STUDY OF MECHANISMS OF THE ENZYMATIC OXIDATION OF THE GRAPE MUST AND OF SOME POSSIBLE ALTERNATIVE SYSTEMS TO SULFUR DIOXIDE FOR ITS PREVENTION

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UNIVERSITAT ROVIRA i VIRGILI

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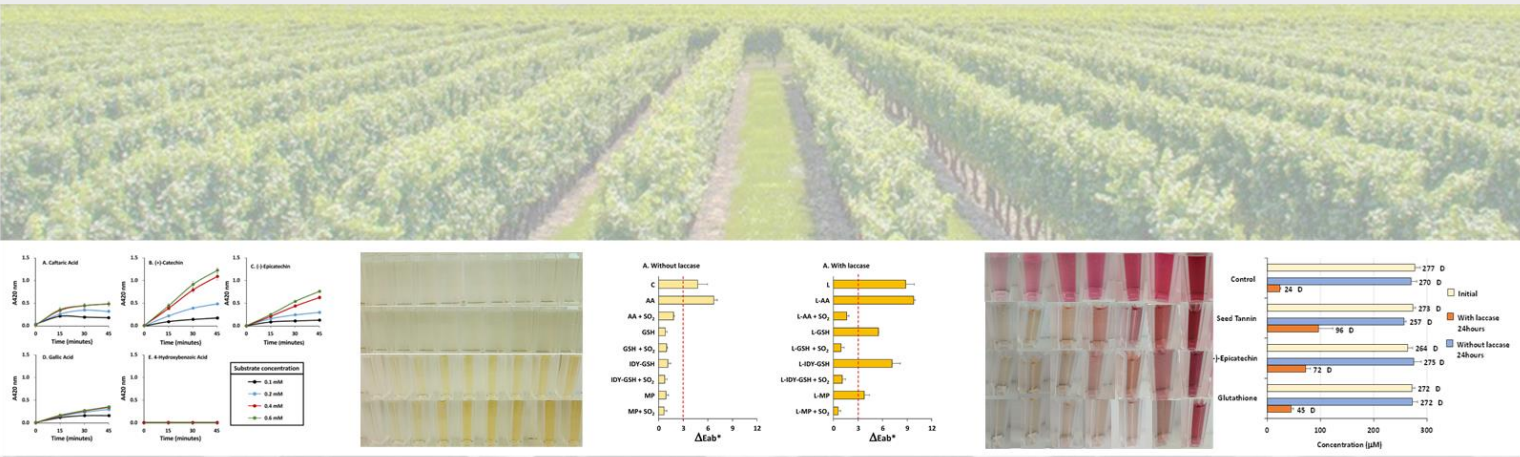

DOCTORAL THESIS

2023

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Study of mechanisms of the enzymatic oxidation of the grape must and of some possible alternative systems to sulfur dioxide for its prevention

Doctoral Thesis

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Fernando Zamora Marín i Joan Miquel Canals Bosch, professors titulars del Departament de Bioquímica i Biotecnologia de la Facultat d'Enologia de la Universitat Rovira i Virgili,

FAIG CONSTAR que aquest treball, titulat "**Study of the mechanisms of the enzymatic oxidation of the grape must and some possible alternative systems to sulfur dioxide for its prevention**", que presenta el **Pol Giménez Gil** per a l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament Bioquímica i Biotecnologia d'aquesta universitat.

Tarragona, 2 de Maig del 2023

Dr. Fernando Zamora Marín

Dr. Joan Miquel Canals Bosch

The development and analysis of this doctoral thesis have taken place mainly in the research laboratories of the Departament de Bioquímica i Biotecnologia of Universitat Rovira i Virgili under the direction of professors Joan Miquel Canals and Fernando Zamora.

Likewise, the oxidation kinetics of the musts were carried out in the experimental winery Mas dels Frares de la Facultat d'Enologia de la Universitat Rovira i Virgili.

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I. Introduction

1. THE GRAPE BERRY AND THE GRAPE MUST

1.1. The grape berry

The grape berry is a non-climacteric fruit produced by the *Vitis vinifera* plant, which belongs to the Vitaceae family and the genus Vitis. As a highly cultivated crop around the world, it holds significant agricultural value and boasts over 10,000 distinct varieties of Vitis vinifera (Parihar and Sharma, 2021). The grape berry of *Vitis vinifera* is the primary source of grape must, which, after undergoing to alcoholic fermentation, becomes wine.

1.1.1. Grape berry development

After flowering, fertilization and fruit set, the grape berry grows following a double sigmoid curve, which includes to three developmental stages (**Figure 1**). Moreover, there are some changes in the matrix of grape berry that make it more adaptable in each stage of the ripening.

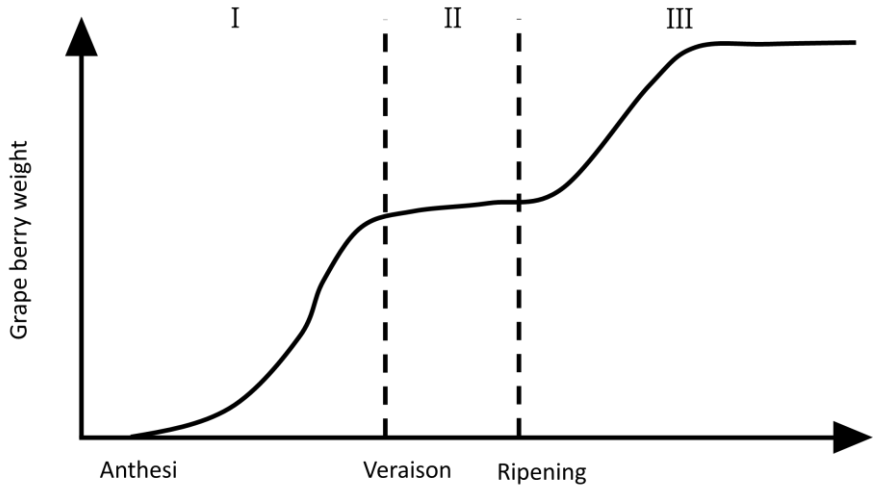


FIGURE 1: STAGES OF GRAPE BERRY GROWING

After fertilization of the flower, in stage I, grape berry growth from an ovary is due to cell mitotic division and cell expansion. In a period approximately of 4-6 weeks post-anthesis, cell division ceases and only cell expansion subsists. During this stage, the

enlarged walls of the ovary become the pericarp and the first stage concludes with the formation of all tissues that constitute the seed. Once the pericarp is formed, it has a clear composition of three distinct cell layers: the epicarp that corresponds to the cuticle and the skin; the mesocarp that corresponds to the flesh; and finally, the endocarp where the seeds are included (Houel *et al.*, 2013).

Stage II is included in the initial stages, where the seed formation concludes and ends at veraison, with slow growth during this period. It is during veraison that the chlorophylls in the grape skin are degraded and the phenolic compounds that contribute to the skin, flavonols and anthocyanins pigmentation are synthetized.

In the last period of grape growing, stage III, three changes linked to veraison appear: (i) the formation of pigments, yellow or red, depending on the colour of the cultivar, (ii) the grape berry growth reinitiating that after veraison is due to cell growth and not to cell multiplication, and (iii) the firmness of the grape berry becomes gradually softer (Houel *et al.*, 2013).

1.1.2. Grape berry structure

Once formed, the grape berry contains five primary structures. The first one is the cuticle, the external zone where there is the pruin, a waxy compound that covers the skin surface. Wine fermentation is due to microorganisms, yeasts, settle on this waxy substance. In depth, the epicarp comprises the epidermis and hypodermis. First, the epidermis is composed of the layer with longitudinal cells that contains the highest concentration of anthocyanins (in red grapes) (Navarro, Botía and Romero, 2021), and second, the hypodermis is has a mixture of longitudinal and rectangular cells.

In the mesocarp, where the flesh is found, the largest cells of the grape berry are concentrated, and the highest concentration of water, sugars, and acids. In this area, polygonal cells dominate, however, elongated radial cells are found near the center of the berry (Ribéreau-Gayon, *et al.*, 2006a).

In berries, the endocarp, which corresponds to the cell layers in contact with the seeds, plays a critical role in protecting the seed during development and dispersal. The providing of additional nutrients and moisture to the developing seed (Dardick and Callahan, 2014).

In the last period of growth of the grape, stage III, the increase in sugar concentration and the lower firmness of the skins give opportunity to different organisms, especially filamentous fungi, to infect the grape berry. From the point of view of viticulture, one of the main diseases is grey rot, caused by ***Botrytis cinerea****,* a filamentous and necrotrophic phytopathogenic fungus. This fungus excretes metabolites, among them, which are indicators of its presence: glycerol, gluconic acid (oxidation of aldehyde function of glucose) and high molecular weight polysaccharides (as a β1-3 and β1-6 glucans). In addition, *Botrytis cinerea* also produce the laccase enzyme (Hong *et al.*, 2012; Cinquanta *et al.*, 2015; Armijo *et al.*, 2016).

The following three maturation concepts can be regarded as related to stage III: (i) Physiological maturity refers to the moment in a vine's reproduction cycle when the seed embryo is fully developed, and the vine is prepared to produce the next generation of plants (Bigard, 2018) (ii) The technological maturation of the wine grape is the stage of the vine in which a correct balance is reached between the sugar and the acidity of the grape (Nogales-Bueno *et al.*, 2014) (iii) The phenolic and aromatic maturation of the grape, which is achieved when the grape reaches the adequate concentration and extractability of phenolic compounds. The main phenolic compounds are anthocyanins and condensed tannins (very important in red grapes), and the enrichment of the suitable content of aromatic volatile compounds and their precursors. This last concept of maturity is probably the most important in oenology, because it conditions completely: the colour, mouthfeel and aromas of the final wines (Hellín *et al.*, 2010; Meléndez *et al.*, 2013).

1.2. The grape must

Grape must is a buffered matrix, composed mainly of water, sugars, carboxylic acids and macromolecules as polysaccharides, proteins and their derivates. Grape juice also contains some cations such as potassium, calcium, iron and copper that can be free or complexed with proteins, pectins, phenols and some carboxylic acids (Clark *et al.*, 2015), and small amounts of inorganic anions. The total amount of each compound will vary

depending on the variety of vine, on the terroir, vine management and finally the ripening level.

Once the grapes are harvested and carried to the winery, winemaking begins. In the case of red wines, the bunches are usually destemmed, crushed, and pumped to the tanks to start the fermentation/maceration process. In contrast, in the case of white wines, the grapes are pressed to obtain the grape juice without the presence of skins and seeds. This process can be performed directly pressing the bunches, pressing after crushing the bunches or even pressing after destemming and crushing. The type of the press and the fractionating of the grape must, during pressing, has a huge importance on the final quality of the grape must. Currently, closed pneumatic presses are the most used ones, and normally the high-quality wine is only elaborated with the first extracted fraction (between 60-65 % of the original weight of the grapes) (Kerslake, Longo and Dambergs, 2018).

When the white grape must is obtained, it undergoes the settling process to separate by sedimentation all the solid particles. This process is usually favored by adding pectinolytic enzymes and can last around 18-24 hours.

As is well known, the final composition and quality of the grape must depend on the different operations applied to obtain it; probably, the phenolic compound extraction and the solubilization of oxygen are the most important ones. On one hand, an excess of extraction of phenolic compounds would negatively affect the colour, astringency and bitterness of the future wine and also its oxidizability, since phenolic compounds are substrates for enzymatic and chemical oxidation (Giménez *et al.*, 2022). On the other hand, an excess of oxygen would cause browning since it is used by polyphenol oxidases to transform most of the phenolic compounds in brown pigments (melanins) (Qi *et al.*, 2020).

Wineries are very careful to limit oxygen solubilization during all these operations to prevent enzymatic oxidation and its consequences in wine colour (browning). However, the earth atmosphere contains 21% (v/v) of oxygen and consequently it is very difficult to avoid completely its presence. For that reason, wineries add sulfur dioxide to the grape must because this additive (E220) is a powerful inhibitor of polyphenol oxidases.

Sulfur dioxide is also used for many other purposes, especially by its antiseptic properties.

The mechanism by which polyphenol oxidases act and the different tools to prevent their action will be discussed later.

2. PHENOLIC COMPOUNDS IN GRAPE AND WINE

Phenolic compounds or polyphenols exist in most plant tissues as secondary metabolites, acting as interactors between the plant and its biological environment (Baur and Sinclair, 2008), and playing the role of antioxidants (Heim, Tagliaferro and Bobilya, 2002). The basic structure of phenolic compounds is an aromatic ring carrying one or more hydroxyl moieties. However, polyphenols are usually composed of multiple phenol rings within a single structure. **Figure 2** shows the general classification of phenolic compounds.

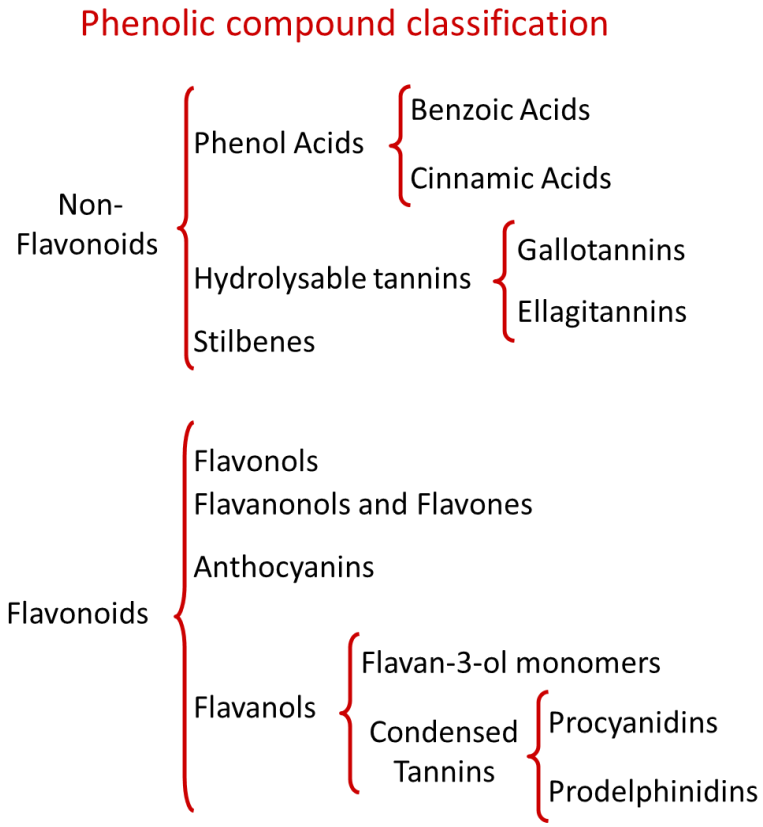


FIGURE 2: PHENOLIC COMPOUNDS CLASSIFICATION ADAPTED FROM ZAMORA, 2003A

I. Introduction

Phenolic compounds are usually classified into two main groups: non-flavonoids and flavonoids. These compounds are extracted from the grape during the first stages of winemaking.

2.1. Non-flavonoids

Non-flavanoids compounds can be classified by their carbon skeleton such as hydrolyzable tannins, phenol acids, benzophenones, xanthones, secoiridoids, stilbenes and coumarins (Quideau *et al.*, 2011). Although the main groups of non-flavanoids in wine are the phenol acids (benzoic and cinnamic acids) and stilbenes. Phenol acids are components of hydrolyzable tannins and some of them can be decarboxylated by some microorganisms, thus being the origin of the volatile phenols (Chatonnet, Dubourdieu and Boidron, 1995).

2.1.1. Phenol acids

Phenol acids are characterized by having a carboxylic acidic group coupled directly to the benzene ring (benzoic acids) or through a vinyl group (cinnamic acids).

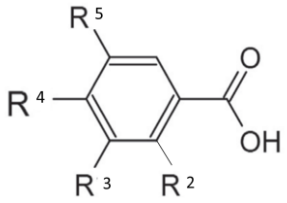
Phenolic acids are present in skins, pulp, seeds, and stems of the grapes. These acids had the tendency to decrease during grape maturation, reaching very low values in the last stages of ripening (Kluba and Mattick, 1978). Furthermore, these acids are available mainly as their tartaric acid esters, caffeoyl-tartaric acid (caftaric acid) and p-coumaryl-tartaric acid (cutaric acid). Both esters are the major substrates for enzymatic browning in grape must (Singleton *et al.*, 1985; Cheynier V. & Van Hulst M., 1988).

Benzoic and cinnamic acids, two groups of phenolic acids that can be separated based on their structural disposition.

2.1.1.1. Benzoic acid (BA) and Hydroxybenzoic acids (HBA), and its derivates.

Table 1 shows the main benzoic acids presents in grape must and wine.

TABLE 1: HYDROXYBENZOICS ACIDS (ADAPTED FROM (PASCAL RIBÉREAU-GAYON *ET AL.*, 2006)

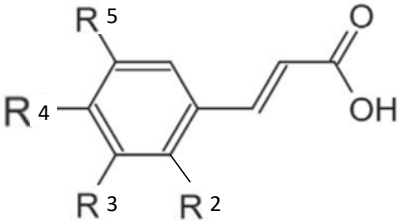
Hydroxybenzoic acids	R_2	R_3	R_4	R_5	Structural formula
Benzoic acid	H	H	H	H	
Vanillic acid	H	OCH_3	OH	H	
Gallic acid	H	OH	OH	OH	
Syringic acid	H	OCH_3	OH	OCH_3	
p -Hydroxybenzoic acid	H	H	OH	H	

The C6-C1 linkage is a prevalent occurrence within various structures, such as 4-hydroxybenzoic acid, vanillic acid (4-hydroxy-3-methoxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid), and syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid). These compounds are commonly present in their free forms in wine (Vanhoenacker *et al.*, 2001), with gallic acid serving as the primary representative and being detected in concentrations ranging from 100 to 230mg/Kg of grape (Chira *et al.*, 2008). It should be noted that the origin of these compounds is not solely attributed to grape extraction, as they also arise from the hydrolysis of hydrolyzable tannins that are esters of gallic acid and/or ellagic acid (Moreno-Arribas and Polo, 2009). As a result, wines that have been treated with oak will contain additional phenolics from this source. Phenolic acids can also be formed by degradation of anthocyanins (Brouillard, 1982).

2.1.1.2. Hydroxycinnamic acids (HCA), cinnamic acid (CA), and their derivates.

The main representation of these compounds is showed in **Table 2**.

TABLE 2: HYDROXYCINNAMIC ACIDS (ADAPTED FROM (PASCAL RIBÉREAU-GAYON *ET AL.*, 2006))

Hydroxycinnamic acids	R_2	R_3	R_4	R_5	Structural formula
Cinnamic acid	H	H	H	H	
Ferulic acid	H	OCH_3	OH	H	
Caffeic acid	H	OH	OH	H	
Sinapic acid	H	OCH_3	OH	OCH_3	
p - Coumaric acid	H	H	OH	H	
o- Coumaric acid	OH	H	H	H	

The origin of these compounds can be traced back to the pulp of the grape berry, predominantly in the form of tartaric acid esters, with free forms being of negligible proportion (Ong- and Nagel, 1978; Kennedy, Saucier and Glories, 2006). Their structure

consists in a simple phenylpropanoid C6-C3 group. These compounds exist in both *cis*- and *trans*-configurations, but the *trans*-conformation is the predominant form due to its stability (Monagas, Bartolomé and Gómez-Cordovés, 2005). These highly oxidizable components of grape juice are partly responsible for the browning of white must (Cheynier V. & Van Hulst M., 1988 Cheynier V. *et al.*, 1989). Caftaric acid (caffeoyl tartaric), coutaric acid (coumaric tartaric), and fertaric acid are the most abundant hydroxycinnamates in grape juice prepared with minimal skin contact ; (Cheynier V. & Van Hulst M., 1988; Singleton *et al.*, 1984) 20 – 100 ppm. Among the hydroxycinnamic acids, caftaric acid constitutes around 50% of the total content of these compounds, with a magnitude order of approximately 200mg/Kg of grape, followed by p-coumaric acid and ferulic acid (Monagas, Bartolomé and Gómez-Cordovés, 2005; Chira *et al.*, 2008).

The impact on wine colour offered by the hydroxybenzoic acids is practically null compared to the hydroxycinnamic acids since they play a very important role in copigmentation, described as an intensification of anthocyanin colour by the presence of other phenolic compounds (Somers, Verette and Pocock, 1987; Dimitrić Marković, Petranović and Baranac, 2000).

