH determination method in white wines has been described in the literature (Márquez et al., 2018).

4.9 Relationships between characterisation of wines and •OH production

4.9.1 Regression model

To determine the sources of the differences in •OH production (**Y** matrix) in the white wines, chemical characterisation of these wines was performed and modelled in a multivariate way with the •OH concentration.

The main goal of the chemical characterisation was to include a wide range of variables to relate them with •OH production. To that end, wines from different production processes, geographical areas and grape varieties were chosen. The concentrations of the studied compounds (flavonols, flavan-3-oles, hydroxybenzoic acids, hydroxycinnamic acids, free SO_2, total SO_2, proton concentration, organic acids and metals) constituted 60 independent variables (**X** matrix) per wine. A PLS algorithm was used to perform the analysis. Due to the validation and quality parameters of the model, 3 factors were selected, whose cumulative variance was 85% and whose SEC was 0.003 (µmol/L). The model demonstrated good correlation between the predicted values and the experimental values of •OH produced for all the wines studied, with a coefficient of linearity (R^2) of 0.952 (Figure 2S in the supplementary material). In the PLS model, the relative contributions of the independent variables to •OH production were analysed by a regression vector (Figure 1).

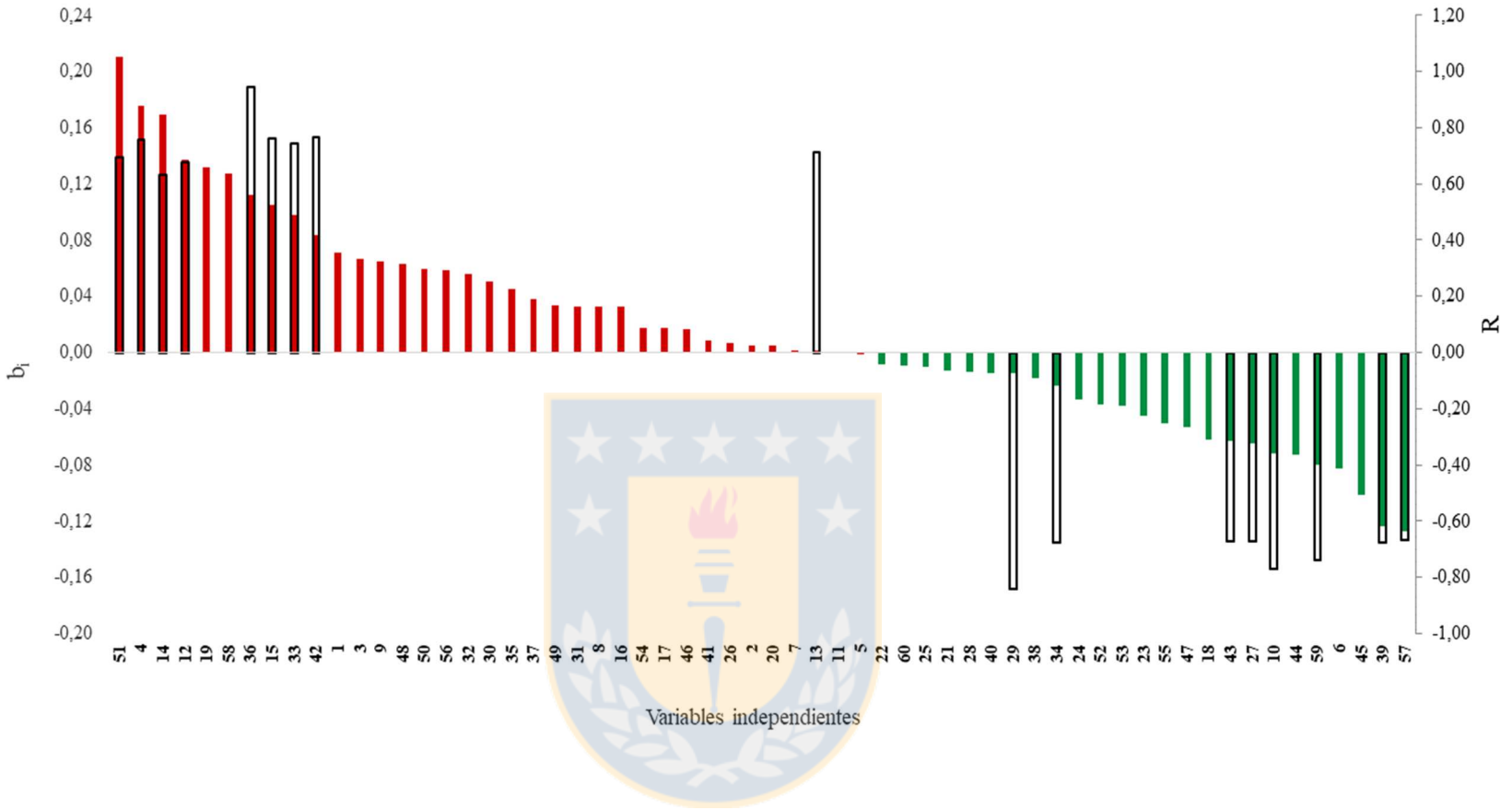

Figure 1: The regression vector of the multivariate model of white wines. The grey bars correspond to the values of the coefficient of the regression vector (bi), and the white bars correspond to the linear correlation coefficient (R) of the variables (Source: elaborated by the author).

From the results obtained, the absolute values of the variables that most contribute to the regression model were classified as antioxidant and pro-oxidants, based on their contributions to •OH production. The ten variables demonstrating the greatest antioxidant effect are 57, 39, 45, 6, 59, 44, 10, 27, 43, and 18, with negative values for b_i. The ten variables demonstrating the greatest pro-oxidant effect are 51, 4, 14, 12, 19, 58, 56, 15, 33, and 42, with positive values for b_i.

4.9.2 Linear correlation

The individual relationship between each independent variable and the dependent variable may be non-linear or related to other variables. As a result, the relationship between these individual variables is not always evident. The 60 variables were individually correlated with •OH production. Of the compounds that demonstrated an antioxidant effect, eight compounds exhibited an inverse linear relationship with •OH production (variables 57, 39, 59, 10, 27, and 43). This finding means that these variables displayed a direct antioxidant effect on •OH production. However, there are variables that were linearly correlated with •OH production (variables 34 and 29), but the values of b_i for these variables were lower than the most significant variables in the multivariate model. This finding means that the antioxidant effect of these variables was linearly dependent upon their concentrations, but that their global effect was not relevant to total •OH production. On the other hand, there were other variables that did not exhibit an inverse linear relationship with •OH production but were relevant to the multivariate model (45, 6, 44, and 18). This effect could be associated with different kinds of interactions. From these analyses, the variables that satisfied both criteria—low values for b_i and a low value of R (Table 2) — were chosen as antioxidants.

Table 2: Variables most important for the chemical oxidation process in white wines, which satisfy both criteria (high values for R and b_i), based on the relationships between the independent variables and •OH production. A high a linear correlation corresponds to high R values, and relevant variables in the multivariate model have high b_i values (Source: elaborated by the author).

	Both criteria	**Linear correlation**	**Multivariate model**
Antioxidants	*cis*-Piceid Ellagic acid Gallic acid Proanthocyanidins * Glucose * $[H^+]$	Syringic acid Gentisic acid	Flavan-3-ol dimers Flavan-3-ol monomers Succinic acid Caftaric acid
Pro-oxidants	Malic acid *p*-Coumaric acid Caffeic acid % Prodelphinidin Procyanidins B1 * Mn * Cu * Zn	Fe	GRP *cis*-Resveratrol

Of the compounds that showed a pro-oxidant effect, eight exhibited a direct linear relationship with •OH production by both criteria (variables 51, 4, 14, 12, 36, 15, 33, and 42). In other words, these variables have a direct pro-oxidant effect on •OH production. However, variable 13 was linearly correlated with •OH production but was not be an important variable in the multivariate model. This means that the pro-oxidant effect of this variable was linearly dependent on its concentration, but its global effect was not relevant to total •OH production. On the other hand, variables 19 and 58 did not show a direct linear relationship with •OH production but were relevant to the multivariate model. These two variables were not individually relevant, but they were relevant in the matrix of real wine. This is the primary reason the regression models were made based on data from real wines, which allowed observation of the interactions between the compounds and their integral functions in the oxidative process of white wines. Variables that satisfied both criteria—high b_i values and a high R value (Table 2)—were chosen as pro-oxidants.

According to the literature (Danilewicz, 2014; Elias & Waterhouse, 2010; Oliveira et al., 2011), the main pathway for chemical oxidation of wine is related to the Fenton process. In these systems, the Fenton reaction promoted by DHBs (Salgado et al., 2018) could play an import role (Danilewicz et al., 2008; Elias & Waterhouse, 2010; Márquez et al., 2018). Different types of DHBs, as well as other phenolic compounds, have been reported to act as pro-oxidants in the Fenton reaction in several matrices (Melin et al., 2015; Tony et al., 2009), though the antioxidant properties of phenolic compounds have also been reported (Perron & Brumaghim, 2009). In fact, there are several reports of different types of phenolic compounds that can exhibit dual behaviour, depending on the conditions and matrices (Castañeda-Arriaga et al., 2018).

Given the structure of the phenolic compounds that exhibit an antioxidant or pro-oxidant effect. Phenolic compounds with a 1,2-DHB structure (caffeic acid, procyanidins, prodelphinidin, and *cis*-GRP) can easily chelate Fe(III), reducing it to Fe(II), and promoting the Fenton reaction. In contrast, DHBs without adjacent hydroxyl groups (gentisic acid, syringic acid, and *cis*-piceid) do not chelate Fe(III), and the oxidative process is decreased. Moreover, organic acids with adjacent hydroxyl groups (tartaric and malic acid) can also chelate Fe(III), forming a labile complex with the metal; in a later step, this complex can be coordinated with 1,2-DHB, promoting the Fenton reaction. To corroborate this, speciation calculations with pro-oxidant and antioxidant compounds, representative of the wines, that can or cannot chelate Fe(III) were performed.

4.10 Speciation calculations

To calculate speciation with Fe(III), a model system that included the pro-oxidant compounds tartaric acid, malic acid, and caffeic acid (1,2-DHB), as well as the antioxidant and succinic acid, and gallic acid (1,2,3-THB), was used. Average concentrations of the concentrations found in real wines were used, allowing the combination to act as a model wine. In addition, the pH was varied from 2.8 to 3.6, based on values observed in real wines. The analysis of the relationship between the metal complexes (MC) and pH is shown in Figure 2.

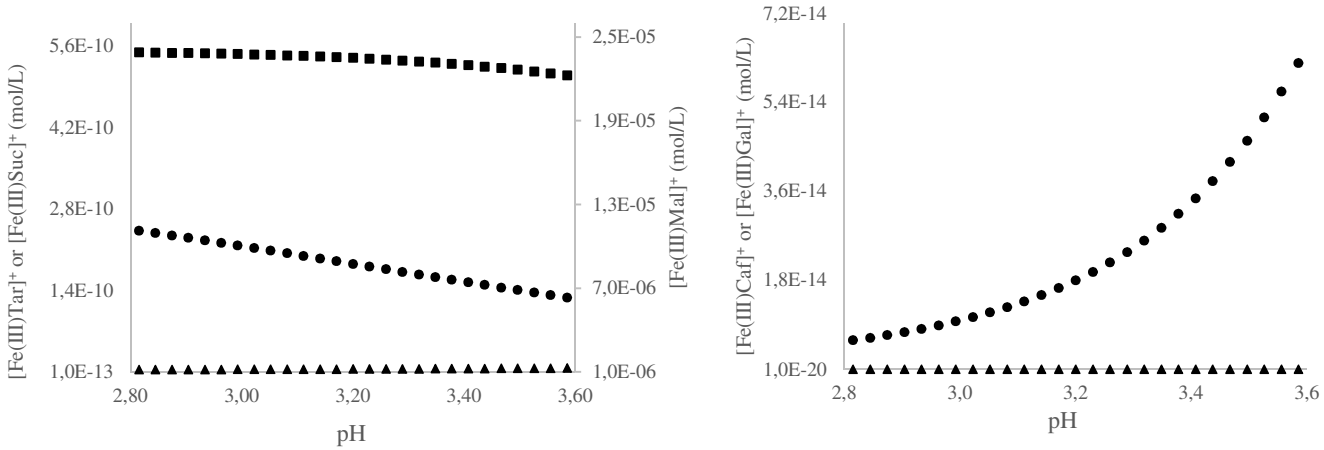

Figure 2: Formation of metal complexes between Fe(III) and representative ligands present in white wines. (a) Organic acids complexes were represented by ■:[Fe(III)Malate]$^{+}$, ●: [Fe(III)Tartrate]$^{+}$, and ▲: [Fe(III)Succinate]$^{+}$, (b) Phenolic compounds complexes were represented by ●: [Fe(III)Caffeate]$^{+}$ and ▲: [Fe(III)Gallate] $^{+}$ (Source: elaborated by the author).