Hydroxycinnamic acids can be decarboxylated by some microorganisms, especially *Brettanomyces*, to form vinyl phenols, which can be subsequently reduced to produce ethyl phenols (Chatonnet, Dubourdieu and Boidron, 1995). Both, vinyl and ethyl phenols are called volatile phenols and contribute negatively to wine aroma. In addition, vinyl phenols can react with anthocyanins to form pyranoanthocyanins (Schwarz, Wabnitz and Winterhalter, 2003; Rentzsch, Schwarz and Winterhalter, 2007).

2.1.2. Hydrolyzable tannins

Hydrolysable tannins are classified into two subfamilies, ellagitannins and gallotannins. These tannins are not normally present in grape must or wine. However, ellagitannins are constituents of oak wood and therefore they can be released from the oak barrels or other oak alternatives (Aron and Kennedy, 2008). In contrast, gallotannins are derived from tara (*Caesalpinia spinosa*) (Aguilar-Galvez *et al.*, 2014) and from galls, produced mainly in oak by wasps (Versari, Du Toit and Parpinello, 2013).

The use of commercial tannins can be also a source of ellagitannins and gallotannins (Pascual *et al.*, 2016) and therefore they can be presents in wine when these additives are used in winemaking (Vignault *et al.,* 2018). Both ellagitannins and gallotannins exert a significant contribution to the wine astringency (Gombau *et al.*, 2019). The name of hydrolysable tannins for gallotannins and ellagitannins is due to the fact that they can be hydrolyzed by acid treatment, leaving either gallic acid and/or ellagic acid.

2.1.2.1. Gallotannins

Gallotannins are polymers whose structures are based on a D-glucose molecule, wherein the hydroxyl groups are partially or completely esterified with either gallic acid monomers or gallic acid structures (Hagerman *et al.*, 1998). Tannic acid is the commercial name for gallotannin extract comprising mixtures of polygalloyl quinic acid ester or polygalloyl glucoses (Pascual *et al.*, 2016). **Figure 3** shows the chemical structure of a gallotannin (decagalloylglucose).

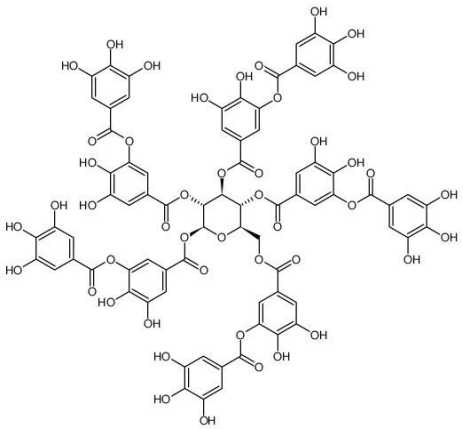


FIGURE 3:CHEMICAL STRUCTURE OF GALLOTANNINS (DECAGALLOYLGLUCOSE)

The presence of gallotannins in wine is attributed to their inclusion as oenological tannins, aimed at different purposes as protecting botrytized must from oxidation or to prevent overfining (Vignault *et al.*, 2018). Regarding their organoleptic contribution, the gallic tannins have an acid, slightly astringent and very bitter taste (Vivas, 1997). The main sources of commercial gallotannins are nut galls and tara (Versari, Du Toit and Parpinello, 2013).

I. Introduction

2.1.2.2. Ellagitannins.

Ellagitannins are polymers of ellagic, gallic and/or hexahydroxydiphenic acids (Versari, Du Toit and Parpinello, 2013; Vignault *et al.*, 2019). To be more precise, a nonahydroxyterphenoyl unit (NHTP) is esterified in positions 2, 3 and 5 with a C-glycosidic bond, while an open-chain glucose is esterified in positions 4 and 6 with a hexahydroxydiphenoyl unit (HHDP) forming the chemical structure of ellagitannins (Quideau, 2005). Ellagitannins have a variety of biological properties which include antioxidant capacity (Hosu *et al.*, 2014). The main sources of commercial ellagitannins are oak and chestnut (Vignault *et al.*, 2018). **Figure 4** shows the chemical structure of ellagitannins (Adapted from Navarro *et al.*, 2021).

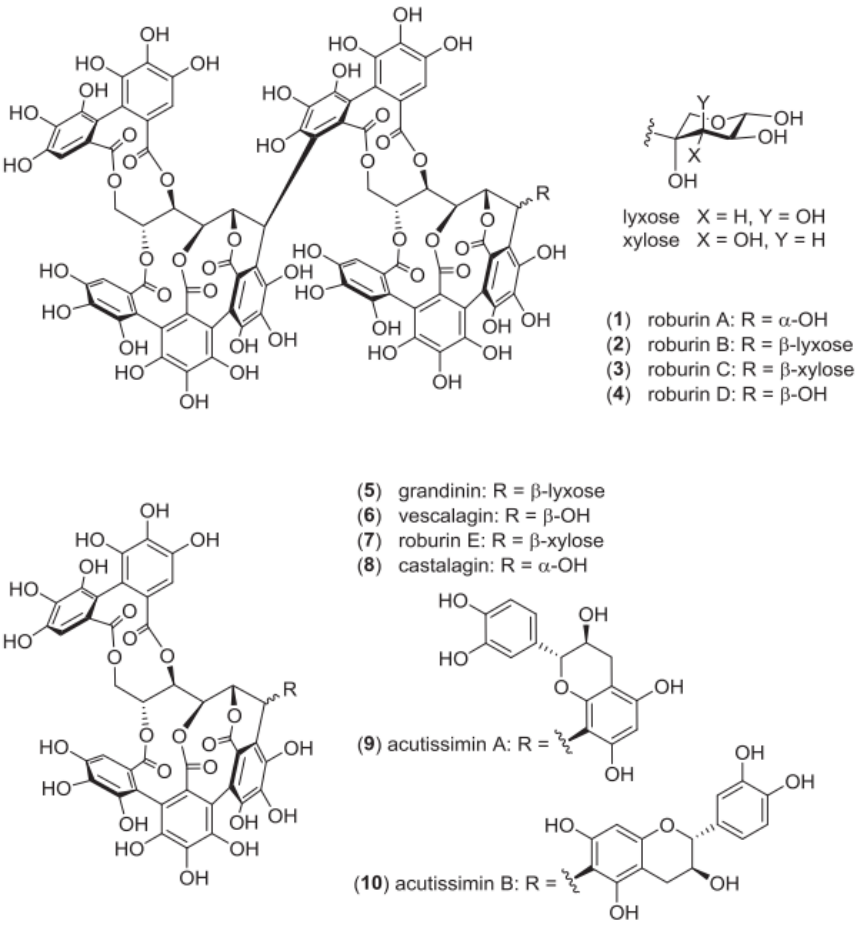


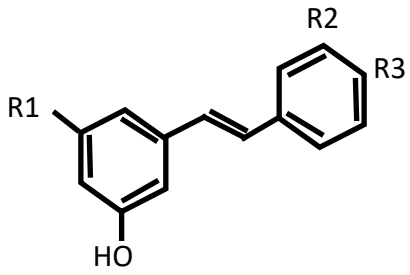
FIGURE 4: CHEMICAL STRUCTURE OF ELLAGITANNINS

The ellagitannin composition of extracts from the duramen depends on the species of oak. All four monomeric (castalagin, vescalagin, and the C-lyxoside and C-xyloside conjugates of vescalagin: grandinin and roburin E) and four dimeric (roburin A, B, C, and D) ellagitannins are present in the three species of European oak, while the American species have practically no dimers (Vivas & Glories, 1996).

2.1.3. Stilbenes

Stilbenes is a phenolic compound group whose biological function seems to be related to defense mechanisms of grape berries against fungal attacks (Langcake and Price, 1976). Its chemical structure is shown in **Table 3**.

TABLE 3: CHEMICAL STRUCTURE OF STILBENES ADAPTED FROM ZAMORA, (2003A)



Stilbene	R1	R2	R3
Resveratrol	OH	H	OH
Piceid	OGlc	H	OH
Astringin	OGlc	OH	OH
Resveratroloside	OH	H	OGlc
Piceatannol	OH	OH	OH

Its location in the grape is limited to the skins (Jeandet, Bessis and Geutheron, 1991). From the point of view of colour and/or any other organoleptic property, the stilbenes are of no importance. However, they have acquired great scientific importance due to the possible beneficial effects that resveratrol and other stilbenes (Lamuela-Raventos and Waterhouse, 1999) appear to have on human health (Versari *et al.*, 2001).

2.2. Flavonoids

The name of Flavonoids comes from the Latin term *Flavus* which means yellow although not all the pigments included in that family have this colour.

I. Introduction

This group of phenolic compounds includes compounds with structure C_6-C_3-C_6 such as flavones, flavonols, flavanones, anthocyanidins, dihydroflavonols, isoflavones, chalcones, flavan-3-ols monomers and proantocyanidins. All flavanoids have the chemical structure showed in **Figure 5**.

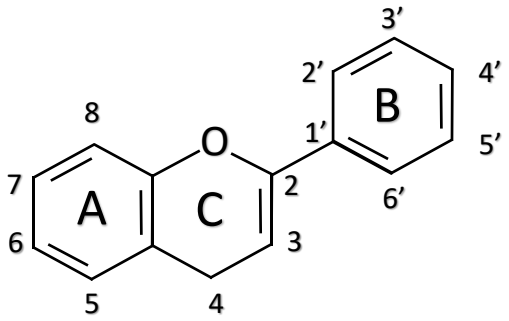


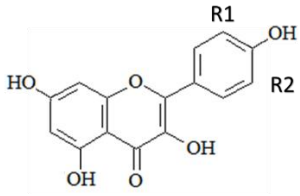
FIGURE 5: CHEMICAL STRUCTURE OF FLAVONOIDS

The flavonoid's structure is characterized by two benzene cycles bonded by an oxygenated heterocycle, derived either from the 2-phenyl chromone nucleus (flavones and flavonols) or the 2-phenyl chromanone nucleus (flavanones and flavanonols). The basic flavonoid chemical structure is the flavan nucleus, consisting of 15 carbons atoms arranged in two benzene rings (A and B) linked by a heterocyclic oxygen-containing pyrone ring, pyran ring or pyrrole ring.

2.2.1. Flavonols.

This family of compounds, as belonging to the group of flavonoids, have the basic chemical structure but with the specificity of having a double bond between positions 2 and 3 and an oxygen (a ketone group) in position 4 of the C ring, like flavones, but with the presence of hydroxyl group at the position 3.

Figure 6 shows the general structure of flavanols.



Kaempferol: R1=R2=H
Quercitin: R1=OH, R2=H
Myricetin: R1=R2=OH
Isorhamnetin: R1= OCH_3, R2=H

FIGURE 6: CHEMICAL STRUCTURE OF FLAVANOLS

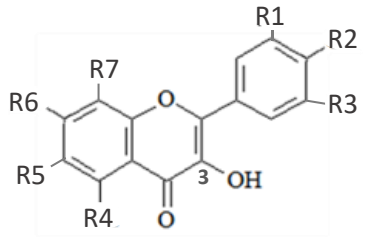
These compounds are present in the skins of both red and white grapes, contributing as yellow pigments to the grape skin colour.

The glycosylated forms (in position 3) are the most abundant in grapes. However, during the winemaking process, heterosides can be partially hydrolyzed to leave the free aglycone. Their concentration ranges between 1 to 3 ppm in white wines, and it can reach until 100 ppm in red wines (Makris, Kallithraka and Kefalas, 2006).

2.2.2. Flavones, flavononols and flavonones

2.2.2.1. Flavones.

Flavones exhibit a chemical structure analogous to that of flavonols, but lacking the hydroxyl group at carbon 3, being therefore weak free radical scavengers. These compounds are yellow pigments and are present in grape skins. **Figure 7** shows their general structure.



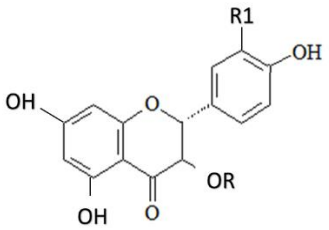
Apigenin: R1=R3=R5=R7=H, R2=R4=OH
Baicalein: R1=R2=R3=R7=H, R4=R5=R6=OH
Luteolin: R1=R2=R4=R6=OH, R3=R5=R7=H
Diosmetin: R1=R4=R6=OH, R2=OCH_3 R3=R5=R7=H
Tangertin: R1=R3=H, R2=R4=R5=R6=R= OCH_3

FIGURE 7: CHEMICAL STRUCTURE OF FLAVONES

Within grapes and wine, apigenin and luteolin represent the chief flavones that have been identified, whilst astilbin and engeletin represent the primary flavanonols, according to Monagas *et al.*, 2005.

2.2.2.2. Dihydroflavonols or flavononols.

These compounds lack the double bond between carbons 2 and 3 in the C ring present in flavones and flavonols (Gonçalves *et al.*, 2018). **Figure 8** shows their general structure.



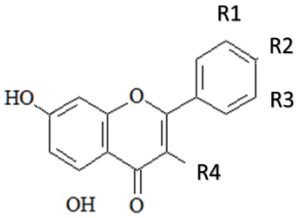
Astilbin: R1=OH, R=Rh
Engeletin: R1=H, R=Rh

FIGURE 8: CHEMICAL STRUCTURE OF DIHYDROFLAVONOLS OR FLAVONONOLS

The main flavononol is astilbin and its concentration range may vary between 0.5 to 15 mg/L (Landrault *et al.*, 2002).

2.2.2.3. Flavanones.

The only difference that distinguishes flavanones from other flavonoids is the absence of a double bond and the presence of a chiral center at carbon 2. They may occur as hydroxylated, glycosylated, and *O*-methylated derivatives**. Figure 9** shows the general structure of this group.



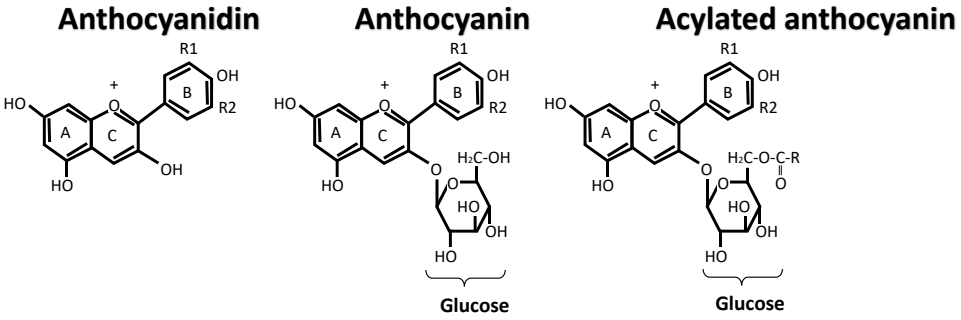
Erioditol: R1=R2=OH, R3=R4=H
Naringenin: R1=R3=R4=H, R2=OH
Hesperetin: R1=OH, R2= OCH_3, R3=R4=H

FIGURE 9: CHEMICAL STRUCTURE OF FLAVONONES

2.2.3. Anthocyanins

Anthocyanins (from the ancient Greek *anthos* and *kyanos* blue) are responsible for the bluish-red colour of the skin of red grapes and also of the colour of red wine (Ribéreau-Gayon, 1964; Glories, 1984a). As the other flavanoids, they have the same basic

structure composed of one heterocyclic benzopyran ring (C ring), one fused aromatic ring (A ring), and one phenyl constituent (B ring). Anthocyanins differ from other flavanoids in that their heterocycle has delocalized positive charge when they are in flavylium form, and in that they can have hydroxyl or methoxyl substitutions in the lateral phenyl B ring. **Figure 10** shows the chemical structure of the anthocyanins.



Delphinidin: R1=OH, R2=OH **Cyanidin**: R1=OH, R2=H **Petunidin:** R1=OH, R2= OCH_3
Peonidin: R1=OCH_3, R2=OH **Malvidin:** R1=R2=OCH_3

FIGURE 10: CHEMICAL STRUCTURE OF ANTHOCYANINS

Only glycosylated forms exist naturally and specifically in Vitis vinifera there are only the monoglycosides in position 3. However, in other species of the genus Vitis, we can find diglycosides in position 3 and 5 (Ribéreau-Gayon, 1959). In addition, the glucose of the glicosilated forms can be acylated with acetic, coumaric or caffeic acids (Fong, Webb and Kepnert, 1974; Kamsteeg *et al.*, 1978; Shi, Lin and Francis, 1992; Gómez-Alonso, García-Romero and Hermosín-Gutiérrez, 2007). Consequently, in *Vitis vinifera* these pigments can be in form of 3-*O*-monoglucoside, called anthocyanin, and in form of acetylated anthocyanin. However, anthocyanins can be hydrolyzed in the wine, releasing glucose and the corresponding aglycone, called anthocyanidin.

There are different anthocyanins depending on the substituents R1 and R2. In nature there are other anthocyanins such as pelargonin, although in the genus Vitis only the 5

indicated in the **figure 10** are found, being malvidin the one with the highest concentration in the majority of grape cultivars.

2.2.3.1. Equilibrium of anthocyanins in function of pH.

Another aspect to consider is the fact that anthocyanins present a chemical equilibrium depending on the pH between their different chemical forms, which greatly conditions the colour of these pigments (Brouillard, 1982; Glories, 1984b). **Figure 11** illustrates the changes between the different forms of the anthocyanins in function of the pH.

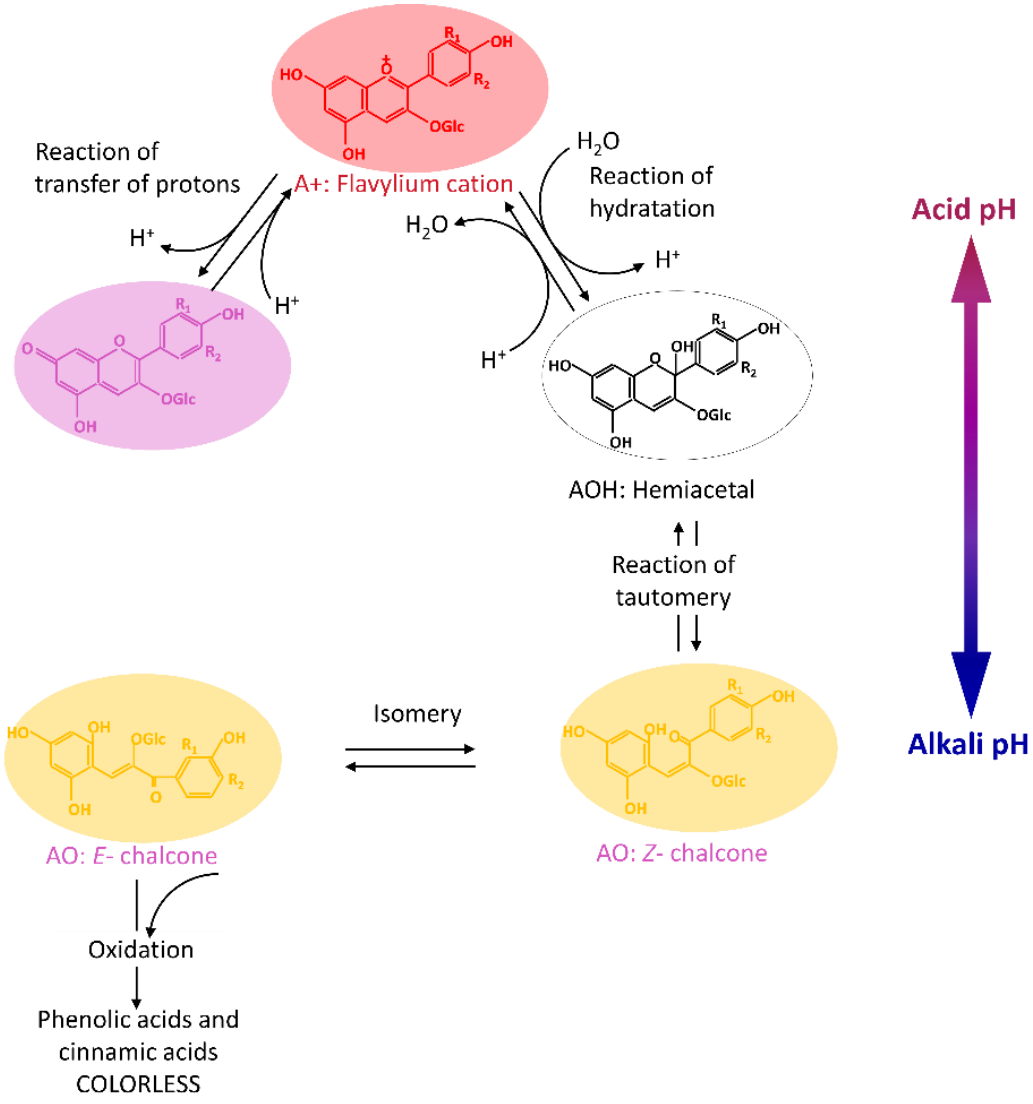


FIGURE 11: EQUILIBRIUM OF ANTHOCYANINS IN FUNCTION OF PH

Thus, at very acidic pH, the majority form of anthocyanins is flavylium cation, which presents a red coloration. However, when the pH of the medium increases, the flavylium

cation is transformed into the quinone base of a violaceous colour and into the colorless hemiacetal form. Both reactions occur simultaneously, according to their equilibrium constants. On the other hand, the hemiacetal can be transformed into the *cis* and *trans* chalcones that have a slight yellow colour (Ayabe *et al.*, 2010; Paulino, Pereira and Basílio, 2022). This last transformation is strongly favored by high temperatures (Furtado *et al.*, 1993). Finally, the trans chalcone can be oxidized, giving rise to phenolic acids (Brouillard, 1982). All these reactions are reversible with the sole exception of the oxidation reaction that would lead to the irreversible loss of colour in the wine (King *et al.*, 1980). Therefore, the stability of the colour of the wine will be highly compromised if the storage temperatures are high since the formation of chalcones and their subsequent oxidation are highly favored.