Figure 2 shows that the complexes of organic acids with Fe(III) are present at a higher concentration than those of complexes with phenolic compounds. The calculated concentrations of malic, tartaric, and succinic acids was approximately $2.4x10^{-5}$ mol/L, $2.2x10^{-10}$ mol/L, and $4.0x10^{-12}$ mol/L respectively (Figure 2a). In addition, the Fe(III) complex with caffeic acid reached a concentration of $2.3x10^{-14}$ mol/L, and the complex with gallic acid reached a concentration of $5.2x10^{-21}$ (Figure 2b). It should be noted that almost all the Fe(III) is chelated by the organic acids (especially malic acid). Thus, the Fe(III) is available in solution. These species can react with 1,2-DHB reducing Fe(III) to Fe(II), which is available to react with H_2O_2 by the Fenton reaction. The reactivity of 1,2-DHB with Fe(III) chelated by organic acid has been reported in the literature (Oviedo et al, 2003), and the reduction of Fe(III) by 1,2-DHB inside other stable complexes has also previously been studied (Yang et al., 2014).

4.10.1 Discussion of contribution of the variables

Minor contribution of the variables to the oxidative process

a) Variables that only contributed in linear correlation

As mentioned in section 3.3, there were variables that were only linearly correlated with •OH production (Table 2, high R values) but were not relevant to the multivariate model (low b_i values). Among these variables were Fe, syringic acid and gentisic acid.

Total iron has been linearly correlated with a pro-oxidant effect, a phenomenon that has been reported in multiple studies on model wine systems (Danilewicz & Standing, 2018; Elias & Waterhouse, 2010; Kreitman et al., 2013). However, it should be mentioned that iron is complexed with different components of wine (mainly malic acid, tartaric acid and some phenolic compounds). Therefore, a relationship between all of the iron species and •OH production is not observed. This is logical considering that several iron species are not available to promote the pro-oxidant effect. In contrast, there are stable complexes that sequester iron, thus preventing the oxidative process. Due to this dual role of iron species formed in wine, total iron does not exhibit a direct pro-oxidant relationship with •OH production.

Gentisic and syringic acids also only shows a linear correlation with •OH production, but these are not relevant to the multivariate model. This finding may be observed because these compounds only reduce the efficiency of the oxidation process. Unlike the 1,2-DHBs, these compounds do not form complexes or reduce Fe(III). Therefore, their reduction in •OH production is minor, since they do not eliminate free radicals or iron from the system but, rather, only make the oxidation process less efficient.

b) Variables that only contributed to a multivariate model

In addition, there were variables that were only relevant to the multivariate model (Table 2, high b_i values), but for which it was not possible to establish a linear correlation with •OH production (low R values). Among these variables are GRP, *cis*-resveratrol, flavan-3-ols dimers and monomers, succinic acid, and caftaric acid. This means

that these variables play an indirect role in the oxidative process. In the literature, there is evidence for the participation of GRP in the oxidation of wine, promoted by fungal enzymes, where the oxidation product is quinone-GRP, which could polymerize and produce the browning of the wine (Comuzzo & Zironi, 2013). This reaction has not been reported by chemical oxidation, though it has been proposed that the oxidation of GRP is carried out in the presence of metals due to its 1,2-DHB structure (Salgado et al., 2018).

The pro-oxidant effect of resveratrol at low concentration has been reported (de la Lastra & Villegas, 2007; Singh et al., 2016). These concentrations are similar concentration range to those found in wines (less than 100 µmol/L). At low concentrations, resveratrol is not a free radical scavenger. Rather, the phenoxy radical formed by the radical reaction is stabilised by a conjugated double bond (by a pathway similar to that of *p*-coumaric acid, which is covered in the next section) that promotes the polymerisation and browning of wine (De la Lastra & Villegas, 2007).

The flavan-3-ol dimers and flavan-3-ol monomers variables (Figure 1, variables 44 and 45) possessed significant values for bi. However, these variables include several other variables (catechin, epicatechin, epigallocatechin, etc.) that did not possess significant values for bi or R. Therefore, the antioxidant effect of flavonols occurs mainly synergistically (Ren et al., 2017).

Succinic acid exhibited significant values only for bi as an antioxidant. The iron chelated by this organic acid is not significant (Figure 2). In addition, covariation of this variable has been reported with sugar concentration (primarily glucose), pH, titratable acidity, oxygen and SO_2 (Coulter, Godden, & Pretorius, 2004). Consequently, the antioxidant effect of succinic acid in the multivariate model could be the result of the effect of all of the aforementioned variables.

Finally, caftaric acid has been reported as an antioxidant with high antioxidant activity in several matrices (Newair et al., 2017). In addition, it has been reported that caftaric acid reacts faster than other phenolic compounds as a free radical scavenger (Villaño et al., 2007).

Variables the most contribute to the oxidative process

To understand the effect on •OH production of the variables that contribute most to the oxidative process, both criteria are considered (positive high values for R and b_i). Notable among these variables are the 1,2-DHBs and organic acids with adjacent hydroxyls. A proposal for the pro-oxidant effect of these compounds is depicted in Figure 3. Previously, in section 3.4 (Figure 2a), it was shown that organic acids (mainly malic acid) can chelate Fe(III) (Figure 3, step 2), though these types of complexes are labile (Deiana et al., 2002) and the 1,2-DHBs can be coordinated (Figure 2b) with this type of complex (Figure 3, step 3). In step 4, Fe(III) is reduced to Fe(II) by an inner sphere mechanism, while 1,2-DHB is oxidised to quinone (Figure 3, step 5) (Salgado et al., 2018).

p-Coumaric acid reacts by a different pathway without complex formation, since it possesses only one hydroxyl group attached to the aromatic ring. In spite of this, the *p*-coumaric acid has a branch with a double bond in the -para position, which allows stabilisation of the phenoxyl radical and subsequent oxidation (Figure 3, step 6) (Li et al., 2012). Therefore, in steps 3 to 5 and 6, the Fe(III) is reduced to Fe(II), after which Fe(II) will be available for the Fenton reaction (Figure 3, step 1). Hence, organic acids and DHBs with adjacent hydroxyls, together with *p*-coumaric acid, promote the reduction of Fe(III) and, therefore, •OH production. In summary, through of this scheme can be explained the pro-oxidant effect of the variables that most contributed to the oxidative process of white wines (Table 2, both criteria). These variables are malic acid, *p*-coumaric acid, caffeic acid, % prodelphinidin, and procyanidins B1. Other relevant variables with a pro-oxidant effect are the concentrations of Mn, Cu, and Zn, respectively. These metals have been described as catalysts in Fenton-like systems (Tony et al., 2009). The overall concentration of these metals shows a significant linear correlation (Figure 3S in the supplementary material) with •OH production. This is better than the R values for each individual variable that was within the range of 0.631 to 0.763 (Figure 1, white bars). Hence, these metals would be acting as catalysts for promoting •OH production (variables highlights with an asterisk in table 2).

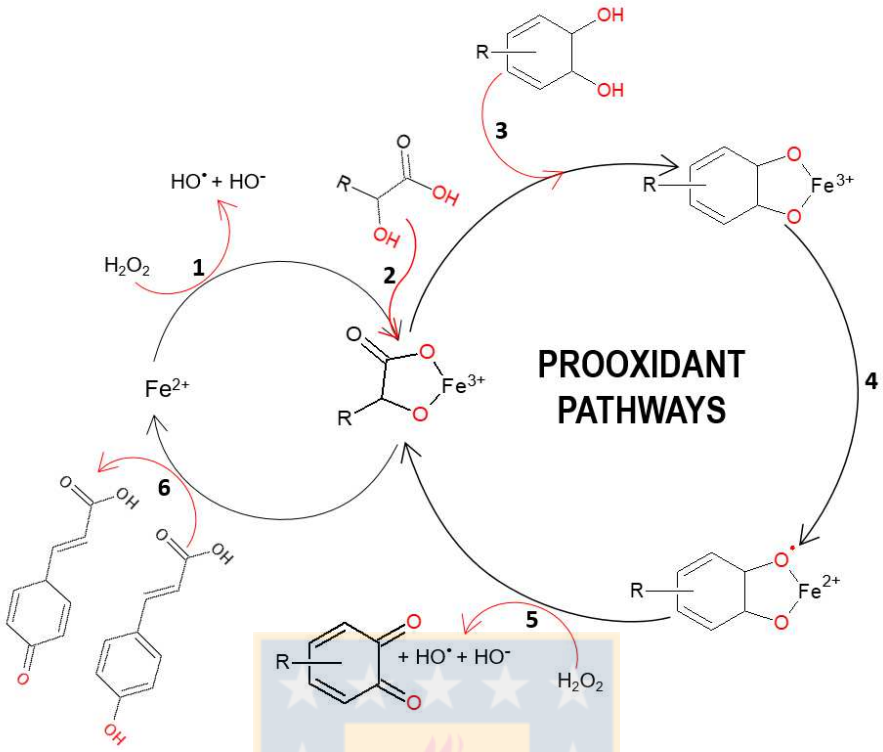

Figure 3: Scheme proposed to explain the possible pro-oxidant pathways of the variables that most contribute to •OH production (Source: elaborated by the author).

Conversely, for the variables that most contribute to the multivariate model and share an inverse linear correlation with •OH production (and have thus been considered to have an antioxidant effect). Both criteria are considered (negative high values for R and b_i). These variables are *cis*-piceid, ellagic acid, glucose, [H^+], gallic acid, and proanthocyanins. The antioxidant effect of these variables was associated with the position and number of hydroxyl substituents and the concentration range in the wines. Based on their antioxidant mechanisms, they can be classified into two groups: (a) phenolic compounds (THB and DHB) with free radical scavenger properties (Cassino et al., 2016; Dalvi et al., 2017), and (b) phenolic compounds with adjacent hydroxyls with the properties of being Fe(III) chelating and redox inert, which remove Fe(III) from the solution (Perron & Brumaghim, 2009) and hence do not promote •OH production. Another relevant variable with antioxidant properties is glucose, which is a reducing sugar whose antioxidant effect can be associated with its redox property. In addition, it is reported in the literature that, at high concentrations of glucose and fructose, oxidative stress in wines was reduced (Pazzini et al., 2015). pH

is also a relevant variable, and the increase in [H^+] causes the oxidative process to be less effective. This is explained by the fact that the Fenton reaction driven by DHBs is the main pathway for the oxidative process, with an optimum pH is 3.4 (Contreras, et al., 2006). At lower pH, the formation of the mono hydroxo-complex with Fe(III) ($Fe(OH)^+$) is not favoured and, therefore, the exchange of H_2O_2 is also less favoured, decreasing $^{\bullet}OH$ production (Salgado et al., 2018). Therefore, the increase in glucose and H^+ concentrations decrease the $^{\bullet}OH$ production, that is, they make the oxidative process less efficient (variables highlights with an asterisk in table 2).

CONCLUSIONS

From multivariate analysis and linear correlations with $^{\bullet}OH$ production, three groups of variables were observed: (a) variables that only show a linear correlation with $^{\bullet}OH$ production, (b) variables that only contribute to the multivariate model, and (c) variables that satisfy both criteria (high values for R and bi).

The group of variables that satisfy both criteria are the variables that contribute most to the oxidative process in white wines. To understand the prooxidant effect of these variables, a scheme for the reduction of Fe(III) by compounds with vicinal hydroxyl (malic acid, caffeic acid, prodelfinidines and procyanidins B1), together with p-coumaric acid has been proposed. In addition the metals Mn, Cu and Zn, would be acting as catalysts in Fenton-like systems, promoting the $^{\bullet}OH$ production.

On the other hand, the variables with higher antioxidant effect can be explained according to two antioxidant mechanisms (a) phenolic compounds with free radical scavenger properties, and (b) phenolic compounds that chelate Fe(III) and are redox-inert, remove the Fe(III) from the solution and hence do not promote $^{\bullet}OH$ production. The variables can be explain by these two mechanisms are cis-piceid, ellagic acid, gallic acid, and proanthocyanidins. Also, the increase in glucose and H^+ concentrations decrease the $^{\bullet}OH$ production, that is, they make the oxidative process less efficient.

SUPPLEMANTARY MATERIAL

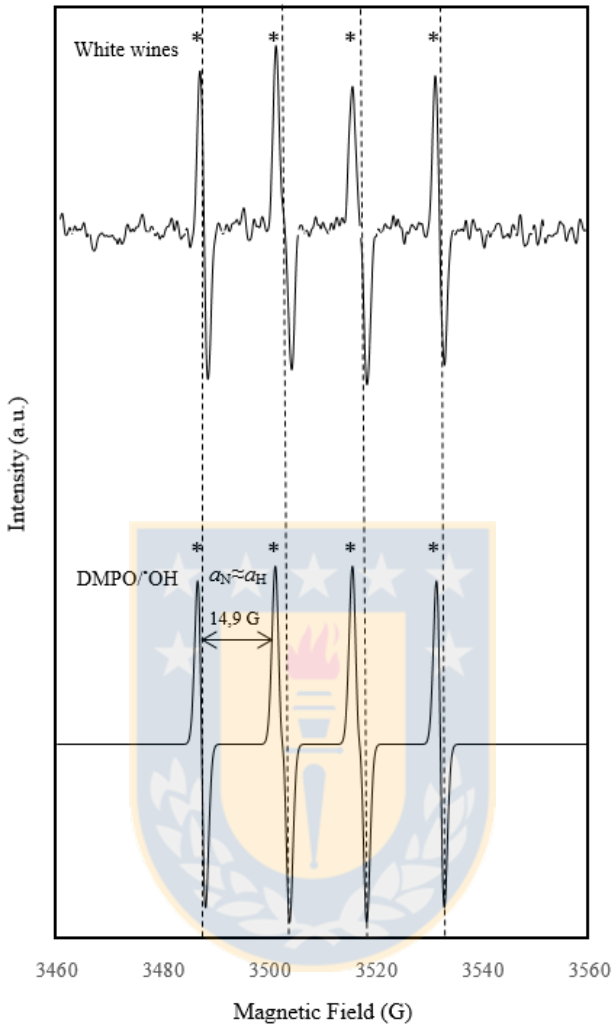

Figure 1S: Identification of EPR signal. The EPR spectrum above corresponds to a representative spectrum for the 18 white wines. The EPR spectrum below corresponds to the DMPO/·OH adduct signal simulated by Bruker's Spin-Fit software (Source: elaborated by the author).