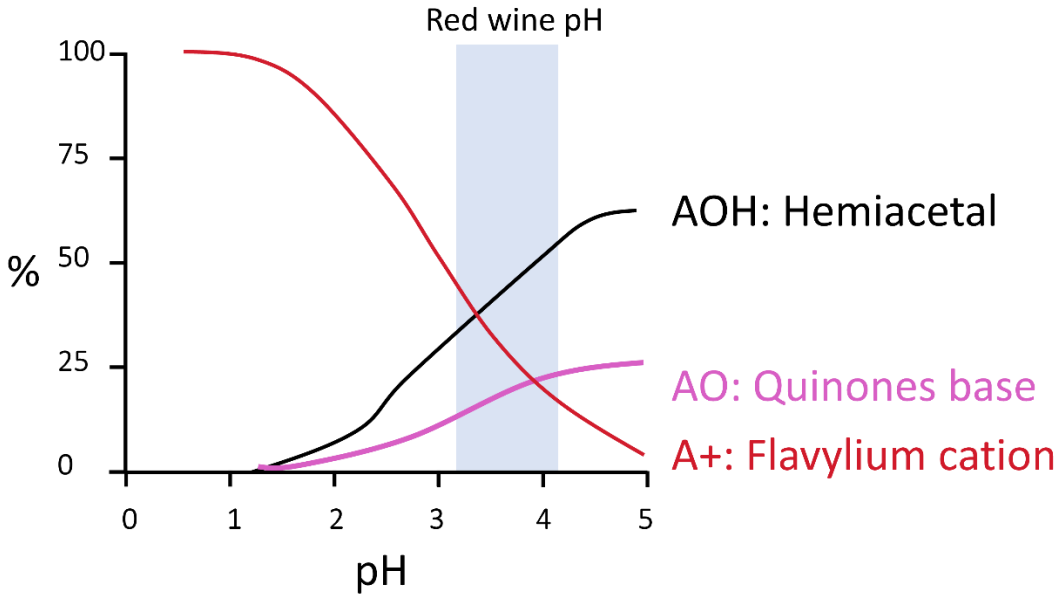


FIGURE 12: THEORETICAL INFLUENCE OF PH ON THE PERCENTAGE OF DIFFERENT FORMS OF MALVIDIN-3-O-GLUCOSIDE

Whatever the pH of a wine (**Figure 12**), an equilibrium among the different forms of the anthocyanins is established. That means that when the pH is very acidic, its colour will be more intense and the red tones will predominate, while when it is less acidic, the colour will be less intense and blue tones will predominate. In fact, the pH of most red wines is normally between 3.5 and 4.1, conditions in which only 20-30% of the anthocyanins would contribute to colour. Likewise, at pH close to 4, wines should not

be more blue than red, which is obviously not true. Fortunately, red wine is a much more complex matrix than a malvidin-3-*O*-monoglucoside solution and other phenomena occur in it that modulate this equilibrium. These phenomena are copigmentation and the formation of new pigments derived from anthocyanins**.** Both phenomena change this pH equilibrium, making that a higher proportion of these pigments contributes to the red wine colour (Brouillard and Dangles, 1994; Francia-Aricha *et al.*, 1997; Boulton, 2001).

2.2.3.2. Copigmentation.

The phenomenon of copigmentation is based on the fact that anthocyanin molecules are flat and can form associations between them or with other molecules, called co-pigments, giving rise to "sandwich" type structures (Boulton, 2001). The bonds between these molecules are of the weak type (Van der Waals, hydrophobic interactions). Within these "sandwich" or "stacking" type structures, a hydrophobic environment is generated that prevents the access of water molecules, in such a way that the nucleophilic attack that the hydroxyl groups would generate does not occur. In this way, the formation of colorless hydrated bases (hemiacetal) is reduced, and the equilibrium shifts towards the formation of colored structures (flavylium cation) (Liao, Cai and Haslam, 1992). Therefore, a higher percentage of anthocyanins than would correspond according to the pH, will contribute to the colour, as long as the adequate co-pigments exist in the medium.

Various types of compounds, such as phenolic acids, flavonoids, some amino acids, nucleotides, etc. can act as co-pigments (Mazza and Brouillard, 1990). The only necessary requirements are that the molecules present a planar configuration and that they also have aromatic rings that allow interactions such as those described.

The most important consequence of copigmentation is the hyperchromic effect that generates a greater intensity of colour than should be expected. This is undoubtedly the main effect of copigmentation, but not the only one, since the colour tone can also be modified (Boulton, 2001; Darias-Martín *et al.*, 2001). Thus, a hypsochromic effect can be produced when there is a displacement of the maximum absorbance of the spectrum towards shorter wavelengths, or a bathochromic effect when the displacement is towards longer wavelengths.

2.2.3.3. Pigments derived from anthocyanins.

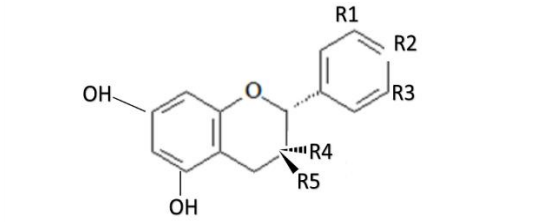
Red wine colour is a truly complex phenomenon involving multiple factors. The established theory supports that the colour of red wine during its youth is due to its concentration in anthocyanins (Brouillard, 1982; Glories, 1984a), although the pH; (Glories, 1984b; Zamora, 2003), the concentration of free sulfur dioxide (Jurd, 1964) and the presence of other phenolic compounds that can act as co-pigments (Mazza and Brouillard, 1990; Boulton, 2001) also participate in a very patent way. Subsequently, as the wine ages, a part of the anthocyanins disappear by oxidation (King *et al.*, 1980), and another part of them gives rise to combinations with proanthocyanidins, also called condensed tannins, which gives rise to new, more stable pigments (Sarni-Manchado *et al.*, 1996; Mateus *et al.*, 2001). Also, according to the established theory, these combinations, which would be responsible for the colour of aged wines, can be generated by direct reaction of anthocyanins with tannins, or by the participation of ethanal (Cheynier, Moutounet and Sarni-Manchado, 2000; Pascal Ribéreau-Gayon *et al.*, 2006). This ethanal would originate in a first phase during alcoholic fermentation, and later during the aging of the wine in barrels or thanks to the application of the micro-oxygenation technique (Boulet and Moutounet, 2000; Kontoudakis *et al.*, 2011). Ethanal, which would act as a link between anthocyanins and tannins, forming the so-called ethyl bridges between molecules, would justify the phenomena of colour stabilization and astringency softening (Cheynier, Moutounet and Sarni-Manchado, 2000; Pascal Ribéreau-Gayon *et al.*, 2006) that are observed when aging red wines in barrels or when applying the micro-oxygenation technique (Llaudy *et al.*, 2006).

In addition, other pigments can also be formed from anthocyanins thanks to cycloaddition reactions with ethanal, private, vinyl phenol and vinyl flavanols (Sarni-Manchado *et al.*, 1996; Bakker and Timberlake, 1997; Francia-Aricha *et al.*, 1997; Fulcrand *et al.*, 1998). These pigments, called pyranoanthocyanins or vitisins, have an orange-red colour, are very insensitive to pH variations, are not discolourable by sulfur dioxide, are highly resistant to oxidation, and are especially present in aged wines. Consequently, pyranoanthocyanins are good candidates to explain the colour of old wines (Zamora, 2003). However, other pigments derived from pyranoanthocyanins have also been described (Mateus *et al.*, 2003; Monagas and Bartolomé, 2009; Zamora, 2013;

Fernandes *et al.*, 2017) making that the elucidation of the chemistry of red wine colour a very complicate subject that remains to be fully understood.

2.2.4. Flavan-3-ols or Flavanols

The term flavan-3-ols or flavanols includes the flavan-3-ol monomers in **Figure 13**, and their polymers known under the name of condensed tannins or proanthocyanidins. These polymers, that are extracted from skins and seed during winemaking, are made from mainly 4 monomeric units: (+)-catechin, (−)-epicatechin, (−)-epigallocatechin and (−)-epicatechin- 3-O-gallate (Terrier *et al.,* 2009; Li and Sun, 2019). Typically manifesting values ranging from 2 to 11g/kg in red grapes, and 1 to 4g/L in red wine (Zoecklein *et al.*, 1999).



(+)-Catechin: R1=R4=H, R2=R3=R5=OH
(+)-Catechin gallate: R1=R2=OH, R3=H , R4=H, R5=gallyl
(+)-Gallocatechin: R1=R2=R3=R5=OH, R4=H
(+)-Gallocatechin gallate: R1=R2=R3=OH, R4=H, R5=gallyl
(-)-Epicatechin: R1=R5=H, R2=R3=R4=OH
(-)-Epicatechin gallate:R1=R2=OH, R3=R5=H, R4=gallyl
(-)-Epigallocatechin: R1=R2=R3=R4=OH, R5=H
(-)-Epigallocatechin gallate: R1=R2=R3, R4=gallyl, R5=H

FIGURE 13: CHEMICAL STRUCTURE OF FLAVAN-3-OLS MONOMERS

However, the chemical composition of the proanthocyanidins from seeds and skins is not identical. Grape seed proanthocyanidins are polymers composed of (+)-catechin, (−)-epicatechin and (−)-epicatechin- 3-O-gallate (Prieur *et al.*, 1994). Grape Skin proanthocyanidins are composed of the same monomers but also contain (−)-epigallocatechin, and the proportion of (−)-epicatechin- 3-O-gallate is much lower (Souquet *et al.*, 1996). Consequently, grape skin tannins are composed of procyanidins and prodelphinidins because their acidic cleavage gives cyanidin and delphinidin, whereas grape seed tannins are composed only of procyanidins. In addition, seed proanthocyanidins have a lower degree of polymerization (mDP) than skin

proanthocyanidins (Prieur *et al.*, 1994). Thus, procyanidins with a larger percentage of galloylation and a lower mDP are solely produced by grape seeds, whereas procyanidins and prodelphinidins with a higher mDP are released by grape skins.

Flavan-3-ols are the main determinants of the astringency and bitterness perception in red wine (Peleg *et al.*, 1999). It has been described that the higher the mean degree of polymerization (mDP) and higher the proportion of (-)-epicatechin- 3-O-gallate of the proanthocyanidins the greater the astringency perception (Vidal *et al.*, 2003; Sun *et al.*, 2013). It is generally accepted that seed proanthocyanidins are more astringent than skin proanthocyanidins because they have a higher proportion of epicatechin-3-O-gallate. In contrast, flavanol-3-ols monomers and proanthocyanidins with lower mDP seem to enhance bitterness perception (Peleg *et al.*, 1999).

As it has been explained in previous sections, during winemaking and aging, the presence of small quantities of oxygen leads to the formation of ethanal from ethanol. The ethanal can in turn react with flavanols to induce the formation of a very reactive carbocations that quickly react either with another flavanol molecule or with an anthocyanin, producing ethyl-bridged flavanol-flavanol or flavanol-anthocyanin oligomers (Timberlake and Bridle, 1976; Atanasova *et al.*, 2002; Kontoudakis *et al.*, 2011).

All these reactions, together with the formation of pyranoanthocyanins, result in a gradual shift in the colour of the wine from the initial purple-red to a reddish-brown. The astringency also diminishes, but the mechanism by which this happens it is not so clear. Theoretically, the formation of ethyl bridges should increase the degree of flavanol polymerization, which in turn should increase the astringency (Vidal *et al.*, 2003). However, astringency decreases during aging, and this fact has been traditionally attributed to the reaction of phenolic compounds with oxygen (Glories, 1984b; Zamora, 2003, 2019). One possible explanation may be that the condensation reactions between anthocyanins and flavanols can diminish astringency (Vidal *et al.,* 2004). Some authors have even suggested that the cleavage reactions of proanthocyanidins as a result of acid catalysis may cause the observed reduction in astringency (Cheynier *et al.*, 2006).

These changes of the wine flavanol composition are affected by several factors such as temperature, pH, and free sulfur dioxide concentration, although oxygen exposure is probably the main determinant (Singleton, 1987; Rivas-Gonzalo, Bravo-Haro and Santos-Buelga, 1995; Atanasova *et al.*, 2002). In fact, the reason that wines are traditionally aged in oak barrels is because the porosity of the wood, the interstices between staves, and the bunghole allow the entry of small amounts of oxygen, which can induce all the aforementioned reactions (Vivas, 1997; Zamora, 2019).

3. THE WINE COLOUR

Color (American English) or colour (Commonwealth English) is the visual perception based on the electromagnetic spectrum. However, colour is not an inherent property of matter since its perception is related to light absorption, reflection, emission spectra, and interference from other objects. The Royal Spanish Academy of Language defines colour as "*The sensation produced by the light rays that impress the visual organs and that depends on the wavelength*". This definition is incomplete since the appreciation of colour is greatly influenced by the environment surrounding the object and the lighting to which it is subjected. The great Leonardo da Vinci (1452-1519) already affirmed that colour was the result of a complex relationship between the observed phenomenon and the conditions of its observation.

In the human eye, colour is perceived in the visible light spectrum with three types of cone cells (trichromacy). Other species may have a distinct range of colour sensitivity because they have a different number of cone cell types or eyes that are sensitive to a different wavelength, such as bees that can discern ultraviolet. Animal perception of colour is originated from different light wavelength that stimulate the spectral sensitivity in cone cell types, which is then processed by the brain, generating our own image of the colour that we perceive.

The first sensation that is perceived from a glass of wine is, without a doubt, its visual appearance. It is precisely the immediacy of the vision that gives capital importance to

its appearance. Its transparency, its brightness and above all its colour are some of the most determining attributes of the quality of a wine (Glories, 1984a), not only because of the obvious implications on its image, but also because they are indicators of other aspects related to its aroma and with its flavor (Peynaud, 1987). Thus, by the colour of a wine we can have an idea of its age, its tannin concentration, its state of conservation, and we can even guess some defects that we will later notice when tasting it (Zamora, 2004). In the specific case of red wines, colour becomes even more important since, as oenologists know very well, red wines endowed with great colour, and above all the ability to maintain it for a long time, are currently the most valued in the market (Lattey, Bramley and Francis, 2010; Valentin *et al.*, 2016). It is a proven fact that colour is one of the key points that determine the price of wine in commercial transactions (Combris, Lecocq and Visser, 1997, 2000).

Figure 14 shows the absorption spectra of a young white wine and a young red wine. The absorbance axe is not dimensioned in order to show both spectra in the same figure, since the red wines have much more absorbance intensity than the white wines.

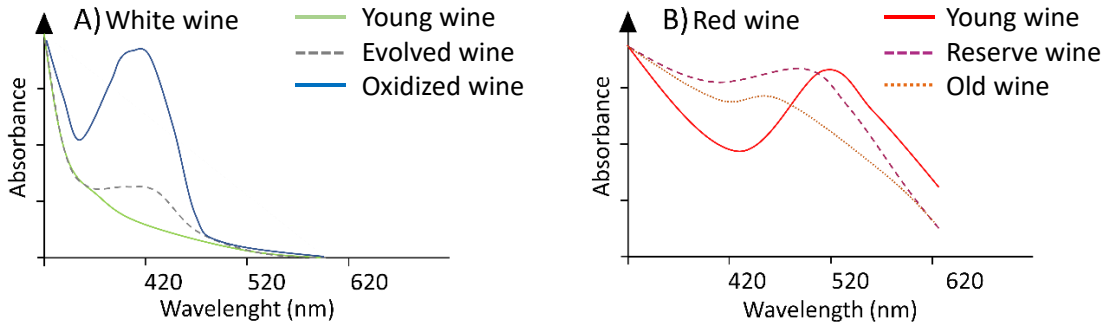


FIGURE 14: ABSORPTION SPECTRA OF A WHITE WINE AND A RED WINE AND THEIR EVOLUTION OVERTIME (ADAPTED FROM ZAMORA, 2003; GUZMÁN-ALFEO, 2010; PHILIPIDIS ET AL., 2017)

In this figure it can be observed that the spectrum of young white wine has no any significant absorbance from 700 nm to 450 nm and starts to absorb in the last zone of visible wavelengths, from 450 nm to 400 nm (**Figure 14A** continuous green line). For that reason, the visual appearance of this wine is slightly yellow. In contrast, the spectrum of young red wine (**Figure 14B** continuous red line) presents a maximum at 520 nm, corresponding to the red colour, and some relatively important yellow and blue

components (420 nm and 620 nm respectively). For this reason, this red wine has an intense red colour with violet hues (Sudraud, 1958).

Figure 14 also illustrates how the absorption spectra of white and red wine evolves overtime. Specifically, the colour intensity of white wines will grow over time, making that its initially slight and bright yellow colour becomes increasingly brown (Skouroumounis *et al.*, 2005). In contrast, the colour of the red wine will lose intensity in the red and blue components and will gain in yellow causing that the original intense purplish-red colour turns firstly into a brick-red hue and after a longer time into brown nuances (McRae *et al.*, 2012; Avizcuri *et al.*, 2016). It is obvious that the rate in which these changes occur is strongly influenced by the storage conditions and the healthiness of the grapes. The higher the temperature and the oxygen availability, the faster the colour deterioration (Arapitsas *et al.*, 2014; Scrimgeour *et al.*, 2015). In addition, the infection of *Botrytis cinerea* in the original grapes, which conditions the presence of the enzyme laccase, would seriously accelerate the wine colour worsening (Steel, Blackman and Schmidtke, 2013; Vignault *et al.*, 2020; Giménez *et al.*, 2022).

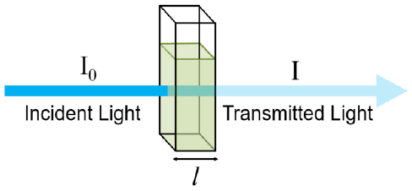
3.1. Measurement of wine colour

From everything stated in the previous section, it can be deduced that the objective determination of the colour of the wine is essential to be able to characterize one of its main organoleptic properties.

There are various systems for measuring the colour of wine, all of them based on its visible absorption spectrum. Visible light represents a very narrow section of this range with wavelengths between 400 nanometers (nm) for blue light to around 700 nm for red light. Shorter wavelengths fall into the ultraviolet region and longer wavelengths are in the infrared region. Each compound has a colour absorbance and reflectance, for example, the observed colour (reflect) at 400nm is yellow-green, but the corresponding colour (absorbed light) at 400nm is the violet. White light is mixture of all the wavelengths in the visible range.

I. Introduction

When light strikes an object, it may be reflected, absorbed, transmitted, or diffracted. Different spectrophotometric parameters can be defined. The first one is **Transmittance,** which compares the intensity of the light that passes through the sample (I) to the intensity observed without the sample (I_0). **Figure 15** and **Equation 1** define this parameter.



EQUATION 1: TRANSMITTANCE

$$Transmittance\ (T\%) = 100\ x\ I/I_0$$

FIGURE 15: SCHEME ON TRANSMITTANCE MEASUREMENT

Transmittance therefore indicates the percentage of the light not retained by the sample and will be 0 % when all the light is retained and 100 % when no light is retained. Evidently, the higher the concentration of a chromophore, the greater the light retention percentage. However, the relationship between the Transmittance and the chromophore concentration of a sample is logarithmic and inversely proportional. For that reason, another parameter is defined, **Absorbance**, which is defined according with **Equation 2.**

EQUATION 2: ABSORBANCE

$$Absorbance\ (A) = 2 - log_{10}(\%T)$$

Absorbance, therefore, gives an idea of the attenuation of the light passing through a solution with a chromophore. An Absorbance value of 0 indicates that all the light passes through the sample (Transmittance = 100 %) whereas a value of 2 indicates that 99 % of the light is retained by the chromophore (Transmittance = 1 %). **Figure 16** illustrates the relation between Transmittance and Absorbance.