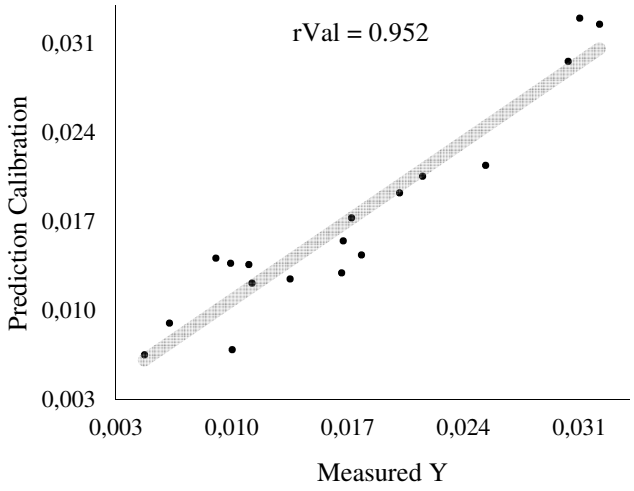

Figure 2S: Correlation between experimental values and values predicted by the PLS-model for white wines (Source: elaborated by the author).

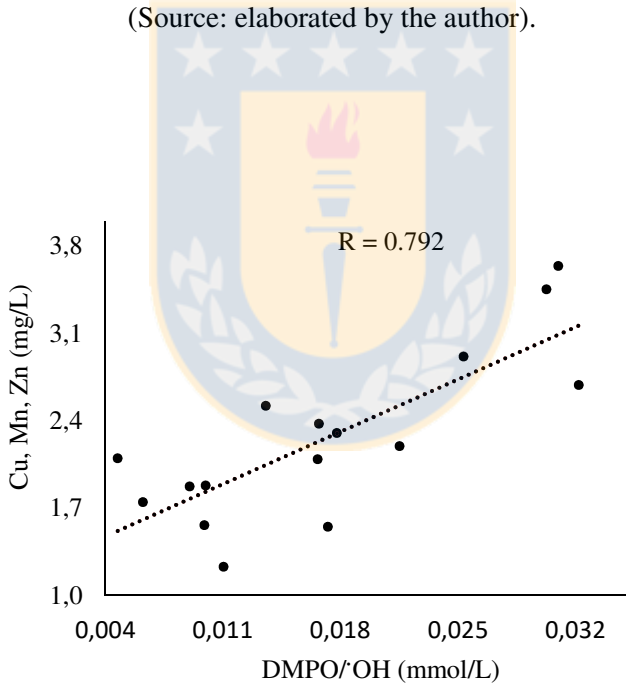

Figure 3S: Synergistic effect of most important metals by multivariate model (Source: elaborated by the author).

Table 1S: Determination of flavonols and hydroxycinnamic acids by HPLC-ESI-MS/MS (Source: elaborated by the author).

	(mg/L)								(µmol/L)	
Wine	**trans-GRP**	**caftaric acid**	**cis-GRP**	**cis-cutaric acid**	**trans-cutaric acid**	**p-coumaroyl-glc**	**trans-fertaric acid**	**cis-fertaric acid**	**Q-3-glcU**	**Q-3-glc**
A	9.91	6.88	0.47	3.19	2.86	7.41	1.14	0.13	0.52	2.84
B	7.62	6.20	0.50	3.68	3.77	4.45	0.65	0.08	1.14	2.11
C	0.46	22.20	0.73	4.53	2.28	10.47	4.81	0.34	2.29	nd
D	10.00	2.92	0.65	1.91	1.44	1.85	0.56	0.06	0.44	1.52
E	13.20	7.97	0.66	4.70	3.73	2.65	0.88	0.15	0.26	1.57
F	4.88	4.65	0.37	3.11	0.89	3.71	0.96	0.14	0.54	0.36
G	13.64	1.98	1.13	1.96	0.88	4.33	0.88	0.10	0.36	1.06
J	2.92	2.10	0.32	1.03	0.54	4.42	1.09	0.10	1.87	0.25
K	4.93	3.35	0.48	1.77	0.65	2.63	1.24	0.15	0.62	0.59
L	6.44	4.33	0.54	2.48	0.88	4.06	0.62	0.12	0.33	0.55
M	0.43	1.52	0.26	0.60	0.76	2.62	0.44	0.04	0.57	nd
N	10.21	3.13	0.39	2.79	2.17	2.86	0.57	0.02	0.30	0.51
O	6.94	3.92	0.42	1.42	1.42	6.92	1.75	0.10	1.40	0.41
P	2.77	1.06	0.85	1.31	0.92	4.57	0.77	0.05	1.15	0.21
Q	6.57	3.32	0.37	4.10	1.19	3.10	0.94	0.18	0.23	1.67
R	6.09	2.02	0.45	0.61	0.72	4.47	0.76	0.13	0.53	1.49
S	1.82	2.83	0.47	1.03	0.92	4.75	0.49	0.04	0.43	0.20
T	0.44	4.49	0.09	2.27	1.33	3.19	0.26	0.08	0.26	nd

*GRP: Grape Reaction Product; glc:glucose; glcU: glucuronide; nd = not detected.

Table 2S: Determination of flavan-3-oles, proanthocyanidins and stilbenes by HPLC-MS-MRM (Source: elaborated by the author).

	Percentage (%) and concentrations (mg/L)																		
Wine	**mDP**	**%Ga**	**%P**	**PC**	**FM**	**FD**	**SB**	**EC**	**GC**	**EGC**	**MG**	**PB1**	**PB2**	**PB 4**	**OD**	**GD**	**t-RG**	**c-RG**	**c-RV**
A	1.37	nd	6.51	4.02	4.37	0.55	0.53	13.4	2.76	nd	45.7	60.9	22.6	2.96	13.6	nd	22.8	48.4	28.9
B	1.20	nd	22.3	3.07	3.03	0.34	0.35	16.3	8.23	0.98	40.6	46.5	28.2	11.1	13.9	0.21	24.4	46.7	28.9
C	1.15	nd	nd	0.18	0.64	nd	0.42	5.66	nd	nd	76.8	nd	nd	nd	nd	100	35.2	56.6	8.20
D	1.16	0.33	20.5	1.14	1.15	0.09	0.11	14.5	6.33	nd	56.7	33.2	45.5	nd	21.3	nd	33.7	66.3	nd
E	1.20	0.28	25.3	5.30	4.55	0.53	0.57	16.1	9.97	0.96	33.2	46.6	19.8	11.8	21.7	nd	26.5	59.7	13.8
F	1.11	nd	30.0	0.54	1.50	0.02	0.33	8.66	7.82	nd	38.1	81.9	nd	nd	13.4	4.74	35.4	39.0	25.6
G	1.26	nd	44.3	3.86	4.82	0.45	0.97	13.6	18.6	2.44	17.8	58.2	19.0	5.27	17.5	nd	12.9	17.6	69.5
J	1.10	0.36	6.56	1.09	2.46	0.03	0.34	5.45	3.07	nd	64.9	66.0	34.0	nd	nd	nd	39.9	42.2	17.9
K	1.52	nd	nd	0.18	0.51	nd	0.06	nd	nd	nd	90.6	nd	nd	nd	nd	nd	47.0	53.0	nd
L	1.10	nd	11.2	0.43	0.83	0.04	0.16	8.75	7.07	nd	68.5	62.7	28.9	nd	8.44	nd	48.9	51.1	nd
M	1.20	nd	nd	0.24	0.41	0.01	0.19	nd	nd	nd	80.6	100	nd	nd	nd	nd	32.9	37.8	29.3
N	2.00	1.62	24.2	6.31	5.92	1.41	0.29	20.9	13.9	1.31	18.0	55.6	21.4	7.22	14.7	1.12	30.2	47.3	22.5
O	1.93	1.57	15.3	4.64	5.60	0.69	0.62	14.5	6.60	0.79	32.0	49.2	19.9	8.22	21.1	1.55	30.4	46.8	22.8
P	1.68	1.37	36.9	2.54	2.52	0.21	0.26	10.0	18.1	2.32	28.7	59.8	15.8	8.03	15.9	0.39	39.7	38.6	21.7
Q	1.67	3.29	nd	0.23	0.72	0.02	0.08	7.61	nd	nd	56.4	60.9	39.1	nd	nd	nd	56.0	44.0	nd
R	1.28	0.34	7.54	0.54	1.93	0.05	0.51	8.79	3.48	nd	45.2	63.6	35.1	nd	nd	1.33	17.4	54.6	28.0
S	1.30	0.05	28.5	2.37	3.56	0.30	0.57	13.0	9.75	2.03	37.9	43.8	22.0	14.9	19.3	nd	19.7	40.9	39.4
T	1.67	0.14	21.6	3.41	3.33	0.42	0.15	11.2	10.2	1.72	26.8	59.9	18.4	5.88	15.8	nd	44.3	55.7	nd

* mDP: medium degree of polymerization; %Ga: %Galloylation; %P: %Prodelphinidin; PC: Proanthocyanidins; FM: Flavonol monomers; FD: Flavonol dimers; SB: Stilbenes; EC: Epicatechin; GC: Galocatechin; EGC: Epigallocatechin; MG: Monoglucosides; PB: Procyanidin; OD: Other dimers; GD: Galolated dimers; RG: Resveratrol glycoside (trans or cis-piceid); c-RV: Cis-Resveratrol; nd = not detected.

Table 3S: Determination of other phenolic compounds by HPLC-DAD (Source: elaborated by the author).

	Concentrations (mg/L)												
Wine	Gallic acid	3,4-DHB acid	Gentisic acid	p-HB acid	Catechin	Vanillic acid	Caffeic acid	Syringic acid	Vanillin	p-coumaric acid	Ferulic acid	Rutin	Ellagic acid
A	5.50	nd	53.32	103.4	0.97	6.17	nd	21.5	1.87	0.34	1.81	8.50	nd
B	4.10	nd	nd	91.2	nd	10.20	0.83	12.9	1.55	1.82	2.02	3.45	nd
C	4.09	11.69	12.75	40.1	2.48	22.08	1.16	23.3	2.37	0.30	nd	1.01	0.82
D	3.29	23.30	20.87	98.1	nd	5.87	nd	7.0	1.87	nd	nd	2.52	0.51
E	4.76	nd	79.57	154.8	nd	10.49	nd	7.8	1.99	2.03	nd	6.73	0.76
F	3.87	nd	22.43	75.2	0.73	nd	0.94	14.6	2.83	nd	1.64	0.38	0.68
G	6.23	nd	27.22	56.6	0.82	nd	2.31	10.8	nd	nd	nd	nd	0.58
J	8.28	15.77	73.28	nd	3.11	12.85	0.89	21.8	3.16	nd	nd	nd	nd
K	8.87	nd	16.11	79.0	0.79	5.79	nd	7.0	nd	0.40	0.56	0.35	2.04
L	5.03	22.44	17.78	101.1	nd	nd	0.65	12.0	1.60	nd	nd	0.15	0.72
M	4.24	nd	nd	nd	2.01	9.37	0.57	14.8	2.90	0.51	0.41	0.31	0.69
N	3.97	nd	24.96	119.0	nd	7.97	1.50	9.2	1.83	nd	nd	1.24	nd
O	12.13	nd	81.62	nd	1.07	1.73	2.40	21.3	1.63	0.37	1.18	nd	1.29
P	1.87	0.60	18.93	48.4	1.84	16.89	0.61	18.5	1.72	nd	nd	1.45	0.28
Q	4.29	nd	42.76	121.9	0.62	nd	1.19	10.6	2.06	0.44	1.74	3.43	0.50
R	4.03	nd	29.37	70.9	nd	nd	nd	13.0	2.35	nd	nd	5.02	255.54
S	5.72	nd	15.67	nd	1.36	nd	0.67	11.8	2.42	nd	nd	nd	nd
T	4.75	3.68	41.85	32.3	1.06	2.43	1.09	6.0	4.54	nd	0.63	nd	371.89

*DHB: dihydroxybenzene; HB: hydroxybenzoic; nd = not detected.

Table 4S: Determination of main organic acids and sugars in wines by HPLC-RI (Source: elaborated by the author).

	Concentrations (mg/L)								
Wine	**Tartaric acid**	**Malic acid**	**Citric acid**	**Succinic acid**	**Lactic acid**	**Acetic acid**	**Shikimic acid**	**Glucose**	**Fructose**
A	2020	1695	165	215	155	290	27.05	2.11	2.19
B	2625	2693	250	180	185	250	26.15	2.54	0.15
C	2775	966	260	200	180	1120	18.30	32.53	90.10
D	3521	1714	120	145	170	310	16.90	1.58	0.30
E	2741	1238	145	200	145	260	22.70	1.26	0.59
F	2638	1531	50	250	375	185	nd	3.36	2.70
G	3188	1741	110	240	235	385	23.65	1.57	0.67
J	2263	1253	nd	90	380	940	nd	38.10	93.65
K	3414	673	80	145	230	595	7.15	0.18	0.07
L	2586	2197	115	200	265	385	10.75	0.63	0.90
M	1560	1285	370	nd	160	550	9.10	25.94	66.83
N	3417	2385	180	235	195	410	25.30	1.91	0.20
O	2427	1254	40	105	120	765	11.20	19.04	69.26
P	3550	2215	280	175	nd	1340	64.50	25.32	85.76
Q	1812	1441	95	465	745	305	27.45	nd	nd
R	1735	1163	420	460	700	nd	19.65	45.54	54.26
S	1786	1163	265	180	nd	1030	6.55	28.92	76.88
T	1835	1215	545	525	165	205	29.05	53.24	61.7

*nd = not detected.