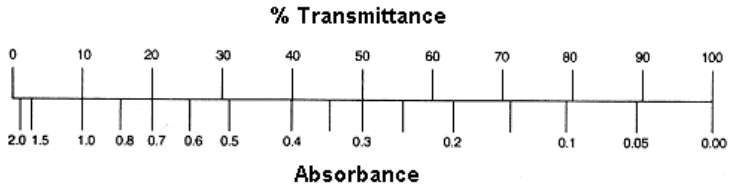


FIGURE 16: RELATION BETWEEN TRANSMITTANCE AND ABSORBANCE

The Beer-Lambert law establish a direct relationship between the concentration of a chromophore and the absorbance of the solution. **Equation 3** illustrates this law.

EQUATION 3: BEER-LAMBERT LAW

$$Absorbance\ (A) = (\varepsilon)\ x\ (c) x\ (l)$$

Where $\boldsymbol{\varepsilon}$ is the molar extinction coefficient, $\boldsymbol{c}$ is the concentration of the chromophore and $\boldsymbol{l}$ the optical path length.

This optical Law (Swinehart, 1962) was described due to the contributions of Johann Heinrich Lambert (Lambert, 1760) and August Beer (Beer, 1852). For that reason, this law is called the Beer-Lambert law in their honour although, regrettably, this name excludes another important contributor as it was Pierre Bourguer (Bouguer, 1729).

There are several systems for measuring the colour, all of them based on its visible absorption spectrum, but probably for the wine colour the most used ones are the measurement of colour intensity and related parameters (Sudraud, 1958; Glories, 1984b, 1984a) and CieL*a*b* coordinates (CIE, 1986; Zamora, 2004).

3.2. Colour intensity and related parameters

The absorbances corresponding to the yellow (420 nm), red (520 nm) and blue (620 nm) components of the colour are measured in a cuvette with 1 mm optical path length for red wines and of 10 mm for white wines. The equations that define this methodology, well known by the oenologists, are shown in **Figure 17**.

I. Introduction

$$\text{Colour intensity (CI)} = F \times (A_{420nm} + A_{520nm} + A_{620nm})$$

$$\text{Hue (h)} = 100 \times \frac{A_{420nm}}{A_{520nm}}$$

$$\text{Yellow component} = 100 \times \frac{A_{420nm}}{CI}$$

$$\text{Red component} = 100 \times \frac{A_{520nm}}{CI}$$

$$\text{Blue component} = 100 \times \frac{A_{620nm}}{CI}$$

FIGURE 17:EQUATIONS FOR CALCULATING COLOUR INTENSITY, HUE, AND THE YELLOW, RED, AND BLUE COMPONENTS OF COLOUR (GLORIES, 1984B, 1984A)

Analytically, one of the ways to determine colour is through spectrophotometry, in which visible light represents a very narrow section of this range with wavelengths between 400 nanometers (nm) for blue light to around 700 nm for red light. Shorter wavelengths fall into the ultraviolet region and longer wavelengths are in the infrared region. Each compound has a colour absorbance and reflectance, for example, the observed colour (reflect) at 400nm is yellow-green, but the corresponding colour (absorbed light) at 400nm is the violet. White light is mixture of all the wavelengths in the visible range.

When light strikes an object, it may be reflected, absorbed, transmitted, or diffracted. This measurement, by spectrophotometer, compares the intensity of the light going through the sample (I) to the intensity observed without the sample (I_0), this effect, the absorbance (Abs) is represented by the amount of light absorbed by the sample.

The result must be multiplied by a factor (F) to refer the measurement to the standard 10-mm pathlength cuvette (Usually F = 1 in white wines and F = 10 in red wines).

The colour intensity, which will give us an idea of how much colour the wine has, will be the sum of all the components (yellow, red and blue). The hue, which indicates the relative importance of yellow over red, will be the quotient between A420nm and A520nm, expressed as a percentage. Finally, the yellow, red and blue components of the colour will be determined as the quotient between each of them with respect to the

colour intensity, also expressed as a percentage. Obviously, these components will express the relative importance of each one of these colours in the overall wine.

This methodology is the most widely used in wineries thanks to the simplicity of its application and the conceptual facility to interpret its results. However, it is an imperfect measurement because it only considers 3 points of the visible spectrum, and it does not consider aspects as important as lighting and observation conditions.

3.3. CieL*a*b* coordinates

In 1976, the *Commission Internationale de l'Éclairage* (CIE) established the norms, which are internationally accepted today, for the correct definition of colour. These norms refer to the characteristics of the illuminates (illuminate D65), to the observation conditions (CIE64 observer, 10^{o} visual field) and to the spectral sensitivity curves of the normal human eye for three conventional light stimuli called X, Y and Z. These tristimulus values represent the three base colors (X: virtual red, Y: virtual green and Z: virtual blue) with which all existing colors can be reproduced through their combination. Therefore, these X, Y, and Z coordinates are a numerical expression that represents the relative proportion of each of the base colors necessary to reproduce, in the eye of the observer, the specific colour of the analyzed object.

The determination of these tristimulus values can be done, as recommended by the (CIE, 1986) by determining the entire spectrum and its parameterization at 10 nm intervals. However, this requires the use of a scanning spectrophotometer and the use of complicated equations (Juarez, Echávarri and Negueruela, 1997), although currently there is equipment that directly performs the calculation. To simplify the determination of tristimulus values, the OIV currently recommends using a simplified method (Ayala, Echavarri and Negueruela, 1999; Pérez-Caballero *et al.*, 2003) that applying the transmittance measurement (expressed as so much per one) only to 4 wavelengths allows a satisfactory precision in the determination of X, Y and Z.

I. Introduction

The simple calculation of these tristimulus values (X, Y and Z) is obtained after reading the transmittance at 450, 520, 570 and 630 nm in a cuvette with a 2 mm optical path, according to what is described in the equations presented in **Figure 18**.

$$X = 19{,}717\ T_{450} + 1{,}884\ T_{520} + 42{,}539\ T_{570} + 32{,}474\ T_{630} - 1{,}841$$
$$Y = 7{,}950\ T_{450} + 34{,}764\ T_{520} + 42{,}736\ T_{570} + 15{,}759\ T_{630} - 1{,}180$$
$$Z = 103{,}518\ T_{450} + 4{,}190\ T_{520} + 0{,}251\ T_{570} - 1{,}831\ T_{630} + 0{,}818$$

FIGURE 18:EQUATIONS FOR THE DETERMINATION OF THE TRISTIMULUS VALUES (ZAMORA, 2003)

The *Commission Internationale de l'Éclairage* (CIE) 1976 also defined the so-called CIEL*a*b* space (**Figure 18**), which tries to represent all the possible colours in a Euclidean sphere.

By means of the three Cartesian coordinates: Y axe: Lightness (L*), X axe: green-red component (a*) and Z axe: blue-yellow component (b*) it is therefore possible to define a colour within this CIEL*a*b* space. These three axes (**Figure 19A**) represent the gradations between opposite colours. Thus, L* goes from white to black, coordinates a* from green to red, and b* from yellow to blue.

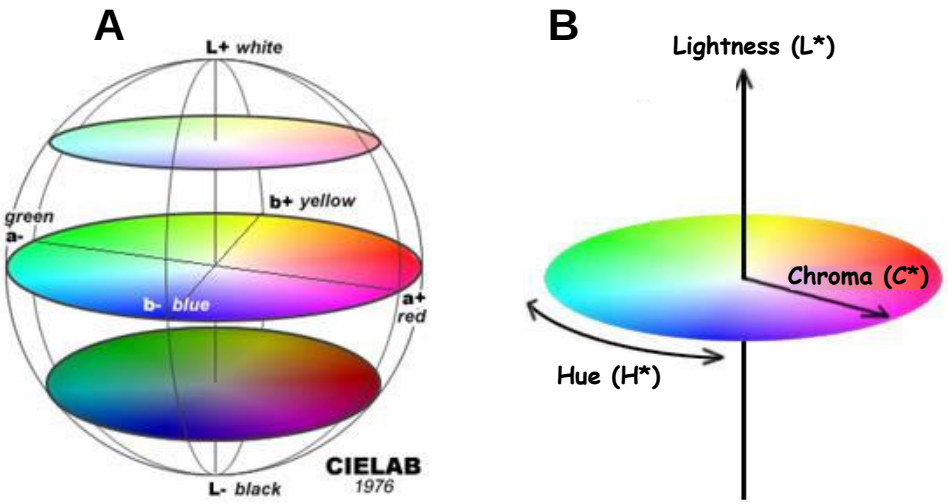


FIGURE 19: EUCLIDEAN SPHERE OF CIEL*A*B*

The calculation of L*, a* and b* is determined from the tristimulus values X, Y and Z according to the equations shown in **Figure 20**.

$$L^* = 116 \sqrt[3]{\frac{Y}{100}} - 16$$

$$a^* = 500 \left(\sqrt[3]{\frac{X}{94{,}825}} - \sqrt[3]{\frac{Y}{100}} \right)$$

$$b^* = 200 \left(\sqrt[3]{\frac{Y}{100}} - \sqrt[3]{\frac{Z}{107{,}383}} \right)$$

FIGURE 20: EQUATIONS FOR CALCULATING THE CIEL*A*B* COORDINATES (ZAMORA, 2003)

To further simplify the expression of colour, the a* and b* coordinates of CIELAB space can be transformed into the spherical coordinates Hue (H*) and Chroma (C*) according to the equations presented in **Figure 21**.

$$H^* = \text{arctg}\,(b^*/a^*)$$

$$C^* = \sqrt[2]{a^{*2} + b^{*2}}$$

FIGURE 21: EQUATIONS FOR CALCULATING THE HUE (H*) AND CHROMA (C*)

Thus, the colour of an object, and obviously that of any wine, can be also defined, within the CIEL*a*b* space, by the three spherical coordinates called L*: Lightness, H*: Hue and C*: Chroma or Saturation. The physical sense of these chromatic spherical coordinates within CIEL*a*b* space is shown in **Figure 19B**.

In short, the chromatic coordinates and the CIEL*a*b* space allow a much more precise definition of the colour of the wine than the standard parameters. However, its application in the wineries has not been extended up to now due to the complexity of its calculation and the difficulty that exists to relate the quality of the colour of the wine with the chromatic coordinates.

However, the current reality indicates that CIEL*a*b* coordinates are widely used to define colour in most of the other human activities and will undoubtedly end up being

imposed in the world of wine as well. In fact, most of the scientific publications use this system, which has also been shown to be extremely useful for obtaining valid information to classify vintages, distinguish varieties or differentiate geographical origins (Almela *et al.*, 1996; Huerta *et al.*, 1998). Likewise, the greater precision that CIEL*a*b* parameters allow has made it possible to consistently relate the usual descriptions of wine colour with certain values of lightness, saturation and hue (Iñíguez *et al.*, 1995; Fan, Liu and Zhang, 2023). Thanks to this, it is even possible to define concepts such as "ruby red" or "brick colour" in physical terms (Hernández *et al.*, 2009).

In addition, CIEL*a*b* coordinates can be also used to determine if the human eye can distinguish or not between two samples of wine. The total colour difference (ΔEab*) is defined as the Euclidian distance between two points in the CIEL*a*b* spherical space according to the equation showed in **Figure 22**.

$$\Delta Eab^* = \sqrt[2]{(L^*_1 - L^*_2)^2 + (a^*_1 - a^*_2)^2 + (b^*_1 - b^*_2)^2}$$

FIGURE 22: EQUATIONS FOR CALCULATING THE TOTAL COLOUR DIFFERENCE (ΔEAB*)

Generally, it is considered that the difference is visible to human eye when ΔEab* > 3 (García-Mariano *et al.*, 2013; Vignault *et al.*, 2019).

4. GRAPE MUST/WINE AND OXYGEN

The atmosphere of the earth contains approximately 21% oxygen (O_2). This gas, which makes possible the life on the planet, play a crucial role in many metabolic and chemical reactions, and is in part responsible of the wine evolution. In fact, Louis Pasteur wrote in his famous book "Études sur le vin" (Pasteur, 1866) the following sentence: "*C'est l'oxygène qui fait le vin; C'est parson influènce qu'il vieillit*". It is therefore obvious that oxygen exerts a great influence on the wine composition and quality and that greatly determines its evolution. The effects of oxygen can be either positive or negative

depending on several factors such as the healthiness of the grapes, the procedures used to make it, the step of winemaking in which oxygen is provided, the grape juice or wine composition and especially the doses of oxygen that the wine receives throughout its life and the procedures used to protect it. This part of the introduction will focus on the basic steps involved in grape must and wine oxidation processes.

4.1. Oxidation and reduction

A chemical oxidation process occurs when a molecule loses electrons, while the opposite, gain of electrons, occurs in a reduction process. It is obvious that when a substance loses electrons, the other one must acquire electrons, forming therefore an oxidation-reduction couple.

In wine, oxygen is the predominant electron acceptor since it can be easily reduced to certain intermediates and eventually to hydrogen peroxide. Molecular oxygen (O_2) is found naturally in a triplet ground state which means that contains two unpaired electrons being therefore a very stable diradical. This limits the reactivity of O_2 since it cannot form bonds by accepting electron pairs. However, the addition of a single electron, which can be originated from reduced transitional metal ions such copper or iron, can overcome this limitation. This leads to an unpaired electron in the resulting negatively charged superoxide radical, and with a second electron transfer would result in a peroxide anion (Miller, Buettner and Aust, 1990; Danilewicz, 2003; du Toit *et al.*, 2006). This phenomenon implies that oxygen engages in several reactions in wine.

4.2. The oxidoreduction potential

The redox potential (oxidation-reduction - E_H) is a measure of the state of oxidation or reduction of a medium. Just like the pH expresses the acid-base state of a solution at a given instant, E_H expresses the level of oxidation or reduction of this same solution. In addition, if the pK indicates the half-neutralization point of an acid/base couple, the equilibrium of a redox couple is expressed by the normal potential (E_o) (Glories and Zamora, 1991).

I. Introduction

The redox potential (E_H) of a solution is defined as the electric potential difference between an unattackable electrode plunging in it and the reference hydrogen electrode, considered equal to zero by convention (Vivas, Zamora and Glories, 1992; Zamora, 2010).

The redox potential (EH), in accordance with thermodynamic considerations, is governed by the expression.

$$E_H = -\Delta G/nF$$

Where **ΔG** represents the variation of Gibbs free energy and indicates the thermodynamic tendency of a medium to be oxidized (EH > O) or reduced (EH < O), **n**, the number of interchanged electrons an **F**, the Faraday's constant.

According to the law of Nernst (Nernst, 1891), the redox potential of a grape must or a wine is regulated by expression.

$$E_H = E_0 + \frac{R \times T}{nF} \times \ln \frac{[\text{Oxidized forms}]}{[\text{Reduced forms}]}$$

Where $\mathbf{E_0}$ represents the normal potential of a redox couple, which means the E_H when the ratio between the oxidized and reduced forms is one. Consequently, E_H depends on the relative concentration between the oxidized and the reduced form of a redox couple. In the case of a solution with only a simple redox couple, the equation is quite easy to interpret. However, it gets devilishly complicated in a so complex medium as the wine is, where several redox couples are acting simultaneously. Moreover, E_H can only detect electroactive redox couples able to interchange electrons with the redox electrodes.

Considering the simplest media, pure water, the only redox couple is that reflected in the following redox reaction:

$$H^+ + O_2 + 4e^- \rightleftharpoons 2H_2O$$

According to that, the development of the Nerst's law would indicate the following equation:

$$E_H = E_o + \frac{R \times T}{4F} \times \ln \frac{[H^+]^2 \times [O_2]}{[H_2O]^2}$$

Considering that for distilled water at 25 ^oC, E_0 = 1.229 V; R = 8.31 J x mol^{-1} x K^{-1} and F = 96,500 C/mol; $[H_2O]$ = 55.55 mol^{-1}; pH = -log $[H^+]$

We obtain the following relation.

$$E_H \text{ (mV)} = 1.177 - 59.15 \text{ pH} + 14.79 \log [O_2]$$

However, as it was, the wine is a quite more complex matrix including several other redox couples. For that reason, the following development of the Nerst's law has been proposed (Vivas, Zamora and Glories, 1992, 1993; Vivas and Glories, 1996):

$$E_H = \frac{R \times T}{nF} \times \ln \frac{[\text{Oxidized forms}]}{[\text{Reduced forms}]} - 59{,}15 \times \text{pH} + B \times \log[O_2]$$

Where the expression RT/nF x ln [(Oxidized forms)/(Reduced forms)] represents the weighted average of the ratios between the oxidized and reduced components of the redox couples of the wine that reflex the redox state of the wine. In that case, the coefficient of log $[O_2]$ is not a constant as it was in the case of pure water since it depends on the stoichiometry of the ensemble of the redox couples using oxygen as electron acceptor.

4.3. Substrates for oxidation in grape must and wine

Grape must contain many substances capable of reacting directly or indirectly with oxygen. Some of these substances, such as ascorbic acid or glutathione, practically disappear in the preliminary stages of winemaking (Singleton, 1987; Giménez *et al.*, 2023; Giménez *et al.*, 2023), while others, phenolic compounds, remain in the wine although they can be partially or totally transformed (du Toit *et al.*, 2006; Pascal Ribéreau-Gayon *et al.*, 2006)

All these transformations, which will be described below, can be performed enzymatically or non-enzymatically and are responsible for browning, an oxidation process that occurs in many foods that increases the brown colour (Friedman, 1996), which often leads consumers to reject them. This problem is especially harmful in the wine industry since is highly vulnerable to browning (du Toit *et al.*, 2006; Oliveira *et al.*, 2011).

Among phenolic compounds, it seems that hydroxycinnamic acids are the main substrates for both enzymatic and non-enzymatic browning (Singleton *et al.*, 1985; Cheynier, Souquet and Moutounet, 1989) although other phenolic compounds can also be oxidized (Oliveira *et al.*, 2011; Steel, Blackman and Schmidtke, 2013; Giménez *et al.*, 2022).

4.4. Enzymatic oxidations

The enzymatic oxidation is mainly catalyzed by a large family of enzymes grouped under the name of polyphenol oxidases (PPOs). Two basic types of phenol oxidase can be found: tyrosinases and laccases that according to the International Union of Biochemistry and Molecular Biology (IUBMB) receive the Enzyme Commission numbers EC 1.14.18.1 and EC 1.10.3.2 respectively.

While laccase oxidizes a wider range of substances, including substituted mono- and polyphenols, aromatic amines, and thiols, tyrosinase mostly oxidized p-monophenols and ortho-diphenols to quinones (Bounegru and Apetrei, 2021). Both enzymes are

responsible for enzymatic browning in grape must and wine, and also in many other foods (Franck, 2007). Tyrosinase is naturally present in grapes (du Toit *et al.*, 2006; Oliveira *et al.*, 2011) whereas laccase is only present when the grapes are infected by grey rot (Oliveira *et al.*, 2011; Steel, Blackman and Schmidtke, 2013).

The mechanism of enzymatic browning is synthetized in **Figure 23**.

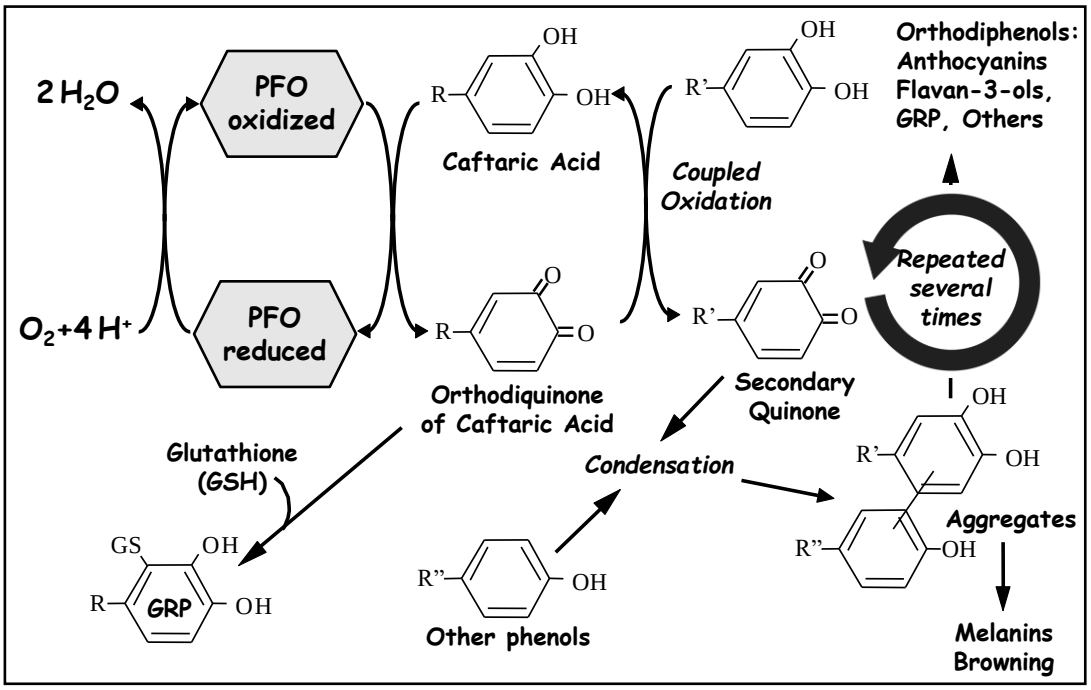


FIGURE 23: MECHANISM OF ENZYMATIC BROWNING

In a nutshell, PPOs employ molecular oxygen to oxidize mostly diphenols through a reaction pathway accelerated by radicals to produce quinones (Claus, 2004; Li, Guo and Wang, 2008). Following a series of processes, these quinones and their derivatives are polymerized to create the brown pigments known as melanins (Queiroz *et al.*, 2008; Oliveira *et al.*, 2011; Claus, 2014). These pigments, which are relatively insoluble depending on their degree of polymerization (Moon *et al.*, 2020), are responsible for increasing the intensity of the brown colour in white wines (browning) and for the precipitation of the colouring matter in red wines (oxidasic haze) (Pascal Ribéreau-Gayon *et al.*, 2006).