Table 5S: Validation parameters for the organic acids and sugars determination method in white wines (Source: elaborated by the author).

Parameters	**Tartaric acid**	**Malic acid**	**Citric acid**	**Succinic acid**	**Lactic acid**	**Acetic acid**	**Shikimic acid**	**Glucose**	**Fructose**
λ detection	210	210	210	210	210	210	210	210	210
Linearity	0.987	0.997	0.991	0.974	0.996	0.970	0.994	0.984	0.986
CV (%)	3.500	2.800	2.950	3.900	2.870	3.190	3.050	2.860	2.830
LOD (g/L)	0.158	0.088	0.012	0.029	0.036	0.046	0.002	0.360	0.058
LOQ (g/L)	0.528	0.294	0.040	0.097	0.120	0.152	0.006	1.201	0.193

Table 6S: Determination of metals, sulphur dioxide and proton concentration by several methods (Source: elaborated by the author).

	(mg/L)								(mmol/L)
Wine	**Al**	**Cu**	**Fe**	**Mn**	**Zn**	**S**	**Free SO_2**	**Total SO_2**	**$[H^+]$**
A	2.23	0.04	0.79	1.00	1.27	90.3	7.7	62	0.692
B	1.65	0.06	0.79	1.16	1.69	115	9.6	71	0.575
C	1.29	0.06	1.62	2.64	1.53	189	12.3	113	1.350
D	0.65	0.06	0.92	0.92	1.11	98.7	12.0	73	0.813
E	0.72	0.32	0.76	0.92	1.28	106	5.6	34	1.320
F	0.70	0.19	1.24	0.94	1.06	97.3	6.6	48	1.100
G	1.05	0.23	1.19	1.38	2.04	167	6.6	52	0.631
J	0.85	0.10	2.38	0.55	0.57	190	11.7	80	1.000
K	0.61	0.11	0.70	0.86	0.78	135	7.5	57	0.955
L	0.57	0.13	1.02	0.76	0.66	125	6.0	55	0.589
M	1.07	0.16	2.01	2.62	0.67	149	14.1	100	0.282
N	1.04	0.03	0.92	0.70	0.84	102	10.7	73	0.550
O	1.87	0.17	0.94	1.16	1.05	188	9.0	147	0.676
P	1.18	0.15	1.40	1.37	1.16	312	13.2	183	0.251
Q	0.74	0.07	1.20	1.37	5.96	135	7.4	52	0.661
R	1.44	0.06	2.39	1.39	0.66	162	5.4	50	0.550
S	0.93	0.08	1.18	1.02	0.77	202	7.5	166	0.389
T	2.26	0.07	2.82	1.26	0.54	314	12.9	217	0.372

Table 7S: Validation parameters for the metals determination method in white wines (Source: elaborated by the author).

Parameters	**Al**	**Cu**	**Fe**	**Mn**	**Zn**	**S**
λ (nm)	396.15	324.75	238.20	257.61	213.86	181.98
Linearity (R^2)	0.999	0.999	0.999	0.999	0.999	0.999
CV (%)	1.33	1.49	0.38	0.56	0.35	0.78
LOD (g/L)	0.020	0.009	0.005	0.014	0.056	0.341
LOQ (g/L)	0.066	0.028	0.015	0.047	0.186	1.136

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CHAPTER 5: Final conclusions

1. The kinetics of radical production was followed for 90 minutes by EPR spectroscopy using DMPO as spin trapping. Where the only identified species was the DMPO/•OH adduct. The samples showed three different kinetic profiles. First, the decay profile for DMPO/•OH adduct concentration. This behaviour has been associated with the presence of an oxidant compound (such as H_2O_2) in the solution that reacts with the DMPO/•OH adduct a reported in the literature. The second profile shows DMPO/•OH adduct with a concentration that was constant along the reaction time and the last profile DMPO/•OH adduct was increased over the assayed reaction period. Hence, for all samples tested, the maximum amount of radical produced was considered.

2. In all the white wines studied, •OH was identified and quantified induced by a short air bubbling period. The oxidation of wine depends on the amount and type of pro-oxidant compounds present in it. Hence, wines with different chemical composition generated significantly different •OH concentrations.

3. Relationships between the geographical origin, type of wine and the grape variety with the •OH production were searched. However, in this study, it was not possible to establish any relationship between •OH production and these three variables. As a consequence, it could be established that the •OH production in the wines did not only depend on the productive process and variety of grape, but also depends on a complex mixture of chemical components.

4. The speciation of Fe(III) in model wines was studied. The effect of tartaric acid, malic acid and SO_2 in wines was analysed. In all of these investigations, no relationship was found with •OH production. At all concentration ranges of organic acids in the wines, iron was completely chelated. Additionally, SO_2 concentration was not significantly different in its antioxidant effects on the wines. This could be explained due to SO_2 can react with H_2O_2 and quinones, reducing the oxidation of wines. Therefore, the antioxidant effect of SO_2 has several pathways and is not necessarily a linear relationship.

5. The total and the individual phenolic compounds concentration were related to •OH production. Five linear relationships between the phenolic compounds and •OH production were found. The concentrations of protocatechuic acid, caffeic acid, and p-coumaric acid have a direct linear relationship with •OH production. Conversely, an inverse linear relationship between the concentrations of gentisic acid and syringic acid with •OH production were found. The linear relationships showed lower or upper limits for linearity. This limit value was called the "critical concentration". Hence, the concentration range of the phenolic compounds is a key parameter that determined the increased or abating of •OH production.

6. A scheme 1 (chapter 3) for the chemical oxidation of white wine has been proposed. This scheme explains the possible pathways of the oxidation of wine through of Fenton reaction. The first step for oxidation is the reaction between Fe(III) and some DHBs, to generate SQ•. Later SQ• is oxidized to quinone by oxygen that is reduced to H_2O_2. Thus, there are H_2O_2 and Fe(II) available for Fenton reaction was performed. The main product of Fenton reaction is •OH. These radicals react with the major components of the wine, damaging its organoleptic properties. On the other hand, the quinone could be reduced to its respective DHB by HSO_3^- that oxidized to SO_4^{2-}. Hence, the Fenton reaction as a pathway of the chemical oxidation of white wines was postulated.
7. The chemical characterization of wines allows a wide range of variables to be included in the multivariate model to relate them to the •OH production. For this reason, the wines were chosen with different production processes, geographical area, and grape variety. The concentration of all these compounds (phenolic compounds, SO_2, pH, organic acids, and metals) constituted 60 independent variables (**X** matrix) for each wine. In the PLS model, the relative contribution of the independent variables in the •OH production was analyzed through the regression vector. In addition, the individual correlations of these variables with the •OH production were considered. Then both criteria were selected (absolute value of R and high bi) to choose the variables that contribute most to the oxidative process and were classified as antioxidants and pro-oxidants, according to their relationship with •OH production.

8. From the multivariate analysis and linear correlations with the •OH production, 3 groups of variables are observed: (a) variables that only show a linear correlation with •OH production (high R values), (b) variables that only contribute to multivariate model (high bi values), and (c) variables that meet both criteria (high values for R and bi). This last group of variables is the most contributing to the oxidative process of white wines. It is proposed scheme 2 (chapter 4) allows to explain the pro-oxidant effect of the variables that most contribute to this study. In addition, the antioxidant effect can be explained according to two mechanisms: (a) phenolic compounds with the sequestering property of free radicals, and (b) phenolic compounds that chelate Fe(III) and are redox-inert.

CHAPTER 6: Complementary research about Fenton reaction

Manuscript accepted and published in New Journal of Chemistry, 2017, 41, 12685-12693.

Experimental and computational investigation of the substituent effects on the reduction of Fe^{3+} by 1,2-dihydroxybenzenes.

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Available in: https://doi.org/10.1039/C7NJ01322A

INTRODUCTION

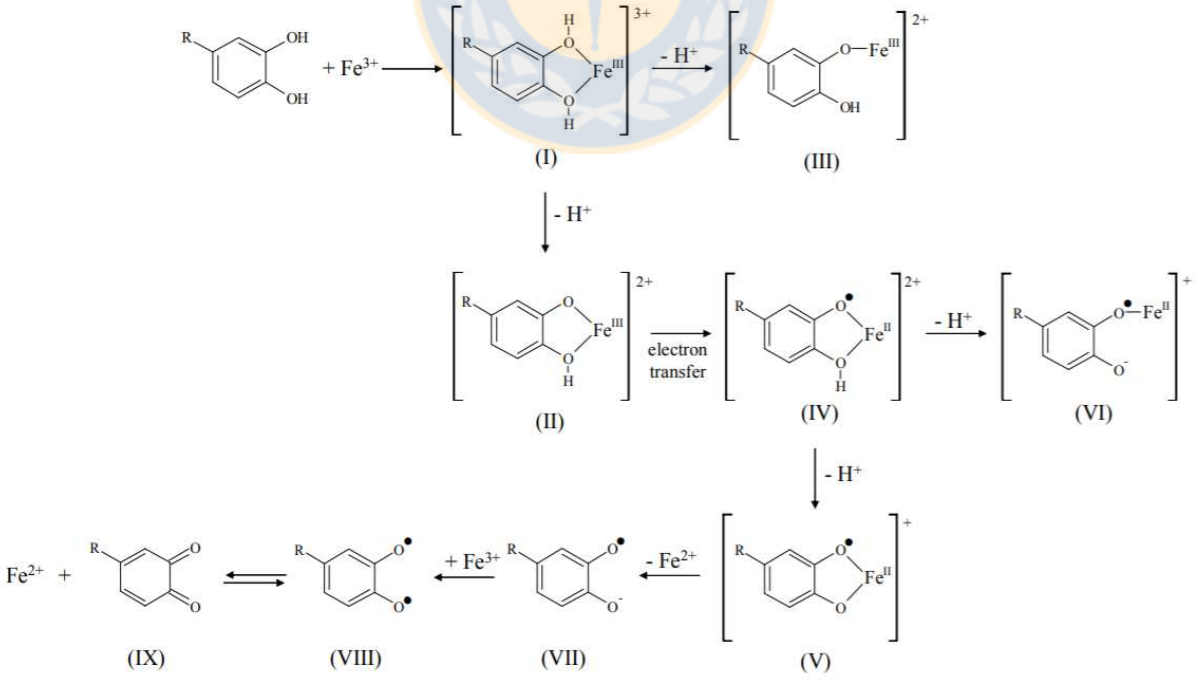
Siderophores are ferric ion-specific chelating compounds produced by bacteria and fungi growing under limited availability of iron. They are low molecular weight compounds (600-1500 Daltons), which chelate ferric iron with an extremely high affinity, and the complex is actively transported for instance across the outer and inner membranes of gram-negative bacteria (Lankford & Byers, 1973). There are two main classes of siderophores: catechols and hydroxamates. Catechol-type siderophores chelate ferric iron via hydroxyl groups, and hydroxamate-type siderophores chelate ferric iron via a carbonyl group with an adjacent nitrogen. After complexation only catechol-type siderophores can reduce Fe^{3+} to Fe^{2+} at acidic pH (Arantes et al., 2011; Goodell et al., 1997).

In microorganisms present in some plants, such as rot fungi, it was shown that oxalic acid is produced for sequestration of Fe^{3+} and lowering of pH in a region close to the hyphae (Hyde & Wood, 1997). These rot fungi are also able to produce catechol-type ligands, which uptake the Fe^{3+} sequestered by oxalic acid and reduce it to Fe^{2+}. These ions further react with enzymatically produced H_2O_2 (Espejo & Agosin, 1991; Koenigs, 1974) to yield ·OH radicals (Fenton reaction), which degrade the wood and the byproducts generated are uptaken by the fungi (Qian et al., 2002; Zhu et al., 2016). In addition, some neurodegenerative diseases, such as Alzheimer and Parkinson, are related to the presence of Fe^{3+} and catecholamines like dopamine, epinephrine, and norepinephrine 9-11 (Bisaglia et al., 2014; Jomova & Valko, 2011; Surowka et al., 2015), highlighting the relevance of understanding the interactions between catechol-like ligands and Fe^{3+}.