Botrytis cinerea laccase is an extracellular glycoprotein multi-copper oxidase enzyme. It is structurally composed of three domains, namely domain 1, domain 2, and domain 3.

Domain 3 is situated in the superficial region of the protein, wherein copper T1 acts as the initiator of oxidation by reducing from Cu (II) to Cu(I), thereby limiting the acceptance of electrons (e^-) from phenol moieties. Subsequently, an electron transfer occurs from copper T1 to trinuclear copper T2/T3a&b, located in domains 1 and 2, respectively, which is reduced to copper ion. Finally, trinuclear copper is responsible for the reduction of $4e^-$ of the molecular reduction of oxygen (O_2) to form two water molecules ($2H_2O$). Concurrently, the oxidation of phenol to phenoxyl radical occurs, which participates in polymerization based on coupling or radical rearrangement (Giardina *et al.*, 2010).

4.5. Strategies to prevent enzymatic browning

The most common solution that winemakers used to protect the grape juice from enzymatic browning is without any doubt the use of sulfur dioxide (Ough and Crowell, 1987; Pascal Ribéreau-Gayon *et al.*, 2006). SO_2 is a powerful inhibitor of both types of PPOs (du Toit *et al.*, 2006; Oliveira *et al.*, 2011; Verma *et al.*, 2018; Vignault *et al.*, 2020; Giménez *et al.*, 2022), although laccase is more resistant to inhibition by this additive than tyrosinase (du Toit *et al.*, 2006; Pascal Ribéreau-Gayon *et al.*, 2006). For that reason, SO_2 is practically indispensable in wine industry.

Nevertheless, sulfur dioxide is currently being demonized by the public opinion and many consumers are demanding for the reduction and even the elimination of this unfriendly additive. There are several reasons for this actual trend but probably the most important ones are its negative effects on the environment (Schöpp *et al.*, 2003) the increasing tendency towards minimal intervention (Massov, 2019) and health (Lester, 1995) since it can cause headaches in sensitive people (Costanigro, Appleby and Menke, 2014).

Owing to all these factors, the present trend in wine production is geared towards reducing or eliminating the use of this antipathetic additive. This has led to the introduction of alternatives to SO_2 to protect grape must and wine against oxidation, such as the addition of ascorbic acid (Pascal Ribéreau-Gayon *et al.*, 2006), protection with an inert atmosphere (Martinez and Whitaker, 1995), protect the wines with

oenological tannins (Vignault *et al.*, 2019, 2020), and more recently, the application of reduced glutathione (Kritzinger, Bauer and Du Toit, 2013; Zimdars, 2020) or dried inactivated yeast rich in glutathione (Gabrielli *et al.*, 2017; Bahut *et al.*, 2020). Other alternatives to SO_2 have been proposed as microorganism inhibitors such as lysozyme (Liburdi, Benucci and Esti, 2014), chitosan (Picariello *et al.*, 2020), DMDC (Costa *et al.*, 2008; Renouf, Strehaiano and Lonvaud-Funel, 2008), or more recently fumaric acid (Morata *et al.*, 2020), but are not developed in this introduction since are not the objective of this work.

Ascorbic acid reduces the oxygen-reactive capacity of polyphenol oxidase enzymes because its direct oxygen consumption is several times faster than that of sulfur dioxide (Pascual *et al.*, 2017; Vignault *et al.*, 2020). However, it should be noted that the use of ascorbic acid can generate hydrogen peroxide after consuming oxygen, which can trigger subsequent oxidations that may affect the sensory quality of the wine (Oliveira *et al.*, 2011). Therefore, the use of ascorbic acid requires the presence of sulfur dioxide to prevent wine oxidation (Barril *et al.*, 2016). Another way to counteract oxygen's effect is by using inert gases, thereby avoiding the action of polyphenol oxidases (du Toit *et al.*, 2006; Pascal Ribéreau-Gayon *et al.*, 2006). Gallotannins and grape seed tannins, in particular, have a protective influence on the colour of white and red wines (Vignault *et al.*, 2019), as they are efficient laccase activity inhibitors (Vignault *et al.*, 2020). Finally, limiting browning can also be achieved through glutathione, which reacts with products generated by the enzymatic oxidation of ortho-diphenols to form 2-S-glutathionyl-caftaric acid, commonly known as grape reaction product (GRP) (Nikolantonaki, Magiatis and Waterhaouse, 2014; Webber *et al.*, 2017). The GRP is not a substrate for tyrosinase, but it can be oxidized in the presence of laccase to form 2,5-di-S-glutathionyl-caftaric acid (GRP2). The oxidation cycle through laccases appears to be unable to oxidize beyond GRP2 under winemaking conditions (Kritzinger, Bauer and Du Toit, 2013). Thus, GSH traps orthoquinones in a colourless form, limiting the formation of brown polymers (Singleton *et al.*, 1985; Kritzinger, Bauer and Du Toit, 2013).

I. Introduction

4.6. Chemical oxidation

Oxidation process occurs by means of the action of the different reactive oxygen species (ROS) which include different highly reactive chemicals formed from diatomic oxygen (O_2). Peroxides, superoxide, hydroxyl radicals, singlet oxygen, and alpha-oxygen are a few examples of ROS.

Within the context of chemical oxidation, it is noteworthy to mention that ROS are also generated due to a loss of electrons from molecular oxygen (O_2), as depicted in **Figure 24**.

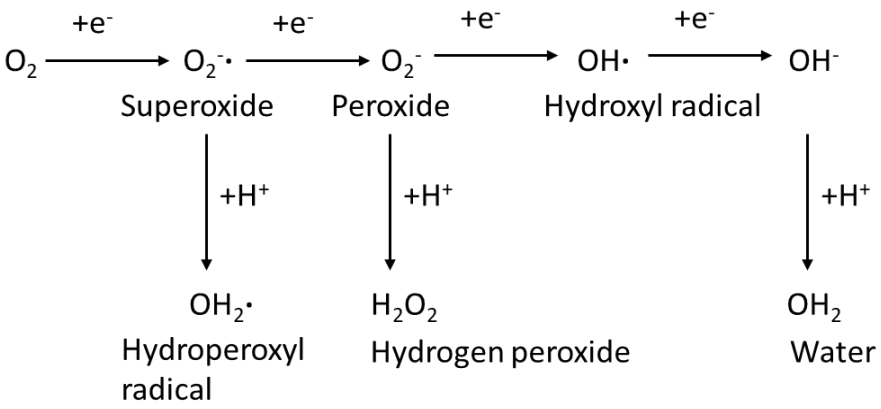


FIGURE 24: PRODUCTION OF ROS THROUGH THE REDUCTION OF MOLECULAR OXYGEN (WATERHOUSE AND LAURIE, 2006)

The generation of ROS, derived from the reduction of oxygen, is produced gradually originating superoxide radical ($O_2^{-}\bullet$), hydroperoxyl radical anion ($OH_2\bullet$), peroxide (O_2^{-}) hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\bullet$), and finally, water (H_2O).

During ROS generation, molecular oxygen (O_2) has a restricted reactivity to organic compounds in their triplet state (with two electrons not paired in separate orbitals) (Khorobrykh *et al.*, 2020) Consequently, numerous studies have indicated the important role played by transition metals in facilitating the production of these ROS, and consequently their importance in oxidation processes. It is important to note that transition metals such as Fe and Cu can be found in the grape must and wine matrices, among others. The concentration range of these metals is not negligible, ranging from

0.16 to 27.8 mg Fe/L, and from <0.002 to 6.83 mg Cu/L (Woldemariam and Chandravanshi, 2011).

The catalysis of molecular oxygen (O_2) consumption is facilitated by Fe (II) or other transition metals, leading to the formation of the superoxide ion, $O_2^{\cdot-}$. According to the Fenton reaction (depicted in **Figure 25),** the transfer of a second electron to molecular oxygen (O_2) results in the production of hydrogen peroxide (H_2O_2).

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot-}$$
$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + {}^{-}OH$$

FIGURE 25: FENTON REACTION AND RECYCLING

The last step of Fenton reaction, between ferrous cations with hydrogen peroxide (H_2O_2) would generate the hydroxyl radical (•OH), an oxidative agent even more reactive than its predecessor.

Fenton's reaction is a phenomenon that exerts an influence on wine and has consequently been extensively examined, as evidenced by the study conducted by Vivas *et al.* in 1993. In their research, the addition of Fe (II) and Cu(I) to wine led to a heightened rate of oxygen consumption. Similar outcomes were achieved in the study conducted by Oszmianski *et al.* (1996), where an increase in the oxidation of (+)-catechins was observed following their combination in a model wine solution. In 2003, Danielwicz noted that interactions between phenols and oxygen only occurred in the presence of transition metal ions.

The process of oxidation that arises from the redox cycle of Fe^{3+}/Fe^{2+} and Cu^{2+}/Cu^{+} in various phenolic compounds, specifically the fraction of ortho-dihydroxybenzenes (catechol ring) or half of 1,2,3-trihydroxybenzene (galloyl group), leads to the production of semiquinone and benzoquinone. Among the phenolic compounds found in wine, some commonly identified ones include (+)-catechin, (-)-epicatechin, gallocatechin, gallic acid and its esters, and caffeic acid. These compounds yield their corresponding

semiquinones and benzoquinone as end products, as illustrated in **Figure 26** (Waterhouse and Laurie, 2006).

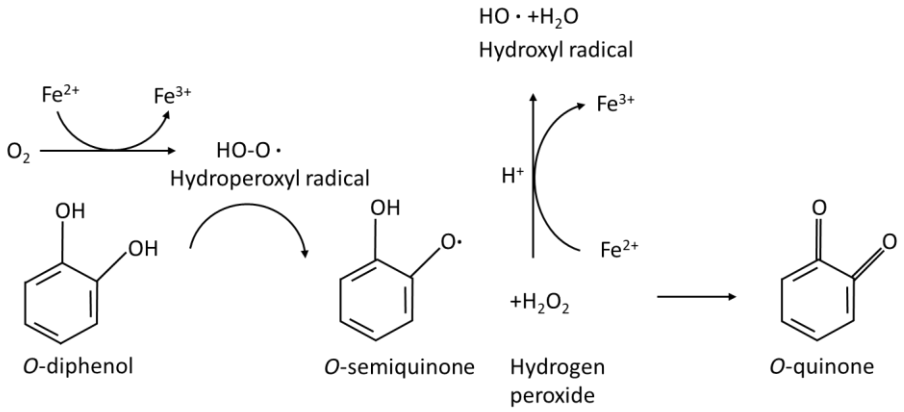


FIGURE 26: *O*-DIPHENOL OXIDATION MEDIATED INDIRECTLY BY FE^{2}+ (WATERHOUSE AND LAURIE, 2006)

These oxidized compounds, semiquinones and benzoquinone, are unstable and generate many reactions, especially with nucleophilic compounds (phenols, thiols and amines) due to their high electrophilic character. When they react with other phenols, dimers and subsequently polymers are produced to form new forms of moiety dihydroxybenzene. In addition, these polymerizations because of multiple oxidations generate a lower redox potential, making these new polyphenols more oxidizable than the initial ones, which originates an acceleration of the polymerization process (Oliveira *et al.*, 2011). All these accelerate reactions occurs in a limited degree in red wines but imply a significant loss of aroma and lead to browning in white wines (Singleton, Trousdale and Zaya, 1979). Fulcrand *et al.*, 1997 demonstrated that the mechanism of polycondensation of (+)-catechin is induced by glyoxylic acid, similarly that ethanal can originate polycondensation with catechins. This reaction generates xanthylium cations that are yellow-coloured (Es-Safi *et al.*, 2000).

Antioxidant compounds have the ability to mitigate such browning effects, whose mechanisms of action will be expounded upon in the ensuing paragraph.

4.7. Antioxidants compounds of wine

Various mechanisms have been proposed to explain how antioxidants act. Among them, removing radicals and donating hydrogen atoms can be highlighted as the main ones. Chelation of metals is another mechanism, which would restrict the accessibility of metal ions to participate in Fenton reactions. It should also be noted that certain compounds exhibit great reactivity with molecular oxygen, which can quickly deplete the available oxygen for other chemical oxidations. Traditionally, the most used antioxidant in grape juice and wine are sulfur dioxide and ascorbic acid. More recently, glutathione, in pure form or as inactivated dry yeasts and different kinds of Bioprotection have been proposed.

4.7.1. Sulfur dioxide

Considering the various mechanisms discussed earlier, sulphur dioxide (SO_2) is a compound that exhibits notable properties including antioxidant, antioxidasic, and antimicrobial activities. When the bisulfite ion is hydrated (HSO_3^-), it reacts with hydrogen peroxide (H_2O_2) to produce sulfuric acid (H_2SO_4). The primary antioxidant function of sulfur dioxide is therefore reacting with hydrogen peroxide and thereby limit the oxidation of ethanol and other hydroxylated compounds (**Figure 27**) (Boulton *et al.*, 1999). Nevertheless, it has been observed that this action is competitive, and high concentrations of sulfur dioxide in models do not necessarily prevent completely the oxidation of other compounds.

Although yeasts can release small amounts of SO_2 during alcoholic fermentation, it is present as a by-product in wine only at very low concentrations. Nevertheless, the levels of this compound that can be naturally present in wine are insufficient to guarantee its properties, prompting the wine industry to employ it as an additive (E-220), not only in winemaking but also in the food industry at large (Schroeter, 1966). Despite its versatility, the current trend is to reduce, and even eliminate its use, owing to its adverse health (Lester, 1995) effects such as headaches (Costanigro, Appleby and Menke, 2014) and allergies (Vally, Misso and Madan, 2009), as well as its negative impact on the environment (D'amico, Di Vita and Monaco, 2016).

I. Introduction

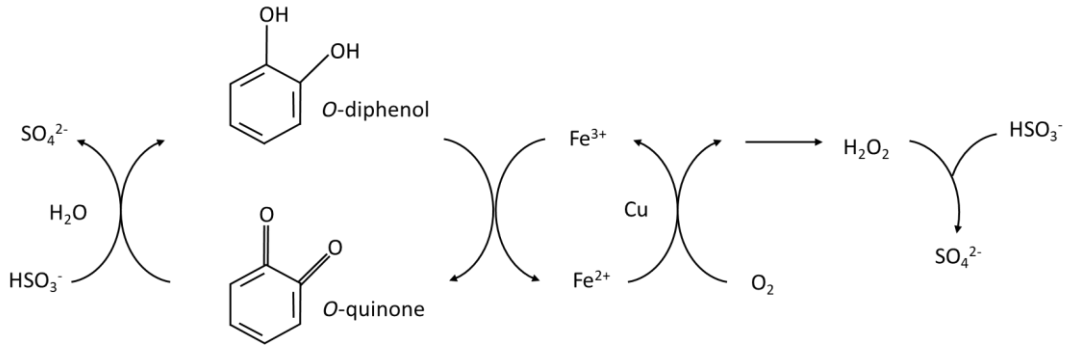


FIGURE 27: THE ROLE OF SULFUR DIOXIDE IN ORTHO-DIPHENOL OXIDATION (DANILEWICZ, 2011)

4.7.2. Ascorbic acid

Ascorbic acid or vitamin C is a ubiquitous compound found in several fruits and vegetables, including grapes, and its concentration is influenced by several agronomic factors such as grape variety and ripening state. Rankie (2002) has reported a concentration range of 10 to 100mg/L upon crushing and pressing. Its use as an additive has been authorized by the OIV up to a concentration of 0.25g/L grape juice or wine (OIV, 2022).

Ascorbic acid reacts very quickly with oxygen making it no longer available for enzymatic and chemical oxidation reactions (Giménez, Just-Borras, *et al.*, 2023). However, the presence of ascorbic acid in wine has been associated with detrimental changes, as reported by (Singleton, 1987; Danilewicz, 2003; Waterhouse and Laurie, 2006). Its negative effect is due to the well-known reaction between ascorbic acid, oxygen, and metal ions, which generates dehydroascorbic acid and hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) (**Figure 28**). To counteract the formation of H_2O_2, sulfur dioxide is typically employed to react with it, forming sulfuric acid, while dehydroascorbic acid is reversibly bound to sulfur dioxide (Barril *et al.*, 2016). For that reason, the employment of ascorbic acid it necessarily needs to be used together with SO_2, so it can only be considered as a complement, but never as an alternative.

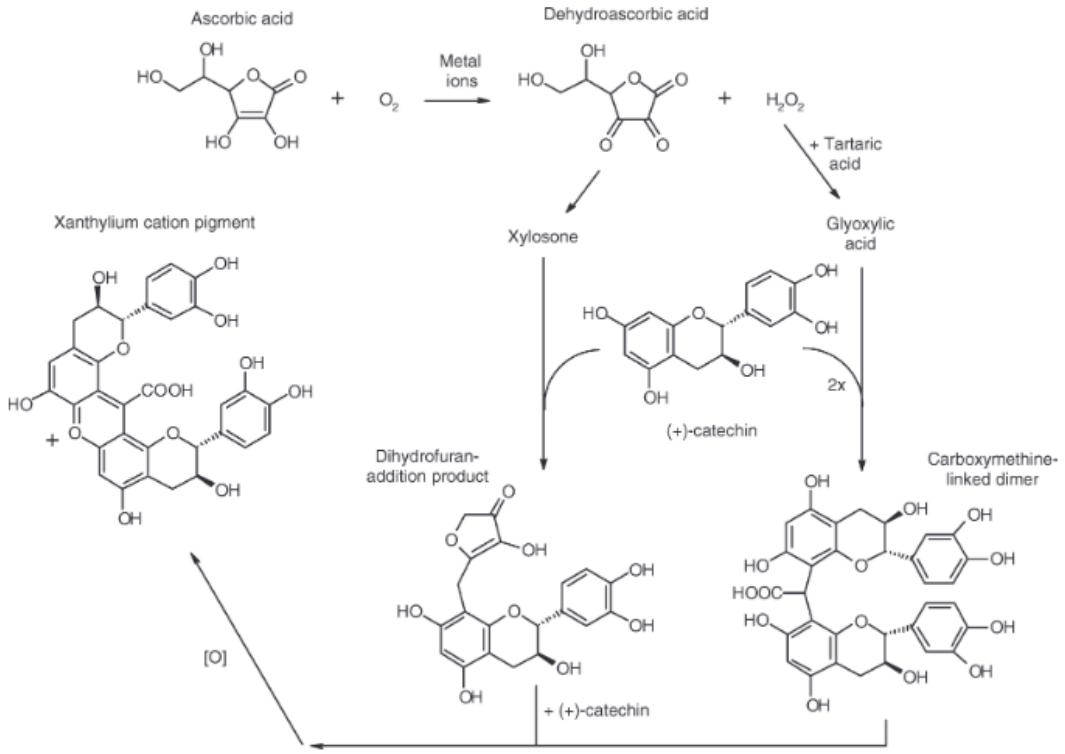


FIGURE 28: SCHEME ASCORBIC ACID REACTION, ADAPTED FROM SONNI *ET AL.*, 2011

4.7.3. Glutathione

Glutathione (GSH) is a sulfur-containing compound, a tripeptide (Glu-Cys-Gly), which is very significant in grape must. It protects grape cells against free radicals by donating a hydrogen atom or an electron. Glutathione reacts with orthodiquinones, result from the oxidation of ortho-diphenols, to form a new product, the grape reaction product (GRP; 2-S-glutathionylcaftaric acid). Thus, glutathione traps the orthoquinones in a colourless form making that the formation of brown polymers is limited (Singleton *et al.*, 1985; Kritzinger, Bauer and Du Toit, 2013; Giménez, Just-Borras, *et al.*, 2023). One of the most studied examples is the reaction of the quinone of caftaric acid with GSH to form the GRP (Singleton *et al.*, 1985; Kritzinger, Bauer and Du Toit, 2013; Nikolantonaki, Magiatis and Waterhaouse, 2014; Nikolantonaki *et al.*, 2018). Subsequently, the colourless GRP, is oxidizable by the Botrytis cinerea laccase but not by grape tyrosinase (Ribéreau-Gayon, Glories, *et al.*, 2006).

It should be noted that orthodiquinones react with varietal volatile thiols, significantly decreasing their positive impact on wine aroma. Since glutathione generates GRP from

orthodiquinones their use not only protect grape must against browning but protect the loss of these varietal aromas as well (Nikolantonaki and Waterhouse, 2012).

The use of glutathione as an additive in winemaking was authorized by the International Organization of Vine and Wine (OIV) in 2015, at maximum concentrations of 20 mg/L (OIV, 2015b, 2015a). This additive is expensive, and therefore, more cost-effective alternatives have been proposed, such as the use of inactivated dry yeast (IDY-GSH), which were also approved by the OIV in 2018.

4.7.4. Bioprotection

Bioprotection consists in the use of living microorganisms or substances produces by them to prevent the growth of spoilage microorganisms of other problems such as oxidation. Bioprotection is a new tendency in current food technology, and it has been introduced in winemaking strategies in very recent years (Pladeau *et al.*, 2019; Krieger-Weber, Heras and Suarez, 2020; Morata *et al.*, 2021; Englezos *et al.*, 2022; Gianvito *et al.*, 2022). Most of the cases, this process focuses on the biological competition among microorganisms, mainly to delay the fermentative processes of *Saccharomyces cerevisiae* in grape musts, or to prevent the growth of undesirable yeasts (as *Brettanomyces*) and bacteria able to spoil the wine.

As it has previously commented, there is a growing interest in avoiding the negative impact of chemicals on human health when consuming wine, trying at the same time to improve the stability and shelf life of this beverage. However, the actual tendency to reduce the use of sulfur dioxide, make wine easier to spoil. According with (Pladeau *et al.*, 2019) define bioprotection as follows: “Bioprotection is presented in oenology as an alternative to the antiseptic role of SO2 in the pre-fermentation phase. It consists of inoculating early on grapes or in the grape must of yeast species known and mastered who establish themselves and colonize the environment to the detriment of the native microbiota present and potentially likely to affect the quality of the grape must. This solution aims to protect the grape musts from microbial risks”. However, (Morata *et al.*, 2021) widen later the concept and consider bioprotection as “the active or passive use of some microorganisms to preserve foods and beverages and to exclude other spoilage

microorganisms, thus avoiding the production of off-flavors, sensory alterations, or even the formation of toxic molecules".

Bioprotection is therefore, a strategic approach aimed at preventing the degradation of wine using various biological tools. It should be noted that bioprotection encompasses multiple mechanisms rather than one. Among the most prominent bio-protection mechanisms used to prevent wine deterioration it can be highlighted the release of organic acids and the competition for oxygen and other nutrients.

The release of organic acids by microorganisms is a technique aimed at establishing a more restrictive matrix with a lower pH, which can be difficult for the growth of some spoilage microorganisms (Rubio-Bretón *et al.*, 2018). Otherwise, the imposition of a selected microorganism can limit the development of others since limiting the availability of some nutrients. In addition, the inoculation of microorganisms with a higher oxygen consumption rate can also inhibit the growth of other spoilage microorganism (Holm Hansen *et al.*, 2001; Englezos *et al.*, 2018) and simultaneously protect the grape must against browning.

As an example of the growing interest in wine bioprotection, several commercial products have already appeared in the market, mainly microorganisms such as *Lactiplantibacillus plantarum* (Krieger-Weber, Heras and Suarez, 2020; Englezos *et al.*, 2022), *Pichia kluyveri* (Englezos *et al.*, 2022), *Metschnikowia pulcherrima*, etc. (Roudil *et al.*, 2019; Gianvito *et al.*, 2022). Although the vast majority of the research related to wine bioprotection has been focused on the antagonistic activity of some microorganisms against others (Ciani and Comitini, 2011; Mas, Guillamón and Beltran, 2016; Pladeau *et al.*, 2019; Simonin *et al.*, 2020) or on the changes in aromatic profiles (both positive or negative) of wines which these microorganisms can produce, including the reduction in alcoholic content and acidification (Sun *et al.*, 2013; Rubio-Bretón *et al.*, 2018; Castrillo *et al.*, 2019; Simonin *et al.*, 2020; Morata *et al.*, 2021; del Fresno *et al.*, 2022), the use of bioprotectants can also provide to the wine many other positive effects. As it was commented above, the use of some non-*Saccharomyces* yeasts has been proposed to protect grape juice from browning because they can consume oxygen very efficiently and therefore reduce its availability for the polyphenol oxidases (Simonin *et al.*, 2020; Windholtz *et al.*, 2021). In that sense, it seems that *Metschnikowia*

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pulcherrima consumes oxygen very effectively (Chacon-Rodriguez *et al.*, 2020) making that some of the initially dissolved oxygen is not consumed by the polyphenol oxidases.

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II. HYPOTHESIS AND OBJECTIVES

II. Hypothesis and objectives

HYPOTHESIS AND OBJECTIVES

Enzymatic browning is a process that occurs in many foods. The increase in brown color is perceived by the consumer as sign of food deterioration. Therefore, in oenology is an important problem to solve since grape juice is very susceptible to develop this process.

Two types of enzymes, namely polyphenol oxidases, are found in grapes and are responsible for this process of browning. These enzymes use molecular oxygen to oxidize diphenolic compounds, to generate semidiquinones and subsequently diquinones. These by-products generated from oxidation, are highly unstable, thus producing a cascade of oxidation reactions with semi-products and reaction products with the rest of compound present in the matrix of grape juice. As these products form structures with a higher degree of polymerization, their intensity of brown coloration increases. In the case of white wines having a less complex phenolic matrix, these complexes are usually interrupted making that the brown polymers remain dissolved into the wine (browning). Conversely, in the case of red wines, the oxidations reach higher levels of polymerization at which their molecular mass increases to the point of instability within the matrix, ultimately resulting in precipitation (oxidasic haze).

To do this, the main objective of this work was to study the enzymatic oxidation mechanisms in the grape must cause by laccase from *Botrytis cinerea*, and what was the involvement in the color of each one of the mechanisms, as well as what were the most effective methods to reduce enzymatic browning. This was the reason for the following working hypothesis:

“Through the development of browning models, it will be possible to better understand the oxidation mechanisms of grape must and propose alternatives to the use of sulfur dioxide for its prevention.”

To study this hypothesis, the following specific objectives were proposed:

1. Development of a synthetic model to study the mechanism of browning caused by laccase activity from *Botrytis cinerea*.

II. Hypothesis and objectives

2. Development of a model using natural white grape must to study the mechanism of browning in grape must from healthy grapes without or with supplementation with laccase from *Botrytis cinerea*.
3. Study of biotechnological tools for reducing the use of sulfur dioxide in white grape must and preventing enzymatic browning.
4. Study of enzymatic oxidation mechanisms of wine anthocyanins.

This research is part of a more general project developed by the Oenological Technology Research Group of the Department of Biochemistry and Biotechnology of the Rovira i Virgili University in Tarragona, Spain (project RTI 2018-095658-B-C3

III. RESULTS

III. Results

CHAPTER I. Study of browning caused by *Botrytis cinerea* laccase.

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Development of a synthetic model to study browning caused by laccase activity from *Botrytis cinerea*

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In viticulture, the most pernicious disease that afflicts grapes is grey rot, caused by *Botrytis cinerea*. Of grave concern to winemakers is the browning caused by the action of laccase on wine quality. Within the wine industry, it is widely acknowledged that preserving the quality of grape juice can be achieved through a variety of measures such as augmenting the dosage of sulfur dioxide, incorporating ascorbic acid, inertizing the atmosphere, introducing oenological tannins, and more recently, increasing the doses of reduced glutathione or employing inactivated dry yeasts that are rich in glutathione.

This chapter pertains to the accomplishment of the first objective, which aimed to formulate a grape must synthetic model for analyzing the optimal conditions of the model for laccase. The investigation established conditions that closely resembled those of grape must, devoid of any interference, to facilitate the analysis of different phenolic compound conformations as substrates and to evaluate the highest production of browning. After characterizing the best browning conditions, an assessment was carried out to determine the efficacy of antioxidants against laccase.

III. Results

Development of a synthetic model to study browning caused by laccase activity from *Botrytis cinerea*.

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ABSTRACT

The aim of this paper is to develop a synthetic model reproducing more realistically the conditions of grape juice to study browning caused by laccase from *Botrytis cinerea*. The laccase browning kinetics were measured by monitoring the increase in absorbance at 420 nm over time in the presence of different substrates – one monophenol: 4-hydroxybenzoic acid; three orthodiphenols: caftaric acid, (+)-catechin and (-)-epicatechin; and one triphenol: gallic acid. The results indicate that orthodiphenols are better substrates than triphenols and that monophenols do not appear to be reactive. Of the orthodiphenols, (+)-catechin showed the greatest browning intensity, followed in decreasing order by (-)-epicatechin and caftaric acid. These results confirm that sulfur dioxide, ascorbic acid and glutathione really do protect grape juice against laccase browning. The effectiveness of ascorbic acid and glutathione also confirm that both antioxidants can be useful tools for reducing doses of sulfur dioxide in winemaking, especially when grey rot is present.

Keywords: Laccase; *Botrytis cinerea*; Browning; Synthetic model; Laccase inhibition.

III. Results

INTRODUCTION

Enzymatic browning is an oxidation process that occurs in many foods. It causes an increase in their brown color (Friedman, 1996), which often leads to rejection by consumers. This problem is of particular concern in the case of winemaking, since grape juice is very susceptible to developing this process (du Toit *et al.,* 2006; Oliveira *et al.,* 2011). The enzymes responsible for browning are polyphenol oxidases, a broad family of oxidoreductases (EC. 1 class, according to the International Union of Biochemistry and Molecular Biology – IUBMB). However, in the case of grape juice only two enzymes really play an important role in enzymatic browning: tyrosinase (EC 1.14.18.1, IUBMB), which is naturally present in grapes (du Toit *et al.,* 2006; Oliveira *et al.,* 2011), and laccase (EC 1.10.3.2, IUBMB), which is only present when the grapes are infected by grey rot (Oliveira *et al.,* 2011; Steel, Blackman & Schmidtke; 2013).

These enzymes use molecular oxygen to oxidize mainly diphenols, through a radical-catalyzed reaction mechanism (Claus, 2004; Li, Guo & Wang, 2008) to form quinones. These quinones and their derivatives are subsequently polymerized through several reactions, forming brown pigments known as melanins (Queiroz *et al.,* 2008; Oliveira *et al.,* 2011; Claus *et al.,* 2014). These pigments, which are relatively insoluble depending on their degree of polymerization (Moon *et al.,* 2020), are responsible for increasing the intensity of the brown color in white wines (browning) and for the precipitation of the coloring matter in red wines (oxidasic haze) (Ribéreau-Gayon *et al.,* 2006a). Both tyrosinase and laccase can oxidize caftaric and cutaric acids, catechin, anthocyanin, flavanols and flavanone as substrates, but laccase acts on a far wider range of substrates than tyrosinase (Oliveira, Silva-Ferreira, De Freitas & Silva, 2011; Steel, Blackman & Schmidtke; 2013). Moreover, when the grapes are affected by grey rot, laccase activity can be much greater than that of tyrosinase in healthy grapes (Steel, Blackman & Schmidtke, 2013; Quijada-Morin *et al.,* 2018). In addition, tyrosinase is so sensitive to sulfur dioxide that a small dose of this additive can inactivate it. In contrast, laccase is more resistant to sulfur dioxide and, unlike tyrosinase, can be present in wine after alcoholic fermentation (Ribéreau-Gayon *et al.,* 2006a).

Grey rot is produced by the development of *Botrytis cinerea* in the grapes, a necrotrophic pathogenic fungus responsible for huge economic losses each year in agriculture, especially in grape and wine production (Steel, Blackman & Schmidtke, 2013). Hill *et al.* (2019) have reported that this fungus causes annual crop losses in New Zealand of up to NZ dollars 2578 per hectare (around 1509 euros or 1855 US dollars). It is responsible for probably the worst blight affecting viticulture because it gives rise to several serious problems such as contamination with non-desirable microorganisms (Barata *et al.,* 2008; Barata *et al.,* 2012; Lleixà *et al.,* 2018), problems of settling and filtration (Villettaz, Steiner & Trogus, 1984; Jadhav & Gupta, 2016), presence of ochratoxin A (Valero *et al.,* 2008; Ponsone *et al.,* 2012), mouldy odors (La Guerche *et al.,* 2007; Steel, Blackman & Schmidtke, 2013) and a worsening of the foaming properties of sparkling wines (Cilindre *et al.,* 2007), which cause the quality of the wine to deteriorate (Ky *et al.,* 2012; Lopez-Pinar *et al.,* 2017). Nevertheless, the damage that laccase causes to the color of the wine is undoubtedly one of the greatest concerns (La Guerche *et al.,* 2007; Ky *et al.,* 2012; Steel, Blackman & Schmidtke, 2013; Vignault, *et al.,* 2019).

The most common solutions that winemakers use to protect the grape juice from the browning generated by polyphenol oxidases are basically to increase the dose of sulfur dioxide (Ribéreau-Gayon *et al.,* 2006a), add ascorbic acid (Ribéreau-Gayon *et al.,* 2006b), use inert atmosphere (Martinez & Whitaker, 1995), add oenological tannins (Vignault *et al.,* 2019; Vignault *et al.,* 2020) and, more recently, to use reduced glutathione (Kritzinger, Bauer & du Toit, 2013; Zimdars, 2020) or inactivated dry yeasts rich in glutathione(Gabrielli *et al.,* 2017; Bahut *et al.,* 2020). Sulfur dioxide acts to inhibit both polyphenol oxidases, although laccase is more resistant to inhibition by this additive than tyrosinase (du Toit *et al.,* 2006; Ribéreau-Gayon *et al.,* 2006a). Ascorbic acid acts by competing with polyphenol oxidases for oxygen, since its direct oxygen consumption rate is several times faster than that of sulfur dioxide (Pascual *et al.,* 2017; Vignault *et al.,* 2020). Nevertheless, it must be considered that ascorbic acid generates hydrogen peroxide after consuming oxygen and its use in wine may therefore cause subsequent oxidations (Oliveira *et al.,* 2011), which can affect the sensory quality of the wine. For that reason, the use of ascorbic acid requires the presence of sulfur dioxide to prevent wine oxidation (Barril *et al.,* 2016). Inert gases make it possible to minimize the presence

of oxygen and thus avoid the action of polyphenol oxidases (du Toit *et al.,* 2006; Ribéreau-Gayon *et al.,* 2006b). Oenological tannins, especially grape-seed tannins and gallotannins, have been shown to be effective inhibitors of laccase activity (Vignault *et al.,* 2020), exhibiting a protective effect on the color of white and red wines (Vignault *et al.,* 2019). Finally, glutathione can limit browning because it reacts with the orthoquinones produced by the enzymatic oxidation of orthodiphenols to form 2-S-glutathionylcaftaric acid, commonly known as grape reaction product (GRP) (Nikolantonaki, Magiatis & Waterhouse, 2014; Webber *et al.,* 2017). GRP is not a substrate for tyrosinase, but it can be oxidized by laccase to form 2,5-di-S-glutathionylcaftaric acid (GRP2). However, it seems that GRP2 cannot be further oxidized by laccase under winemaking conditions (Kritzinger, Bauer & du Toit, 2013). Thus, GSH traps the orthoquinones in a colorless form and as a result the formation of brown polymers is limited (Singleton *et al.,* 1985; Kritzinger, Bauer & du Toit, 2013;).

Given the consequences that the presence of laccase in grapes implies, there is no doubt that the study of laccase activity and how it can be inhibited is of great interest to the wine sector.

Numerous methods have been proposed to determine laccase activity using various substrates such as 2,6-dimethoxyphenol (DMP) (Slomczynski, Nakas & Tanenbaum, 1995), L-3,4-dihydroxyphenylalanine (DOPA) (Saiya-Cork, Sinsabaugh & Zak, 2002; Eichlerová, Šnajdr & Baldrian, 2012), 3,30-dimethylaminobenzoic acid (DMAB) (Matsumura *et al.,* 1987), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Prasetyo *et al.,* 2009), pirocatecol (Perucci, Casucci & Dumontet, 2000), o-dianisidine (Li *et al.,* 2007), o-tolidina (Leatham & Stahmann, 1981), amplex red (Wang *et al.,* 2017), 2,20-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Ruijssenaars & Hartmans, 2004; Eichlerová, Šnajdr & Baldrian, 2012) and syringaldazine (Harkin, 1973; Grassin & Dubourdieu, 1986), but ABTS and syringaldazine are probably the most frequently used. However, all these procedures work with substrates that are not present in grape berries and are usually performed under conditions far removed from those of real grape juice. Moreover, none of these methods really measure enzymatic browning, since they have not considered what happens after the oxidation of orthodiphenols in orthodiquinones and their subsequent polymerization to form melanins.

The aim of this paper is therefore to develop a model to measure the browning caused by laccase activity arising from *Botrytis cinerea* under conditions much closer to those of grape juice and using the substrates naturally present in it. The study also focuses on the inhibitory effect of sulfur dioxide, ascorbic acid and glutathione on the enzymatic browning caused by laccase.

MATERIALS AND METHODS

Chemicals and equipment. All samples and standards were handled without any exposure to light. Polyvinylpolypyrrolidone (PVPP), gallic acid (purity ≥ 97.5%), L-histidine (purity ≥ 99.5%), glycerol (purity ≥ 99.5%), $FeSO_4 \cdot 7H_2O$ (purity ≥ 99%), $NaNO_3$ (purity ≥ 99%), $CaCl_2 \cdot 2H_2O$ (purity ≥ 99%), $MgSO_4 \cdot 7H_2O$ (purity ≥ 99%), ascorbic acid (purity ≥ 99%), reduced L-glutathione (purity ≥ 98%) and syringaldazine (purity ≥ 98%) were purchased from Sigma-Aldrich (Madrid, Spain). L-(+)-tartaric acid (purity ≥ 99.5%), sodium hydroxide (purity ≥ 98%), sodium acetate (purity ≥ 99%), KH_2PO_4 (purity ≥ 99%), $CuSO_4$ (purity ≥ 99%), glycerol (purity ≥ 99.5%), KCl (purity ≥99.5%), NaCl (purity ≥ 99.5%), D-glucose, D-fructose, $Na_2S_2O_5$ (purity ≥ 99.5%), peptone, agar and yeast extract were purchased from Panreac (Barcelona, Spain). Ethanol (96 % vol.) and hydrochloric acid (purity ≥ 36.5 %) were supplied by Fisher Scientific (Madrid, Spain).

The equipment used was as follows: a spectrophotometer UV-Vis Helios Alpha™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); an incubator IPP 260 (DD Biolab, Barcelona, Spain); a centrifuge Heraeus™ Primo™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); and a CB Standard Balance (Cobos, Barcelona, Spain).

Extracellular laccase production. The *B. cinerea* single-spore isolate 213, originally isolated from grapevine leaf in 1998, was selected from the collection of UMR SAVE, Bordeaux (Martinez *et al.,* 2003). It was chosen because of its virulence on grapevine leaves and berries and because it is a *transposa* type strain (Ky *et al.,* 2012; Martinez, Dubos & Fermaud, 2005). The pathogen was cultured on Yeast Peptone Dextrose Petri plates (YPD: 20 g/L of peptone and glucose; 10 g/L of yeast extract and 17 g/L of agar in distilled water) and incubated for about 1 week at 20°C before use. The spores were

then scraped into tubes with vertically solidified YPD and incubated for 4 days under the same conditions. Finally, the spores were extracted from 6 tubes, recovering only the precipitate with 1 mL of saline solution (0.9% of NaCl). This resuspension was added to 125 mL of autoclaved incubation medium (40 g Glucose/L, 7 g Glycerol/L, 0.5 g L-histidine/L, 0.1 g $CuSO_4$/L, 1.8 g $NaNO_3$/L, 1.8 g NaCl/L, 0.5 g KCl/L, 0.5 g $CaCl_2 \cdot H_2O$/L, 0.05 g $FeSO_4 \cdot 7H_2O$/L, 1 g KH_2PO_4/L and 0.5 g $MgSO_4 \cdot 7H_2O$/L) and incubated with agitation (150 rpm) for 3 days. Finally, the broth was added to 1.4 L of autoclaved incubation medium and incubated with agitation (150 rpm) for 2 days. A solution of 3 g of gallic acid in 40 mL autoclaved water was then added and left to incubate for 5 more days. This medium containing laccase activity was decanted, filtered through an ash-free filter paper and frozen at -80^{o}C until use.