Much effort has been devoted to study the process of Fe^{3+} reduction by polyphenols. The model compounds most investigated were catechol derivatives (Agui□r et □l., 2007; Kristinová et □l., 2009; Linert et □l., 1991; Ment□sti et al., 1976; Xu & Jordan, 1988). Several mechanisms have been proposed in the literature to account for the Fe^{3+} reduction by 1,2-dihydroxybenzene (1,2-DHB). The most accepted mechanism is shown in Scheme 1. In the first reaction step the catechol forms 1:1 complex with Fe^{3+} (species I). This type of bidentate complex was proposed by Hider et al. (Hider et al., 1983) for catechol and by Kristinová et al. (Kristinová et al., 2009) for three carboxylic acids derived from catechol: caffeic, ferulic, p-coumaric acids. The complexes further suffer deprotonation to yield

species II. This process is followed by an inner-sphere electron transfer from ligand to metal leading to formation of the semiquinone-Fe^{2+} complex, i.e, a hydroxyphenoxyl radical (species IV), as proposed by Kristinová et al. (Kristinová et al., 2009) for the carboxylic acids and by Hynes and O'Coinceanainn (Hynes & O'Coinceanainn, 2004). The reaction then proceeds again by deprotonation to yield an ortho-semiquinone radical V (Hynes & O'Coinceanainn, 2004). This reactive radical finally loses Fe^{2+} leading to species VII and is oxidized either by Fe^{3+} (Hynes & O'Coin□e□n□inn, 2004; Kristinová et □l., 2009) or oxygen in one or more steps (Kristinová et al., 2009) to yield the biradical VIII, in equilibrium with quinone IX.

A reaction intermediate with absorption maximum at around 700 nm was detected and assigned by some authors to species II (Ch□rkoudi□n & Fr□nz, 2006; Hynes & O'Coin□e□n□inn, 2004; Kristinová et □l., 2009; Perron & Brumaghim, 2009) and by others to species IV (R. C. Hider et al., 1983; Perron & Brumaghim, 2009; Powell & Taylor, 1982) Although not considered in the literature, deprotonation of species I could lead to a monodentate complex of Fe^{3+} (species III). Similarly, the monodentate complex between Fe^{2+} and the semiquinone (species VI) could be formed upon deprotonation of species IV.



Scheme 1: Proposed mechanism for the reduction of Fe^{3+} by 1,2-DHB (Source: elaborated by the authors).

The stability constants of the complexes of transition metals with 4-substituted catechols were shown to depend on the electron withdrawing ability of the substituent (Jameson & Wilson, 1972). In particular, Nurchi et al. (Nurchi et al., 2009) investigated the equilibria of iron (III) with catechol and its 4-nitro derivative by potentiometric, spectrophotometric and NMR spectroscopy. These authors found that the inductive and resonance properties of the nitro substituent led to a general decrease in the complex formation constants. This background motivated us to make a systematic study of the substituent effect of the reaction between 4-substituted catechols and Fe^{3+} in acid medium. To this purpose we obtained kinetic and spectroscopic information on these reactions by means of the stopped-flow technique. A reaction mechanism is proposed with the aid of the effect of the electron-withdrawing ability of the substituent on the rate constants of the three observed processes. Density functional theory (DFT) calculations of molecular structures, vibrational frequencies, thermodynamic properties and absorption spectra of the relevant species proposed in the reaction mechanism helped us to understand the participation in the reaction mechanism of mono- and bidentate species with different protonation degree.

EXPERIMENTAL

6.1 Materials and methods

Catechol, 4-methylcatechol, 4-ethylcatechol, 4-terbuthylcatechol, 3,4-dihydroxybenzonitrile and ferric nitrate ($Fe(NO_3)_3 \cdot 9H_2O$) were purchased from Sigma-Aldrich; whereas 3,4-dihydroxybenzoic acid, 3,4-dihydroxy-benzaldehyde, 4-nitrocatechol, and nitric acid (HNO_3) were purchased from Merck. All solutions were prepared with deionized water, under Argon atmosphere. Besides, all reactions were carried out at an ionic strength of 0.50M KNO_3.The pH of each solution used was adjusted using a 3 Start Thermo Orion pH meter.

6.2 Kinetic Studies

A Hi-Tech Scientific SFA-20 Rapid Kinetics stopped-flow accessory was employed. The absorbance was measured with a sensitive detection system used for flash-photolysis experiments (Berkovic et al., 2010). Briefly, the analysis light from a 150 W Xe arc lamp was passed through a monochromator (PTI1695) and detected by a 1P28 PMT photomultiplier. A 10 mm path length quartz cuvette was employed. Decays typically represented the average of 5 signals and were digitized by and stored in a 100 MHz Rigol DS1102E oscilloscope.

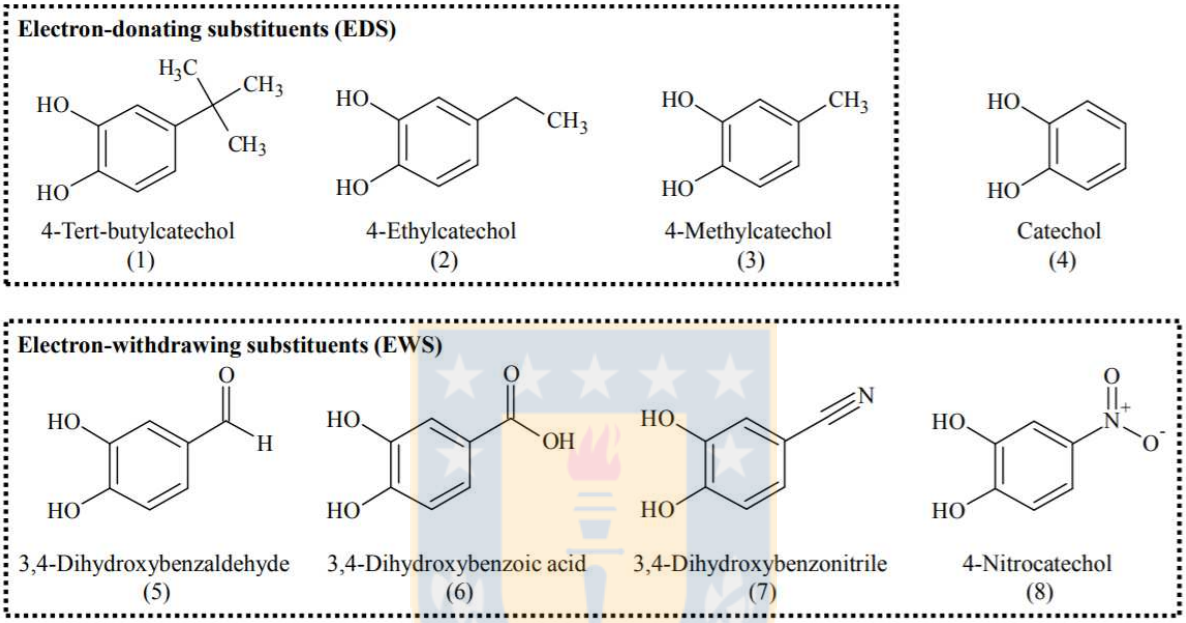
6.3 Quantum-chemical calculations

The equilibrium structures, harmonic vibrational frequencies, total electronic energies, Gibbs free energies and electronic transitions of the radical and radical cation intermediates were calculated at the O3LYP/6-311++G(d,p) level of the DFT (Cohen & Handy, 2001). This hybrid three-parameter functional is formed by the local exchange functional OPTX (Hoe et al., 2001) and the well-known correlation functional LYP (Lee, et al., 1988). Solvent effects were accounted for by using the conductor-like polarizable continuum model, CPCM (Barone & Cossi, 1998), with a relative permittivity for water of 78.3553. The time-dependent density functional theory (TDDFT) was employed to estimate the vertical excitation energy and the oscillator strength *f* for each transition. This last magnitude provides information on the band intensity and depends on electronic, Frank-Condon and spin factors. Up to forty electronic transitions were required to cover the experimental absorption spectra of the species investigated. The oscillator strength was estimated for II, III, IV, V, VI, VII and VIII species (Scheme 1). Note that species II and IV cannot be distinguished in the calculations because they only differ by an internal charge transfer, i.e., they have the same charge and multiplicity. For all calculations the Gaussian 09 package was employed (Frisch et al., 2009).

RESULTS AND DISCUSSION

6.4 UV-visible absorption spectra

The substituted 1,2-DHB studied here are shown in Scheme 2.



Scheme 2: Structure of the substituted 1,2-DHB investigated in this work (Source: elaborated by the authors).

Solutions of Fe^{3+} containing 1,2-DHB with electron donating substituents (EDS) show immediately after the mixture an absorption band at around 700 nm and an additional band at around 400 nm very likely assigned to the formation of quinones (see Figure 1a for 4-tert-butylcatechol). After 1 minute the absorption band at around 700 nm disappears and the band around 400 nm increases. The absorbance changes observed during the first minute after mixing Fe^{3+} and catechol are also similar (Figure S1, ESI). However, in the absorption spectra of mixtures of Fe^{3+} with 1,2-DHB with electron withdrawing substituents (EWS), the absorption bands at around 400 nm and 700 nm almost remain unchanged during the first minute (see Figure 1b for 3,4-dihydroxybenzonitrile).

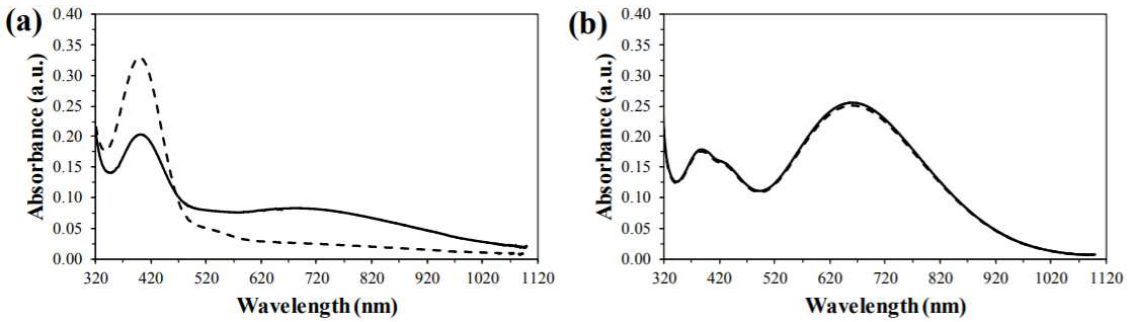


Figure 1: UV-visible absorption spectra of the mixtures of $1x10^{-4}$ mol/L of Fe^{3+} and $1x10^{-2}$ mol/L of (a) 4-tert-butylcatechol and (b) 3,4-dihydroxybenzonitrile. Solid lines (-) indicates the UV-visible absorption spectra taken immediately after the mixture and dashed lines (--) show the spectra of the 1 minute after the mixture (Source: elaborated by the authors).

To obtain information on the evolution of the fast reactions between Fe^{3+} and 1,2-DHB, the kinetics was studied with the aid of a stopped-flow accessory coupled to a spectrophotometer.

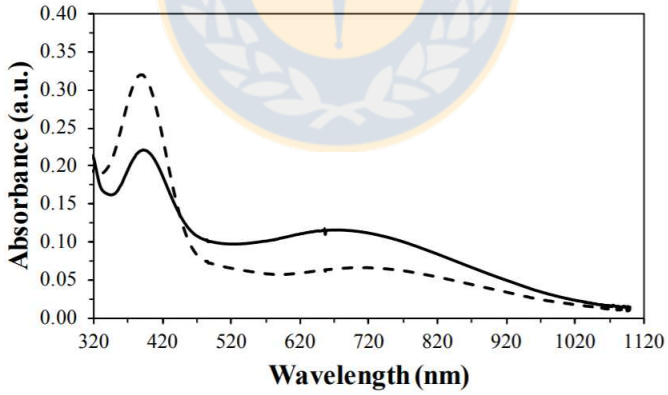


Figure S1: UV-visible absorption spectra of the mixtures of $1x10^{-4}$ mol/L of Fe^{3+} and $1x10^{-2}$ mol/L of catechol. Solid lines (-) indicates the UV-visible absorption spectra taken immediately after the mixture and dashed lines (--) show the spectra of the 1 minute after the mixture (Source: elaborated by the authors).

6.5 Stopped-flow experiments

The absorbance changes (ΔA) detected in the stopped-flow assays are relative to the final mixture of the reactants. Two different kinetic profiles were observed. For the 1,2-DHB with EDS and catechol above a certain substituent-dependent wavelength, the typical kinetic profiles show a fast increase in absorbance followed by a slower decay (see Figure 2a for 4-tert-butylcatechol at 720 nm, black line). This behavior can be associated with the formation of an intermediate presenting a higher absorbance than the final products within the first hundreds of microseconds. This species decays in the seconds time-scale. The kinetic profiles at two different wavelengths for 1,2-DHB with EWS in the whole wavelength range and for 1,2-DHB with EDS and catechol at shorter wavelengths show an initial fast decrease of absorbance, followed by an increase and then a slower decay (see Figure 2a for 4-tert-butylcatechol at 500 nm, yellow line and Figure 2b for 3,4-dihydroxybenzonitrile at 680 nm). The spectral kinetic profiles for all the 1,2-DHB are shown in Figure S2 (ESI).

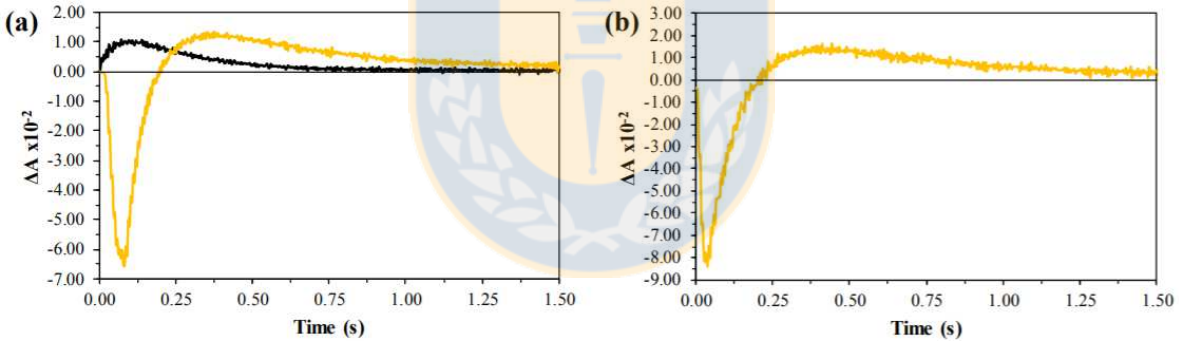


Figure 2: Kinetic profile obtained for the mixture at pH = 3.0 of $1x10^{-4}$ mol/L of Fe^{3+} and $1x10^{-2}$ mol/L (a) 4-tert-butylcatechol at 720 nm (black line) and 500 nm (yellow line), (b) 3,4-dihidroxybenzonitrile at 680 nm (Source: elaborated by the authors).

The kinetics profiles in the 300-800 nm wavelength range were adjusted to equation (1).

$$\Delta A = a_{(\lambda)}(exp^{-bt}) + c_{(\lambda)}(exp^{-dt}) + e_{(\lambda)}(exp^{-ft}) \quad (1)$$

Where the pre-exponential factors a(λ), c(λ), and e(λ), are wavelength-dependent and the apparent rate constants b, d, and f are wavelength-independent.

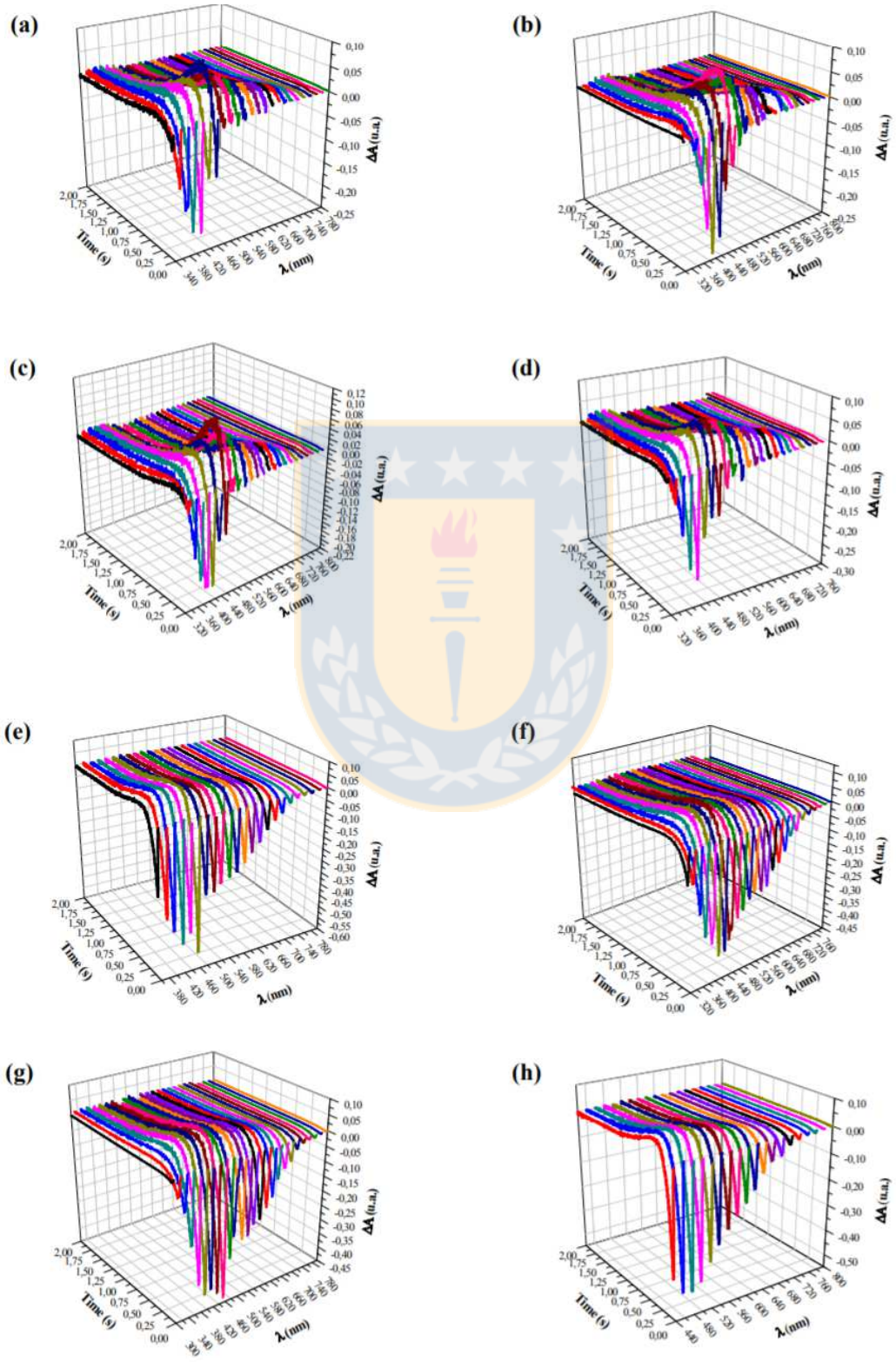


Figure S2: (a) 4-tert-butylcatechol, (b) 4-ethylcatechol, (c) 4-methylcatechol, (d) catechol, (e) 3,4-dihydroxybenzaldehyde, (f) 3,4-dihydroxybenzoic acid, (g) 3,4-dihydroxybenzonitrile and (h) 4-nitrocatechol.

To investigate the substituent effect on the apparent rate constants b, d, and f, the Hammett Equation (2) was used.

$$\log({}^{k_X}/_{k^o}) = \rho\sigma \quad (2)$$

Where kX is the rate constant for substituent X and k° is the corresponding rate constant for X=H (catechol in our case). The σ parameters are a measure of the electron-withdrawing ability of the substituent on the aromatic ring. For the 1,2-DHB the σ parameters were obtained by adding reported values in meta (σm) and para (σp) position (Hansch et al., 1991). The reaction constant (ρ) measures the sensitivity of the reaction to the electronic effect. This constant is independent of the substituent (Peres et al., 2010). Figure 3 shows the Hammett plots for the rate constants b (first process), d (second process), and f (third process).

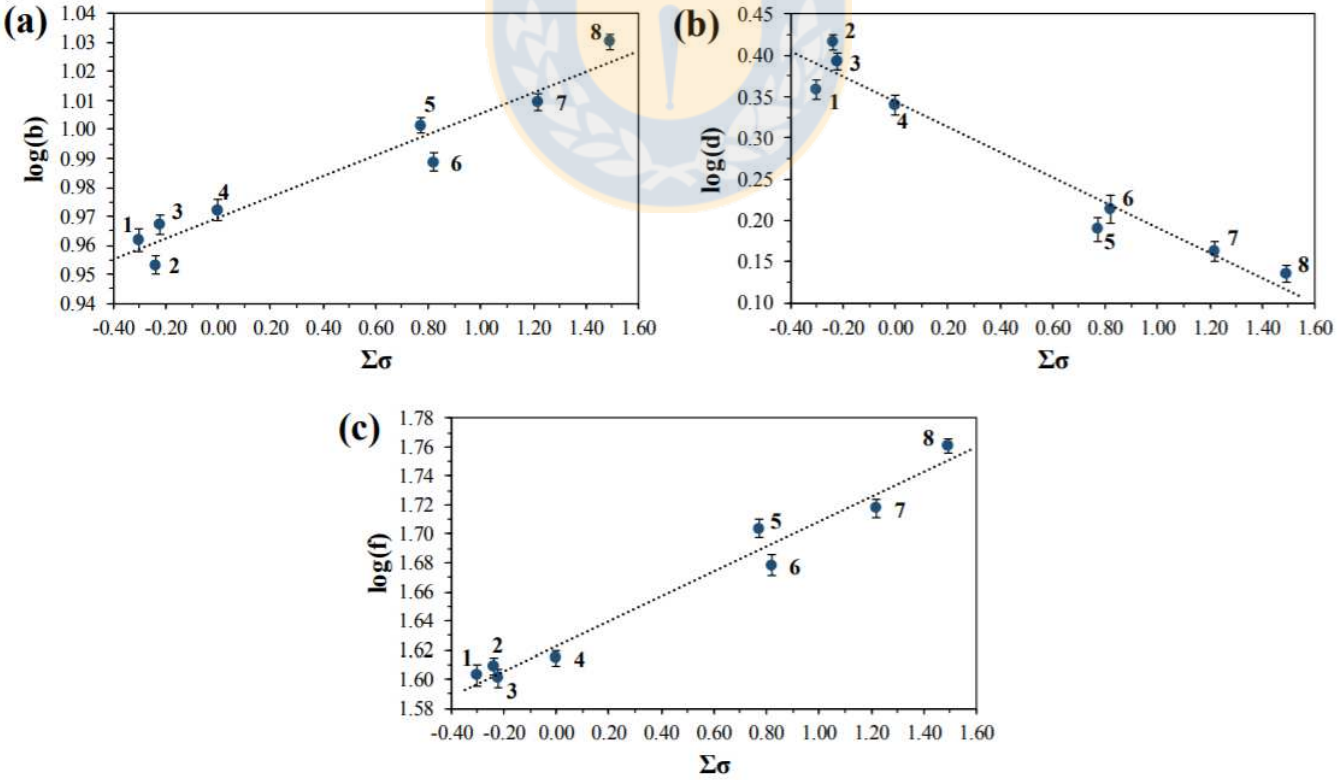


Figure 3: Hammett plots for rate constants: (a) first process, (b) second process, and (c) third process for 1: 4-tert-butylcatechol, 2: 4-ethylcatechol, 3: 4-methylcatechol, 4: catechol, 5: 3,4-dihydroxybenzaldehyde, 6: 3,4-dihydroxybenzoic acid, 7: 3,4-dihydroxybenzonitrile, and 8: 4-nitrocatechol (Source: elaborated by the authors).

The slope of the straight line in Figure 3a, $\rho = 0.036 \pm 0.002$ ($r = 0.970$), indicates a slight substituent effect, being the reaction favored for those 1,2-DHB with EWS. Ishida et al. (Ishida et al., 2004) showed that the first pKa value for substituted catechols correlate with Hammett parameters, and that electron withdrawing substituents significantly stabilize the monoanionic species of free catechols. Thus, if the same behavior is valid for the Fe^{3+} complexes of 1,2-DHB, the first process could be associated with a deprotonation step. Our data seem to indicate that deprotonation either takes place simultaneously with formation of the iron (III) complex or that it is a much faster event.

The slope of the Hammett plot in Figure 3b is $\rho = -0.15 \pm 0.01$ ($r = 0.975$), which means that the process is sensitive to the substituent and that it is faster for 1,2-DHB with EDS, where the electronic density on the $^{-}$OH groups is higher. This result makes us assign the process to the intramolecular electron step from (II) to (IV).

From the positive value of the slope of the Hammett plot shown in Figure 3c for the slowest step, $\rho = 0.085 \pm 0.003$ ($r = 0.985$), we can conclude that this process is moderately favored for 1,2-DHB with EWS. Thus, since the Fe-O bond is expected to be weaker in 1,2-DHB with EWS, the rate determining step of the slowest process should be the transformation of species (IV) into (VI). This step also involves a deprotonation, and the dependence of its rate on Hammett parameters is in line with that reported by Ishida et al. (Ishida et al., 2004) for the first pKa value of substituted catechols.

According to the previous discussion, the highest concentration of species (II) is expected to be present at the times corresponding to minimum (t_{min}) or maximum (t_{max}) of those profiles with the shape of Figures 2a or 2b, respectively. The maximum concentration of species (IV) should occur at the t_{max} for those kinetic profiles with the shape of Figure 2a. Figure 4 shows the absorbance of species (II) and (IV) relative to the absorption spectra of the final mixtures at the times of their highest concentrations obtained from the kinetic profiles for a typical 1,2-DHB

with an EDS substituent (Figure 4a), catechol (Figure 4b), and a typical 1,2-DHB with an EWS substituent (Figure 4c).