Laccase activity assays. Laccase activity was determined using an adaptation of the syringaldazine test method (Grassin & Dubourdieu, 1986). Five mL of the medium containing laccase were added to 0.8 g of PVPP (to remove phenolic compounds that can cause interference), stirred and centrifuged for 10 minutes at 8,500 rpm. One mL of the supernatant was introduced into a plastic spectrophotometer cuvette to which were also added 1.4 mL of buffer solution (8.2 g/L of sodium acetate in deionized water, pH 5.5) and 0.6 mL of syringaldazine solution (60 mg/L of syringaldazine in ethanol 96 %). This solution was then homogenized by inverting the cell, and absorbance was measured at 530 nm every minute for 5 minutes (including a time measurement at 0 minute). All analyses were performed in triplicate. By definition, a laccase unit (LU) corresponds to the amount of enzyme that catalyzes the oxidation of a micromole of syringaldazine per minute. The following equation was used to calculate laccase activity by using the slope of the line obtained via a calibrating linear regression (ΔA) expressed in absorbance units/minute: Laccase activity = 46.15 × ΔA µmol. L^{-1}. min^{-1} = 46.15 × ΔA LU.

Preparation of synthetic grape juice model solution to measure browning. A solution containing 100 g/L of D-glucose, 100 g/L of D-fructose and 4 g/L of tartaric acid adjusted to pH 3.5 with sodium hydroxide was used as the synthetic grape juice model solution for all the browning assays.

Laccase substrates. Five phenolic compounds representing the main phenolic compounds present in wine were used. These included one triphenol: gallic acid; three orthodiphenols: caftaric acid, (+)-catechin and (-)-epicatechin; and one monophenol: 4-hydroxybenzoic acid. All these were supplied by Sigma-Aldrich (Madrid, Spain). Stock solutions (2 mM) of each substrate were prepared in synthetic grape juice model solution.

Browning measurements.

Volumes of 0, 50, 100, 150, 200, 250, 300, 350 and 400 microliters of the different substrate stock solution (2 mM) were introduced into 1 mL spectrophotometer microcuvettes of 10 mm optical path length. The final substrate concentration in the reaction media was therefore 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 mM respectively. Then, it was added the volume of the medium containing laccase activity (around 30 microliters depending on the batch) needed to obtain a final laccase activity of 2 U/mL in the total experimentation volume. Finally, the definitive volume of the reaction media was adjusted to 1 mL with the synthetic grape juice model solution. After stirring to homogenize and dissolve the oxygen, the absorbance at 420 nm (A420) was measured at time 0, 15, 30 and 45 minutes. The slope of the regression straight line was determined in order to express the intensity of browning. All the experiments were performed in triplicate.

Determination of kinetics parameters (V_{max}, $K_{0.5}$ and N_a) in a grape juice model solution.

The Michaëlis-Menten plot was depicted for each substrate in order to visualize the kinetics of browning for each substrate. An attempt was also made to represent the Lineweaver-Burk plot, but the results showed that the kinetics were not Michaelian but allosteric. Consequently, the Vmax was determined empirically, and the concentration of substrate needed to reach ½ of Vmax ($K_{0.5}$) was determined by representing $Log_{10}[V/(Vmax-V)]$ versus $Log_{10}S$ (Hill plot) (Tsao & Madley, 1972). According to the Hill plot, a regression straight line was obtained that can be used to obtain $K_{0.5}$ and the Hill number. $K_{0.5}$ corresponds to the expression $10^{-B/A}$, in which B is the intersection point and A is the slope of the regression straight line. The Hill number, which indicates the

degree of cooperativity, corresponds to the slope of the regression straight line (A). When the Hill number is greater than 1, this indicates the existence of positive cooperativity.

(-)-Epicatechin was selected as the substrate for all the other experiments because its browning kinetics were the most appropriate for obtaining suitable values for A420 nm (between 0.5 and 1.0 units) at the highest substrate concentration after 45 minutes.

Influence of pH and ethanol concentration on the kinetic parameters of browning caused by laccase. Similar experimentation using only (-)-epicatechin as substrate were performed to determine the influence of pH and ethanol concentration on the browning process caused by laccase. For this purpose, different synthetic grape juice models with pH of 3.0, 3.5, 4.0, 5.0 and 6.0 (adjusted with sodium hydroxide) were used. Similarly, ethanol was added to the original synthetic grape juice model (pH = 3.5) to obtain final ethanol concentrations of 0, 5, 10 and 15 % (v/v).

Determination of the inhibitory effect of sulfur dioxide, ascorbic acid and glutathione. Analogous testing assays applying only (-)-epicatechin as substrate were performed to determine the inhibitory effect of the three most frequently used wine antioxidants: SO_2, ascorbic acid and reduced glutathione. To this end SO_2 (in the form of $Na_2S_2O_5$) concentrations of 0, 10, 20 and 30 mg/L, ascorbic acid concentrations of 0, 50 and 100 mg/L, and reduced glutathione concentrations of 0, 20, 50 and 100 mg/L were added to the reaction media.

Statistical analysis. All data are expressed as mean values ± standard deviation of three replicates. One-factor analysis of variance (ANOVA) was carried out using the XLSTAT 2017 statistical package.

RESULTS AND DISCUSSION

Kinetic parameters of *Botrytis cinerea* laccase browning for different substrates. Figure 1 shows the changes in absorbance of the different substrates at 420 nm (A420) according to incubation time. These graphs only show data from 0.1, 0.2, 0.4 and 0.6 mM of each substrate to make it easier to visualize the results. Data for other concentrations were used for the kinetic analysis of browning but are not presented in these figures.

All the substrates except 4-hydroxybenzoic acid showed a clear increase in A420 over time. In addition, a clear trend was observed: the higher the substrate concentration, the higher the increase in A420. Some differences were also detected between the various substrates. Specifically, (+)-catechin showed the greatest browning intensity, followed in decreasing order by (-)-epicatechin, caftaric acid, gallic acid and, of course, 4-hydroxybenzoic acid, which, as mentioned earlier, did not react. These data therefore indicate that orthodiphenols are better substrates for browning caused by laccase than triphenols, and that monophenols, or at least 4-hydroxybenzoic acid, do not seem to react.

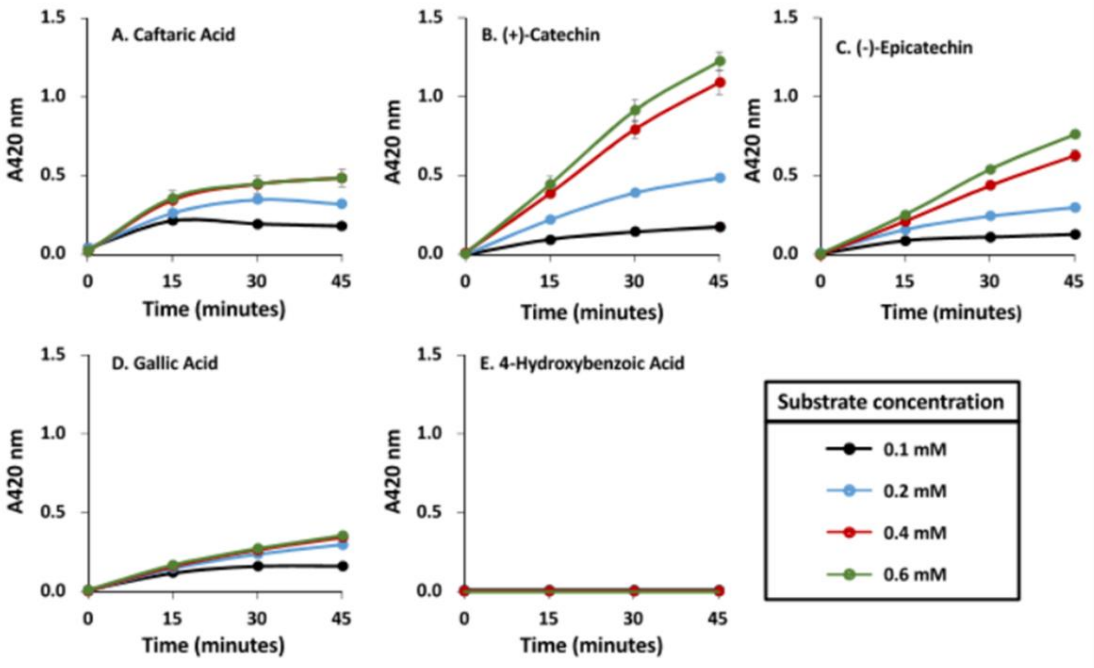

Figure 1. Influence of the type of substrate on the laccase browning kinetics

III. Results

It could be considered unexpected that caftaric acid, which is usually seen as one of the main substrates for laccase browning (Singleton *et al.,* 1985; Cheynier, Trousdale & Singleton, 1986; Zimdars *et al.,* 2017), reacts more slowly than (-)-epicatechin and especially (+)-catechin. It could also be considered surprising that (-)-epicatechin and (+)-catechin, which are epimers with very similar chemical structures, show such different behaviors as regards laccase browning. One possible explanation could be related to differences in their spatial structure. (-)-Epicatechin has a torsional angle between rings B and C, which is higher than (+)-catechin (-45.77^{o} and -31.37^{o} respectively) (Mendoza-Wilson & Glossman-Mitnik, 2006). The less planar structure of (-)-epicatechin might therefore condition its lower reactivity toward laccase. Jarosz-Wilkołazka *et al.,* (2009) have reported that (+)-catechin is oxidized about three times faster than (-)-epicatechin by laccase from *Cerrena unicolor*. Ma *et al.,* (2009) have also reported a faster oxidation of (+)-catechin compared to (-)-epicatechin by laccase from *Rhus vernificera* in organic solvents. In contrast, Quijada-Morin *et al.,* (2017) working with laccases from three different *Botrytis cinerea* strains, have found similar levels of oxygen consumption using (+)-catechin and (-)-epicatechin as substrates. Certainly, our results indicate that (+)-catechin reacts faster than (-)-epicatechin with laccase isolated from the strain used.

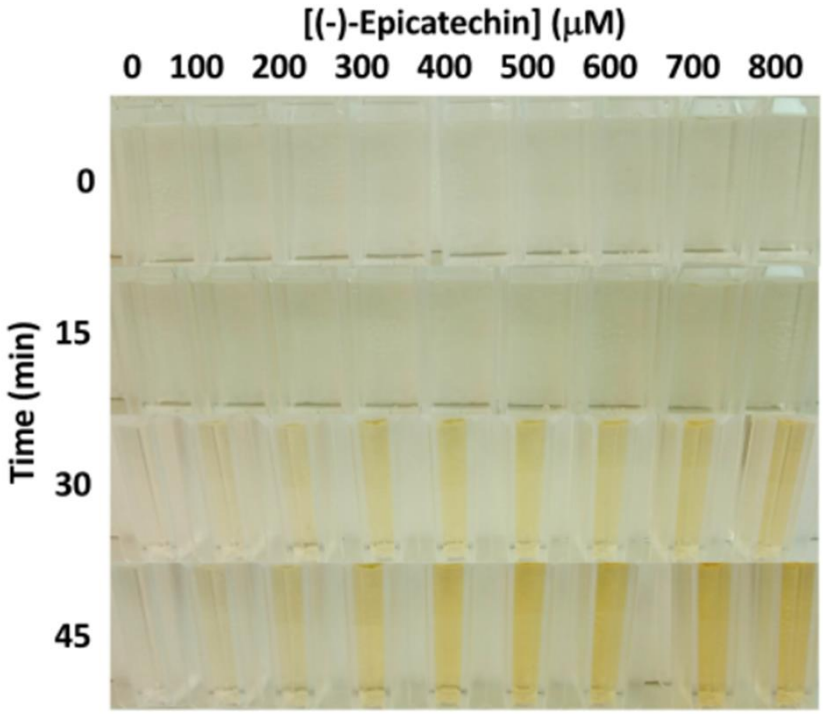

Figure 2. Yellow color development of the synthetic grape juice model solution as a function of (-).epicatechin concentration and reaction time

Given that a picture is worth a thousand words, Figure 2 shows the yellow color developed by solutions with different concentrations of (-)-epicatechin at 0, 15, 30 and 45 minutes. The results are very clear and show that browning increases over time and that it is faster when the substrate concentration is higher.

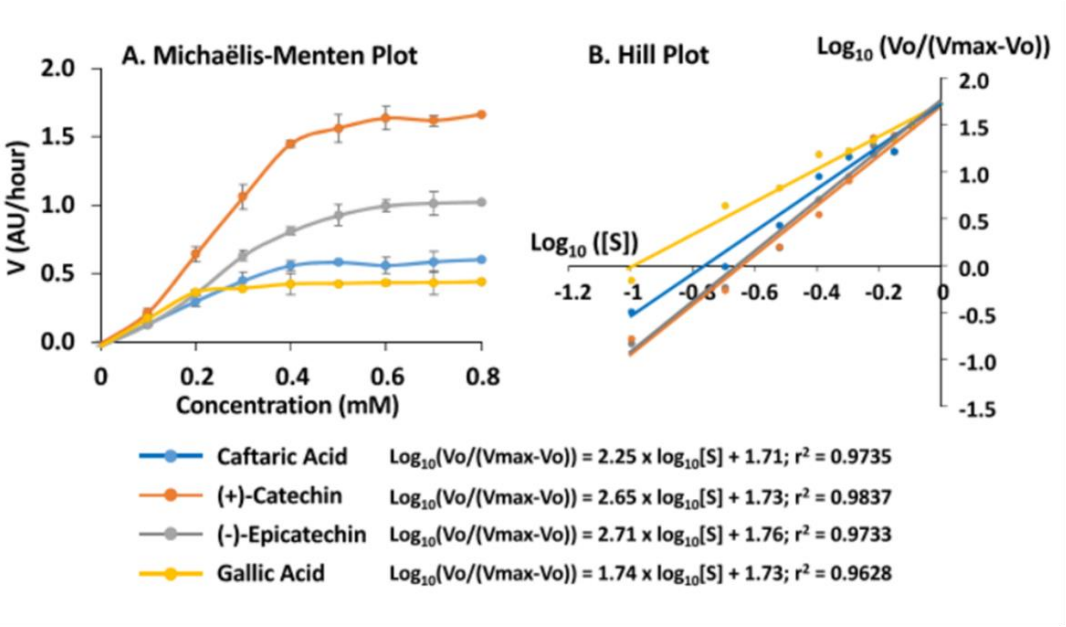

Figure 3. Michaëlis-Menten and Hill plots of diferent substrate

III. Results

Figure 3A shows the Michaëlis-Menten plot for the different substrates studied. As expected, the reaction rate (V) increased for all substrates as their concentration augmented until asymptotic behavior was reached, when the substrate concentration was high enough to saturate the enzyme. The data included in this plot confirm that (+)-catechin causes the fastest browning, followed in decreasing order by (-)-epicatechin, caftaric acid and gallic acid at the same substrate concentrations.

Vignault *et al.,* (2020) have reported classical Michaëlis-Menten kinetics for *Botrytis cinerea* laccase using syringaldazine as substrate to enable the kinetic parameters (Vmax and Km) to be determined using the Lineweaver-Burk approach. However, our data indicate that, under the experimental conditions used, all the plots show a sigmoidal profile that indicates allosteric kinetics. It should be considered that this study has not directly measured laccase activity but browning, a process in which the laccase enzyme obviously participates, but which also involves a subsequent chain of chemical reactions that leads to the formation of melanins (Queiroz *et al.,* 2008; Oliveira *et al.,* 2011). Under these conditions, the final kinetics of browning may be influenced not only by the activity of the laccase enzyme itself, but also by other factors related to the subsequent melanin formation reactions. Under the conditions of this model for measuring browning, the kinetic behavior was at least apparently allosteric. However, other authors have reported allosteric behavior of laccases from different biological origins (Hölker, Dohse & Höfer, 2002; Enaud *et al.,* 2011; Maurya, Nadar & Rathod, 2020), which would support our results.

Whatever the kinetic behavior of *Botrytis* laccase, neither the Lineweaver-Burk nor the Eadie-Hofstee plot show a linear behavior (data not shown), which makes it impossible to determine the kinetic parameters. For that reason, the reaction maximal velocity (Vmax) was determined empirically, and the substrate concentration ($K_{0.5}$) at which the reaction velocity (V) achieves half Vmax was determined using the Hill plot (Tsao & Madley, 1972). Figure 3B shows the Hill plot for the different substrates. All substrates showed reasonable linear regression coefficients with r^2 values between 0.9628 and 0.9837, which indicates that this kind of representation can be used for calculating $K_{0.5}$. All the regression lines of the different substrates cut the ordinate axis at the same intersection point, around 1.73, but present different slopes.

Table 1 shows the browning kinetic parameters for the different substrates. The highest value for Vmax was that of (+)-catechin, followed in decreasing order by (-)-epicatechin, caftaric acid and gallic acid, which more strictly confirms the results shown in Figure 1. Table 1 also shows the $K_{0.5}$ values for the different substrates. (+)-Catechin and (-)-epicatechin showed identical values of $K_{0.5}$, which indicates that *Botrytis* laccase needs similar concentrations of (+)-catechin and (-)-epicatechin to achieve half Vmax, although Vmax for (+)-catechin was significantly higher than for (-)-epicatechin. In contrast, $K_{0.5}$ was significant lower for caftaric acid and especially for gallic acid. The Hill number, which indicates the degree of cooperativity, is also shown in Table 1. Once again (+)-catechin and (-)-epicatechin presented similar values, while caftaric acid and especially gallic acid showed significantly lower values. These data indicate that laccase browning shows positive cooperativities for all the substrates, since the Hill number was higher than 1 in all cases.

Table 1. Kinetic constants of lacasa browning of the synthetic grape juice model solution for the diferent substrates

Substrate	Phenol type	Vmax (AU_{420}/hour)	$K_{0.5}$ (mM)	Hill's number
Caftaric Acid	*o*-Diphenol	0.66 ± 0.04 B	0.173 ± 0.011 B	2.25 ± 0.11 B
(+)-Catechin	o-Diphenol	1.75 ± 0.08 D	0.222 ± 0.015 C	2.65 ± 0.19 C
(-)-Epicatechin	o-Diphenol	1.08 ± 0.06 C	0.223 ± 0.015 C	2.71 ± 0.09 C
Gallic Acid	Triphenol	0.48 ± 0.03 A	0.101 ± 0.008 A	1.74 ± 0.15 A
4-Hydroxybenzoic Acid	Monophenol	nd	nd	nd

Results are expressed as mean ± standard deviation of three replicates. Different letters in a column indicate the existence of statistical difference ($p < 0.05$).

Influence of pH and ethanol concentration on the kinetic parameters of *Botrytis cinerea* laccase browning. Figure 4 shows the influence of pH on the laccase browning kinetics using only (-)-epicatechin as substrate. The Michaëlis-Menten plot (Figure 4A) indicates that pH exerts a very clear effect. The higher the pH the faster the browning kinetics, although no differences were found between pH 5 and 6. These data indicate that the optimum pH for laccase browning of the B213 *Botrytis cinerea* strain is between 5 and 6. Other authors have reported values of optimum pH for *Botrytis cinerea* laccases

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from 3.5 up to 5.5 (Dubernet *et al.,* 1977; Slomczynski & Nakas, 1995; Fortina *et al.,* 1996; Taha *et al.,* 2013). However, it seems that this varies widely depending on the substrate (Mayer & Staples, 2002), fungal origin (Bollag & Leonowicz, 1984) and even the strain (Quijada-Morin *et al.,* 2017).