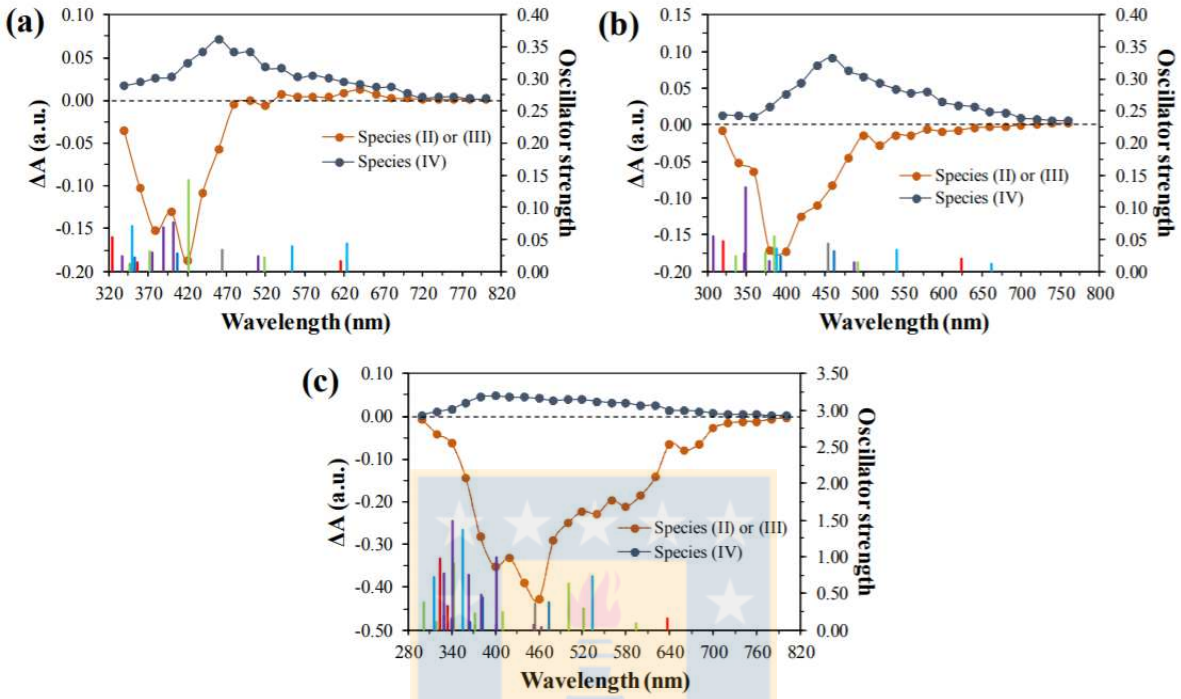


Figure 4: Absorption spectra obtained from the kinetic profiles of the reactions of Fe^{3+} at pH = 3 with: (a) 4-terbutylcatechol at t = 32 (tmin) and 372 ms (tmax), (b) Catechol at t = 28 (tmin) and 388 ms (tmax), and (c) 3,4-dihydroxybenzonitrile at t = 28 (tmin) and 384 ms (tmax). The calculated oscillator strengths are also shown for: species II or IV (green), species III (purple), species V (blue), species VI (light blue), species VII (red), and species VIII (grey) (Source: elaborated by the authors).

6.6 Oscillator strengths of the species involved in the reaction mechanism

To obtain theoretical information related to the experimental absorption spectra the oscillator strengths (f) of several species present in the reaction mechanism were calculated. As mentioned above, species II and IV cannot be distinguished in the calculations. All the calculated oscillator strengths are shown in Table S1, ESI. From the theoretical data, it is possible to assign the wavelength region where the species involved in the reaction mechanism

mainly absorb. We can observe that species number (VI), (VII), and (VIII), and in a much lesser magnitude species (III) (see Table S2 and Figure S3, ESI for the oscillator strengths and wavelengths regions where the species mainly absorb, respectively) absorb above 520 nm. This means that species (VI)-(VIII) are mainly responsible for the absorbance in this wavelength range during the first minute after mixing the reactants (see Figure 1). It is also observed in Figure 1 that 1,2-DHB with EWS present absorb more that those with EDS or catechol itself, in excellent agreement with the much higher oscillator strengths calculated for species (VI)-(VIII) of the 1,2-DHB with EWS.

Table S1: Rate constants obtained from the kinetics profiles for the reaction between Fe^{3+} 1.0x10-4 mol/L and the 1,2-DHBs $1.0x10^{-2}$ mol/L at pH= 3.0 (Source: elaborated by the authors).

	Rate constant (s^{-1})		
1,2-dihydroxybenzenes	**b**	**d**	**f**
4-tert-butylcatecol	9.16 ± 0.16	2.28 ± 0.04	40.07 ± 0.54
4-ethylcatechol	8.98 ± 0.09	2.60 ± 0.07	40.64 ± 0.81
4-methylcatechol	9.27 ± 0.06	2.47 ± 0.05	39.87 ± 0.32
Catechol	9.38 ± 0.08	2.19 ± 0.06	41.14 ± 0.77
3,4-dihydroxybenzaldehyde	10.03 ± 0.11	1.55 ± 0.07	50.55 ± 0.93
3,4-dihydroxybenzoic acid	9.74 ± 0.07	1.63 ± 0.05	47.69 ± 0.95
3,4-dihydroxybenzonitrile	10.22 ± 0.12	1.45 ± 0.06	52.19 ± 0.31
4-nitrocatechol	10.72 ± 0.10	1.37 ± 0.08	57.66 ± 1.05

Table S2: Oscillator strengths for: species II or IV (green), species III (purple), species V (blue), species VI (light blue), species VII (red) and species VIII (grey) (Source: elaborated by the authors).

λ (nm)	4-TC	4-EC	4-MC	CAT	4-CHO	4-COOH	4-CN	4-NO_2
300	0.0347							
302							0.382	
303	0.0512							
304	0.1771	0.1159						
305		0.0550						
306		0.0541				0.332		
307	0.0086							
308			0.0711	0.0551				
309						1.537		
310						0.786		
311						0.247		
313	0.0265							
314						0.417		
315					2.197		0.711	0.311
316	0.0126	0.0398						
319					0.267			
320				0.0468			0.105	
321		0.0137						
322		0.0207	0.0660					
324			0.0511		0.300			
325	0.0531						0.972	
329							0.193	
330					0.766	0.325	0.766	
332						0.297		
333			0.0170					
334							0.335	
335					0.097			
336				0.0243		0.371		1.250
337	0.0242	0.0248						
338					0.516			
339					0.331		0.155	
340					1.363			0.427
341							1.478	1.132
342						0.281		
344							0.914	0.345
345								0.570
346		0.0904	0.0132		0.312			
347	0.0120					0.853		
348		0.0182	0.0564	0.0270				
349				0.1299				
350	0.0712							
353	0.0222							
356	0.0134						1.366	

357	0.0120							
359					0.270			
360			0.1383		0.108	0.202		
361		0.0097	0.0187					0.138
362						0.183		0.289
363		0.1104			0.752		0.752	
365		0.0323	0.0182					
366							0.099	
368			0.0082					
369		0.0423						
370		0.0163						
371								0.520
372	0.0311	0.0118				0.392	0.226	
374				0.0305				
375					0.326			
376	0.0298							
379				0.0168				0.321
381			0.0130		0.478	0.826	0.478	
382		0.0250						
383							0.446	0.312
385				0.0542		0.258		
386			0.0138					
388				0.0352				
389		0.0137						
390	0.0682		0.0505					
391			0.0285					
392		0.0613						0.204
393						0.210		
394				0.0238				
395					0.584			
396						0.280		0.357
399			0.0153					
400			0.0204					
401							0.990	
402	0.0754	0.0177			0.071		0.071	
403		0.0234						
406					0.637			
407	0.0278					0.241		
410							0.240	
419								0.100
421	0.1420							
426					0.662			
427								0.741
436						1.190		
440								0.167
441					0.169			
445		0.0138						
446			0.0147					
453					0.063		0.063	

454				0.0431			0.364	0.150
457								0.131
458					0.347			
459								0.319
461								0.146
462				0.0312				
463			0.0473		0.039		0.039	
464		0.0589				0.327		
465	0.0328	0.0347			0.073			
470		0.0560						
472								0.131
474							0.371	
475			0.0518					
477			0.0343					
479								0.233
485						0.471		
487				0.0139				
492				0.0143				
494					0.952			
498					0.124			0.113
502			0.0230				0.625	
508		0.0237						
510	0.0246							
512						0.121		
519	0.0222							
522							0.289	
533							0.744	0.593
541				0.0334				
546						0.479		
547					0.533	0.397		
549								0.415
552		0.0245						
554	0.0398							
557			0.0353					
594							0.081	
605						0.364		
610					0.226			
616	0.0160							
618		0.0408						
623	0.0440							
624				0.0197				
625		0.0162						
626					0.182			
627								0.497
636							0.158	
647			0.0171					
649						0.135		
656								0.685
658								0.341

662				0.0129				
666					0.088			
676			0.0099					

The theoretical data show that the oscillator strengths for species II (or IV) formed with 1,2-DHB containing EDS and catechol are lower than those containing EWS. This result is also in agreement with the experimental data because we should keep in mind that the absorption spectra shown in Figure 4 are relative to the absorbance of the final mixture.

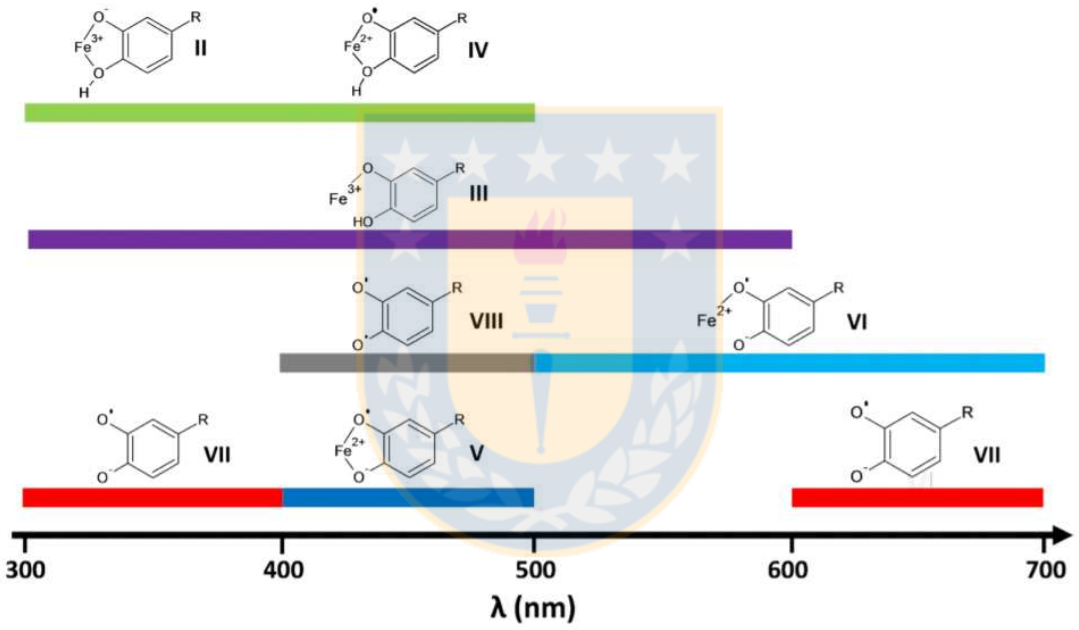


Figure S3: Wavelength regions where the calculated species mainly absorb (Source: elaborated by the authors).

6.7 Structure of reaction intermediates.

Theoretical calculations were performed to investigate whether open (monodentate) or cyclic (bidentate) intermediates are more likely to participate in the reaction mechanism. The optimized geometries of the cyclic and open complexes of Fe^{2+} and Fe^{3+} with 4-methylcatechol are shown in Figure 5. From the difference in Gibbs free energies between the cyclic and open forms of the complexes (see Table 1) we conclude that monodentate Fe^{3+}

complexes are clearly more stable than the bidentate ones, whereas the opposite is found for the Fe^{2+} complexes. Thus, it seems reasonable to propose the formation of a Fe^{3+} monodentate complex, which upon reduction yields a bidentate Fe^{2+} complex.

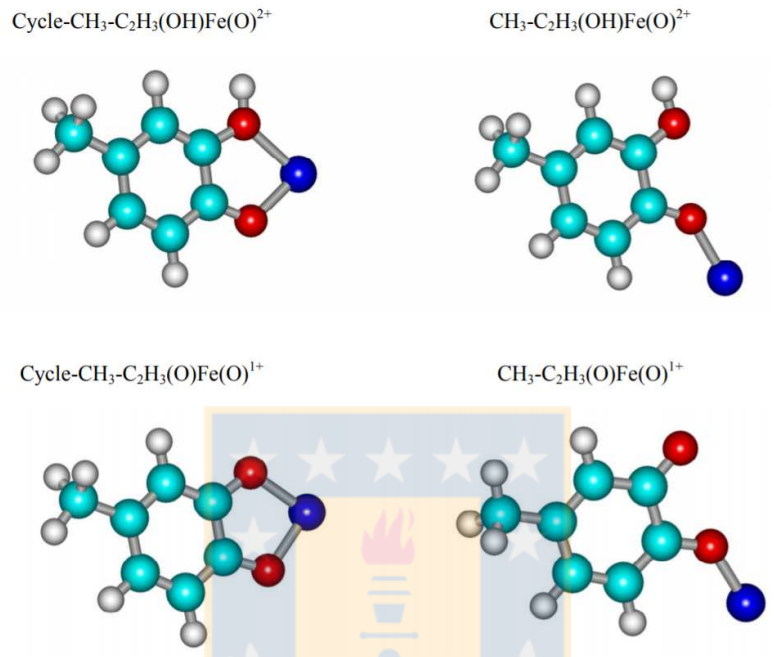


Figure 5: Optimized geometries of the bidentate and monodentate complexes of Fe^{2+} and Fe^{3+} with 4-methylcatechol (Source: elaborated by the authors).

Table 1: Relative Gibbs free energies at 298 K (in kcal/mol) for different R-1,2-DHB-Fe complexes (Source: elaborated by the authors).

R	Cycle-R-$C_2H_3(OH)Fe^{III}(O)^{2+}$- R-$C_2H_3(OH)Fe^{III}(O)^{2+}$	Cycle-R-$C_2H_3(OH)Fe^{II}(O)^{+}$- R-$C_2H_3(OH)Fe^{II}(O)^{+}$
-$C(CH_3)_3$	2.9	-12.0
-C_2H_5	10.9	-13.2
-CH_3	11.5	-23.2
-H	2.5	-23.4
-C(O)H	15.5	-11.3
-C(O)OH	7.6	-11.4
-CN	12.4	-12.4
-NO_2	14.1	-22.6

However, the previous calculations do not take into account that under our experimental conditions (pH = 3), since the pKa of the first -OH group of these molecules is within the range 6-9 (Aydin et al., 1997; Pizer & Babcock, 1977), both -OH groups of the catechol moiety should be protonated. Thus, calculations were also done for species I and II taking 3,4-dihydroxybenzaldehyde as an example of 1,2-DHB with EWS, 4-methylcatechol as representative of 1,2-DHB with EDS, and for the unsubstituted catechol. The optimized geometries of the Fe^{3+} complexes are shown in Figure 6.

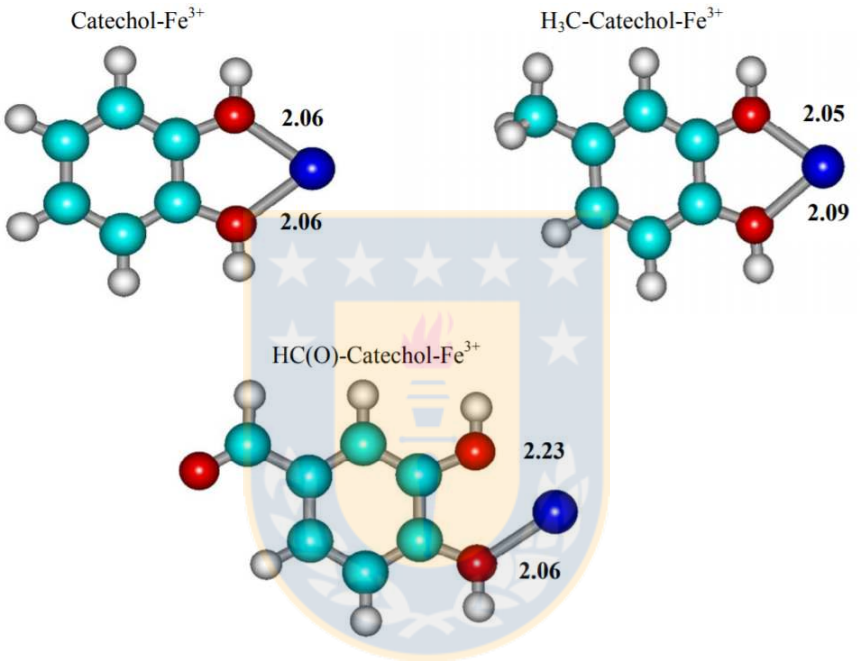
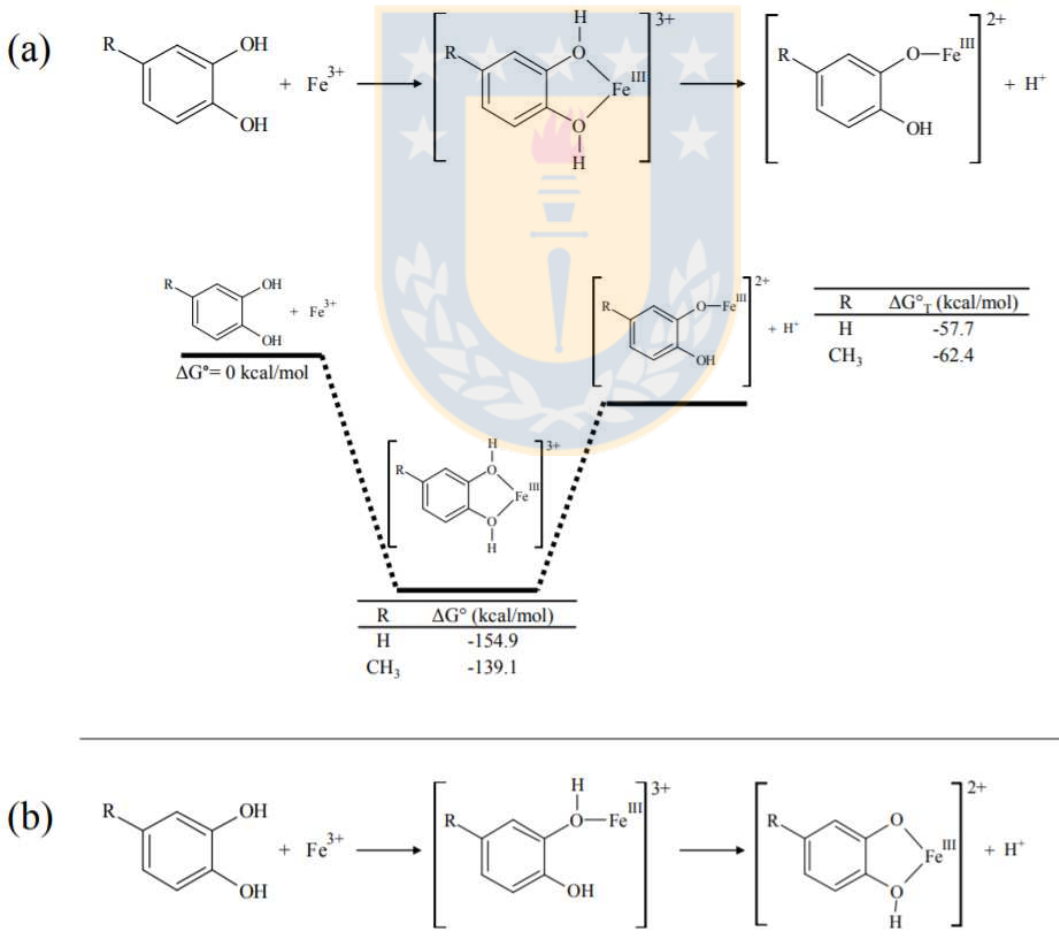


Figure 6: Optimized geometries of the Fe^{3+} complexes. The distances are given in Å (Source: elaborated by the authors).

Note that for 3,4-dihydroxybenzaldehyde, the compound taken as example of 1,2-DHB with EWS, the monodentate is 6.1 kcal/mol more stable than the bidentate complex. On the contrary, Table 1 shows that the bidentate complex of Fe^{3+} is more stable than the monodentate when one of the –OH groups is deprotonated. These results indicate that the relative stability of the Fe^{3+} complexes strongly depends on the protonation of the catechol –OH groups.

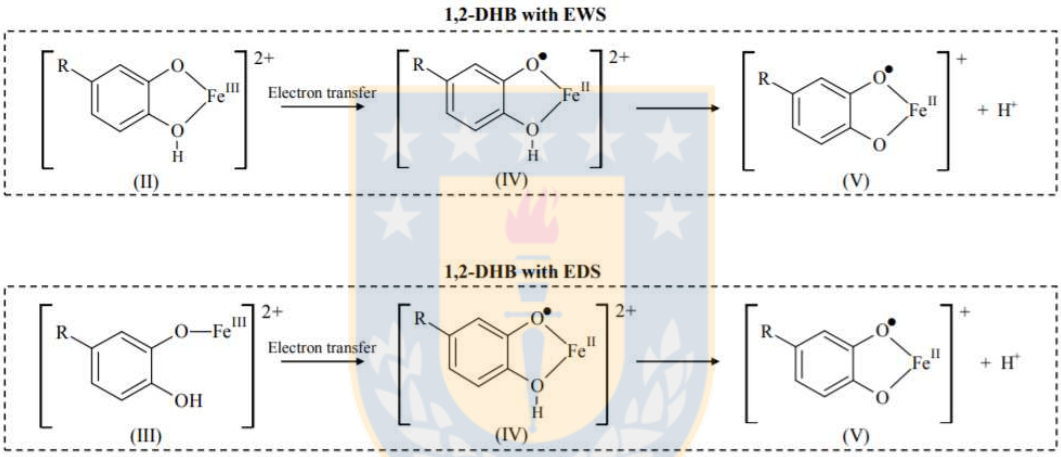
Energy calculations (Scheme 3a for catechol and 4-methylcatechol and Scheme 3b for 3,4-dihydroxybenzaldehyde) show that: i) there is a stabilization of ca. 140-150 kcal/mol upon formation of the complexes between Fe^{3+} and 1,2-

DHB, ii) the more stable complex is cyclic for catechol and 4-methylcatechol, but open monodentate for 3,4-dihydroxybenzaldehyde, and iii) there is an increase of ΔG o upon deprotonation of the complexes in all cases. From these results, it should be very unlikely that once the Fe^{3+} complex is formed the reactions go uphill about 100 kcal/mol to yield the deprotonated complexes, as proposed in the literature for catechol and its derivatives (R. C. Hider et al., 1983; Kristinová et al., 2009). Thus, complexation should not occur before deprotonation and it would be feasible that a concerted mechanism takes place, i.e., formation of the Fe-O bonds and proton loss occur in a single step without involving the participation of complex (I), in complete agreement with the stopped-flow data.



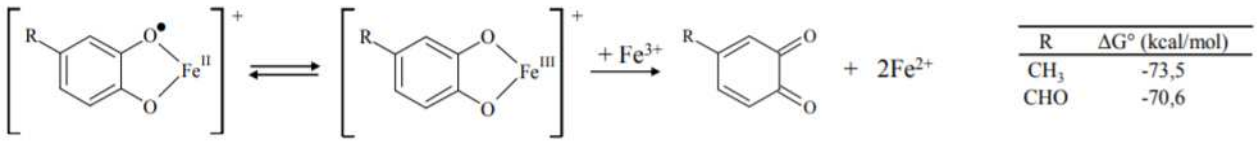
Scheme 3: Energy diagram for the complexation/deprotonation reaction of Fe^{3+} with: (a) Catechol and 4-methylcatechol, (b) 3,4-dihydroxybenzaldehyde. This complex is monodentate for 3,4-dihydroxybenzaldehyde. The most stable species are only depicted in the scheme (Source: elaborated by the authors).

Our DFT data also show that bidentate deprotonated Fe^{2+} complexes are more stable than monodentate complexes. Thus, the second observed kinetic step in the stopped-flow experiments (electron transfer) should be accompanied by a cyclization process in the case of 1,2-DHB with EDS as shown in Scheme 4.



Scheme 4: Electron transfer and deprotonation steps according to the DFT results (Source: elaborated by the authors).

The slowest observed kinetic step corresponds to deprotonation of the Fe^{2+} complex (Scheme 4). DFT calculations were performed in order to obtain energetic information about possible final steps of the reaction. The oxidation of the deprotonated complex by the excess of Fe^{3+} leading to formation of quinones was considered as a possible end step. DFT calculations support the feasibility of this reaction route (see the calculated Gibbs energy for 4-methylcatechol and 3,4-dihydroxybenzaldehyde in Scheme 5).



R	ΔG° (kcal/mol)
CH_3	-73,5
CHO	-70,6

Scheme 5: End step of the reaction of Fe^{3+} with the 1,2-dihydroxybenzenes in the presence of an excess Fe^{3+} (Source: elaborated by the authors).

CONCLUSIONS

The kinetics of the reaction of Fe^{3+} with 1,2-DHB is strongly dependent on the substituent. The absorbance changes could be well fitted to the sum of three exponential terms, being the rate constants associated with these processes affected by the electron-withdrawing ability of the substituent. It was possible to obtain the absorption spectra of the deprotonated Fe^{3+} complexes, as well as those of the Fe^{2+} complexes formed by intramolecular charge transfer.

DFT calculations on mono- and bidentate species with different degree of protonation helped us to evaluate their contribution to the reaction mixture. Energy calculations show that the more stable complex between Fe^{3+} and DHB is cyclic for catechol and 4-methylcatechol, but open monodentate for 3,4-dihydroxybenzaldehyde. These results indicate that the oxidation of catechol by Fe^{3+} should involve different intermediates depending on the substituent. Note that the participation of open monodentate complexes was not considered in the literature (R. C. Hider et al., 1983; Hynes & O'Coin□e□n□inn, 2004; Kristinová et □1., 2009). From the large increase in ΔG^o upon deprotonation, it should be very unlikely the reaction of species I to yield species III, as proposed in the literature for catechol and some of its derivatives (R. C. Hider et □1., 1983; Kristinová et □1., 2009). Thus, we conclude from the experimental and theoretical data obtained here that formation of the Fe-O bonds and proton loss occur in a single step.

Additionally, our DFT data also show that, since bidentate deprotonated Fe^{2+} complexes are more stable than monodentate complexes, the electron transfer process should be accompanied by a cyclization process in the case of 1,2-DHB with EDS.

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Acknowledgment

- Acknowledgment to my family, friends and colleagues.
- Acknowledgment of the financial support provided by the FONDECYT 1160100 project.
- Acknowledgments to the Biotechnology Center at the University of Concepcion (CB-UdeC), Chile.
- Acknowledgments to the Fenton research group at the CB-UdeC, Concepcion, Chile.
- Acknowledgments to the Laboratories of Renewable Resources and Technical Assistance at the CB-UdeC, Concepcion, Chile.
- Acknowledgments to the scholarship for Postgraduate Direction at the University of Concepcion.
- Acknowledgments to the financial support provided by the FONDECYT Nº 1140439.
- Acknowledgments to the Regional Institute of Applied Scientific Research (IRICA) at the University of Castilla-La Mancha, Ciudad Real, Spain.
- Acknowledgments to the Vine and Wine Research Center (IVICAM) of Castilla-La Mancha, Tomelloso, Spain.