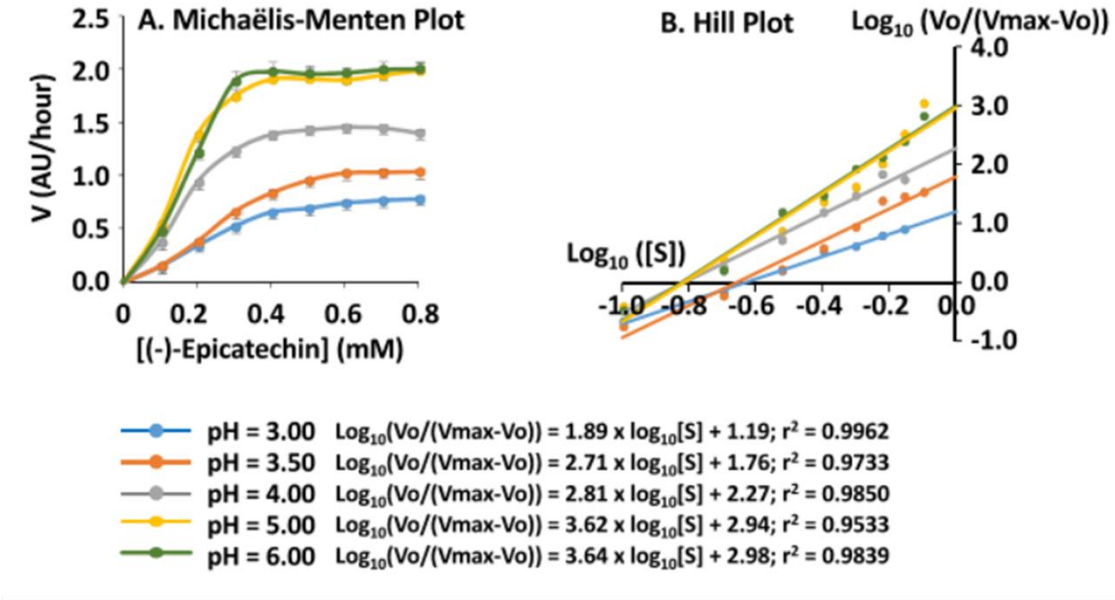

Figure 4. Influence of pH on laccase browning kinetics of the synthetic grape juice model solution using (-)-epicatechin as substrate

Figure 4B shows the Hill plot of laccase browning for (-)-epicatechin at different pH levels. Once again reasonable linear regression coefficients were obtained with r^2 values of between 0.9533 and 0.9962. The observed trend was that the slopes and the Y-intercept points increased when the pH was higher. These data were used to determine how $K_{0.5}$ and the Hill number change depending on pH (Table 2). Data from this table confirm that the higher the pH the higher the Vmax, placing the optimum pH at between 5.0 and 6.0. The values of $K_{0.5}$ tended to decrease in the range between pH 3.0 and 4.0, although this difference was only significant between 3.5 and 4.0. However, $K_{0.5}$ remained stable between pH 4 and 6. These results therefore indicate that the affinity of laccase for (-)-epicatechin increases with pH until reaching pH 4.0. The Hill number also increased with pH until it reached pH 5.0, which would indicate that the degree of laccase cooperativity also increases.

Table 2. Influence of pH on kinetic constants of laccase browning of the synthetic grape juice model solution using (-)-epicatechin as substrate

pH	Vmax (AU_{420}/hour)	$K_{0.5}$ (mM)	Hill's number
3.00	0.88 ± 0.03 A	0.235 ± 0.06 B	1.89 ± 0.07 A
3.50	1.08 ± 0.06 B	0.223 ± 0.015 B	2.71 ± 0.09 B
4.00	1.46 ± 0.07 C	0.155 ± 0.011 A	2.81 ± 0.12 B
5.00	2.00 ± 0.06 D	0.154 ± 0.007 A	3.62 ± 0.10 C
6.00	2.02 ± 0.07 D	0.152 ± 0.005 A	3.64 ± 0.13 C

Results are expressed as mean ± standard deviation of three replicates. Different letters in a column indicate the existence of statistical difference ($p < 0.05$).

Figure 5 shows how the presence of ethanol affects the laccase browning kinetics using only (-)-epicatechin as substrate. The Michaëlis-Menten plot (Figure 5A) shows a curious and surprising result, since the presence of 5 % ethanol activates laccase browning whereas 15 % ethanol did the opposite, with 10 % ethanol displaying similar kinetics to the control without ethanol. Rasekh *et al.,* (2014) have reported that a concentration of 15 % ethanol reduces the laccase activity of *Escherichia coli*. However, to our knowledge there is no information about how ethanol affects laccase of *Botrytis cinerea*. These data are confirmed by the empirically determined values for Vmax (Table3). The fact that small concentrations of ethanol activate the browning caused by *Botrytis cinerea* laccase is a non-negligible aspect to be considered because, when grey rot infects grape bunches, not only does this release laccase but it also favors the development of several microorganisms (Barata *et al.,* 2012; Barata *et al.,* 2012; Lleixà *et al.,* 2018) that can ferment sugars and produce ethanol. In such conditions browning would be favored. Figure 5B shows the corresponding Hill plot, which provides reasonable linear regression coefficients for the different ethanol concentrations with r^2 values of between 0.9755 and 0.9933.

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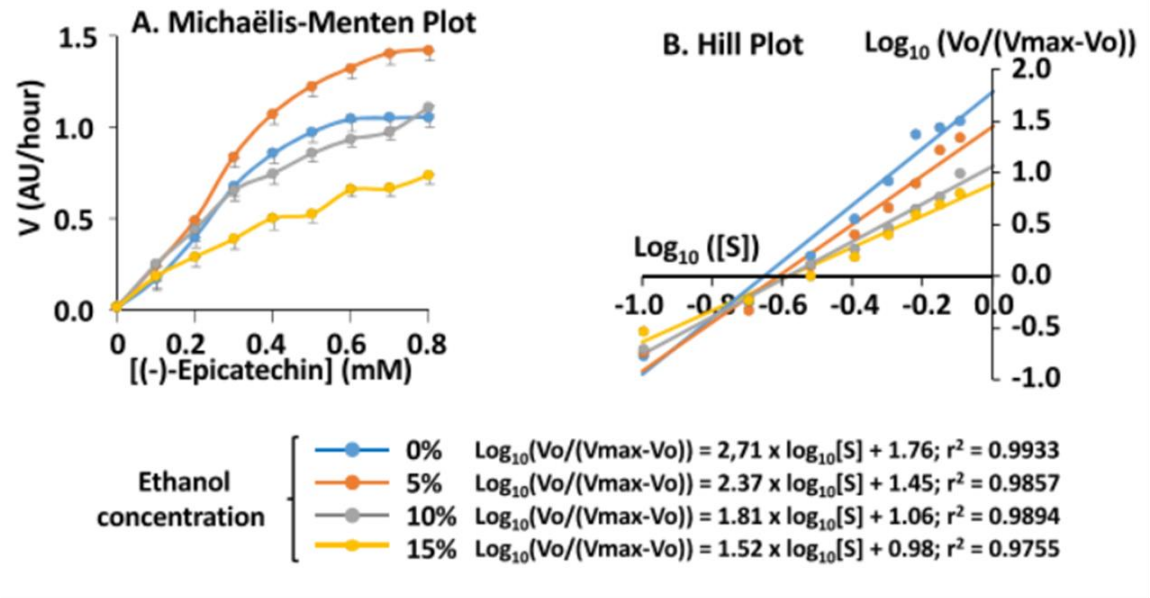

Figure 5. Influence of ethanol on laccase browning kinetics of the synthetic grape juice model solution using (-)-epicatechin as substrate

The values for $K_{0.5}$ increased slightly when ethanol concentration increased, although these differences were only significant between 0 % and 15 %. In contrast, the Hill number tended to clearly decrease. This indicates that the presence of ethanol diminishes the affinity of laccase for (-)-epicatechin and also the degree of cooperativity.

Inhibitory effects of SO_2, ascorbic acid and glutathione on the *Botrytis cinerea* laccase browning kinetic. As mentioned in the introduction, laccase browning is probably the worst problem caused by *Botrytis cinerea* in winemaking and it is for that reason that the wine industry is so concerned about finding suitable tools to minimize its damage. It was therefore decided to study the effect of the main compounds used in winemaking to inhibit laccase browning – sulfur dioxide, ascorbic acid and glutathione – using only (-)-epicatechin as substrate.

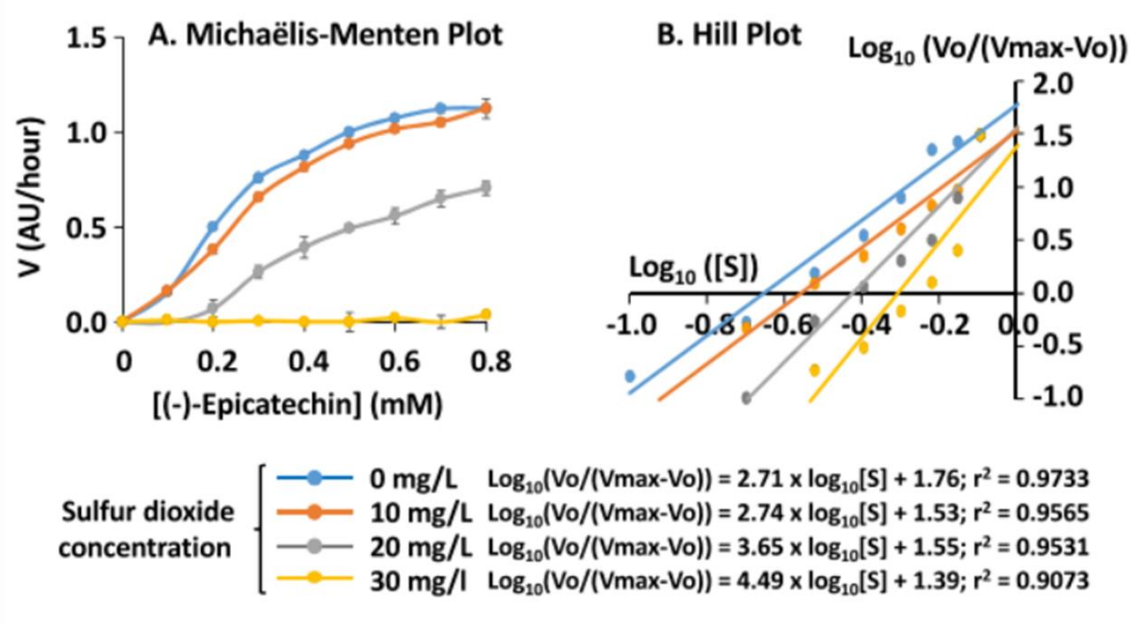

Figure 6. Influence SO_2 concentration on laccase browning kinetics of the synthetic grape juice model solution using (-)-epicatechin as substrate

Figure 6 shows the influence of different doses of SO_2 on laccase browning. The Michaëlis-Menten plot (Figure 6 A) indicates that 30 mg of SO_2/L completely inhibited laccase browning, whereas 20 mg of SO_2/L inhibited around a third, and finally 10 mg of SO_2/L caused hardly any inhibition in the kinetics. This inhibitory effect of sulfur dioxide on laccase activity has been widely described in the literature (du Toit *et al.,* 2006; Oliveira *et al.,* 2011; Verma *et al.,* 2018; Ribéreau-Gayon *et al.,* 2006a; Vignault *et al.,* 2020).

Figure 6B shows the corresponding Hill plot, which provides reasonable linear regression coefficients for the different sulfur dioxide concentrations with r^2 values of between 0.9073 and 0.9733.

The inhibitory effects of SO_2 on laccase browning are confirmed by the empirically determined values of Vmax (Table 4). Considering the obtained values of Vmax, a dose of 30 mg of SO_2/L inhibits laccase browning by 96%, while 20 mg of SO_2/L inhibits by 32%, and finally 10 mg of SO_2/L only inhibited by 1 %. This inhibitory effect of SO_2 on laccase browning is around 100% higher than that described by Claus (2020) in a real grape juice. However, this difference could be due to the fact that real grape juice has many substances capable of combining with SO_2 and thus reducing its effectiveness.

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Table 4. Effects of SO_2 on the kinetic constants of laccase browning kinetics of the synthetic grape juice model solution using (-)-epicatechin as substrate

[SO2] (mg/L)	Vmax (AU_{420}/hour)	$K_{0.5}$ (mM)	Hill's number
0	1.08 ± 0.06 C	0.223 ± 0.015 A	2.71 ± 0.09 A
10	1.07 ± 0.03 C	0.276 ± 0.008 B	2.74 ± 0.06 A
20	0.73 ± 0.21 B	0.376 ± 0.11 C	3.65 ± 1.05 AB
30	0.04 ± 0.02 A	0.490 ± 0.25 C	4.49 ± 2.29 B

Results are expressed as mean ± standard deviation of three replicates. Different letters in a column indicate the existence of statistical difference ($p < 0.05$).

Data from Figure 6B were used to determine how $K_{0.5}$ (Table 4) was affected by the presence of different doses of sulfur dioxide. The values for $K_{0.5}$ tended to increase with the presence of SO_2. This indicates that sulfur dioxide not only decreases the Vmax of laccase browning but also the affinity for its substrate. Table 4 also shows that the presence of SO_2 seems to increase the degree of cooperativity, since the higher the SO_2 concentration the higher the Hill number.

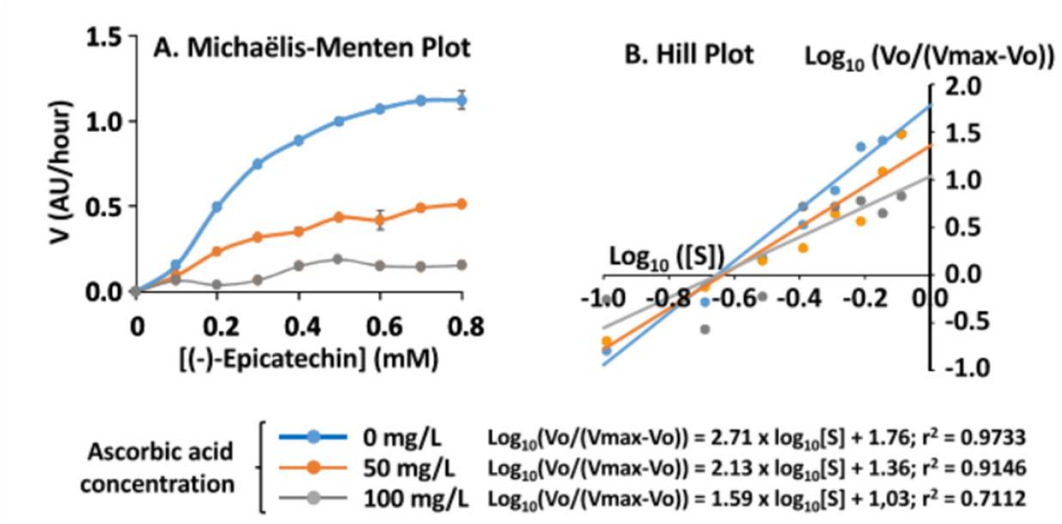

Figure 7. Influence of ascorbic acid concentration on laccase browning kinetics of the synthetic grape juice model solution using (-)-epicatechin as substrate

Figure 7 shows how the presence of ascorbic acid affects laccase browning. The Michaëlis-Menten plot (Figure 7 A) confirms that ascorbic acid really does protect against laccase browning because the supplementation with 50 mg/L and especially with 100 mg/L significant decreases the augmentation of A420 over time. It should be noted that the maximal legal dose established by the OIV for this antioxidant is 250 mg/L (OIV, 2021). The corresponding Hill plot (Figure 7B) provides reasonable linear regression coefficients for the control without ascorbic acid and for when 50 mg of ascorbic acid/L was added (r^2: 0.9733 and 0.9146 respectively). However, in the case of a supplementation with 100 mg/L the linearity worsened (r^2: 0.7112), probably because it was affected by the strong inhibition. All the regression lines cut the abscissa axis at the same intersection point, around -0.65, but show different slopes. Bearing in mind the empirically determined values of Vmax for laccase browning (Table 5), a supplementation with 50 mg of ascorbic acid/L caused 51% of inhibition while 100 mg/L reached 83%.

Table 5. Effects of ascorbic acid on the kinetic constants of laccase browning of the synthetic grape juice model solution using (-)-epicatechin as substrate

[Ascorbic Acid] (mg/L)	Vmax (AU_{420}/hour)	$K_{0.5}$ (mM)	Hill's number
0	1.08 ± 0.06 C	0.223 ± 0.015 A	2.71 ± 0.09 C
50	0.53 ± 0.03 B	0.231 ± 0.011 A	2.14± 0.11 B
100	0.18 ± 0.03 A	0.225 ± 0.032 A	1.59 ± 0.23 A

Results are expressed as mean ± standard deviation of three replicates. Different letters in a column indicate the existence of statistical difference ($p < 0.05$).

The inhibitory effect of ascorbic acid in laccase browning is well known (du Toit *et al.,* 2006; Ribéreau-Gayon *et al.,* 2006b; Vignault *et al.,* 2020) and for that reason it is widely used in wineries, especially when grapes are affected by grey rot (Steel, Blackman & Schmidtke, 2013). The presence of ascorbic acid did not affect $K_{05,}$ probably because it acts to reduce the availability of one of the laccase substrates (oxygen) and does not act directly on the enzyme. The Hill number tended to decrease in the presence of ascorbic acid, which indicates a decrease in the degree of cooperativity.

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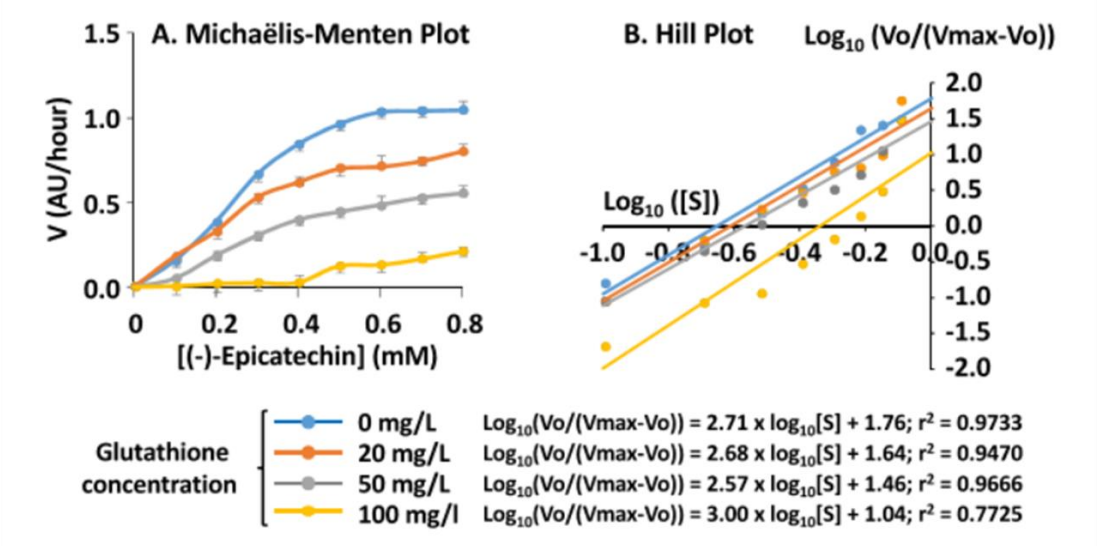

Figure 8. Influence of glutathione concentration on laccase browning inhibition of the synthetic grape juice model solution using (-)-epicatechin as substrate

Figure 8 shows the influence of different doses of glutathione on laccase browning. The Michaëlis-Menten plot (Figure 8 A) confirms that glutathione really does protect against laccase browning and that its protective effect is dose dependent. Indeed, a dose of 100 mg/L almost completely eliminates the yellow color generation in the interval of (-)-epicatechin concentrations between 0.1 and 0.4 mM, and only a slight increase in A420nm was detected at higher concentrations. This protective effect of glutathione against wine browning has been widely reported previously (Makhotkina & Kilmartin, 2009; Oliveira *et al.,* 2011; Kritzinger, Bauer & du Toit, 2013; Zimdars, 2020; Bahut *et al.,* 2020).

The corresponding Hill plot (Figure 8B) provides reasonable linear regression coefficients with r^2 values between 0.9470 and 0.9733, with the exception of the highest dose of glutathione. In this case the linearity worsened (0.7725), probably due to the strong inhibition caused by 100 mg of glutathione/L, similar to what happened with the highest dose of ascorbic acid. It should be noted that all the regression lines are nearly parallel without significant differences between the slopes (from 2.57 ± 0.17 to 3.00 ± 0.71).

Table 6. Effects of glutathione on the kinetic constants of laccase browning of the synthetic grape juice model solution using (-)-epicatechin as substrate

[Glutathione] (mg/L)	Vmax (AU_{420}/hour)	$K_{0.5}$ (mM)	Hill's number
0	1.08 ± 0.06 D	0.223 ± 0.015 A	2.71 ± 0.09 A
20	0.82 ± 0.02 C	0.244 ± 0.006 A	2.68± 0.06 A
50	0.57 ± 0.04 B	0.280 ± 0.019 B	2.57 ± 0.17 A
100	0.22 ± 0.05 A	0.451 ± 0.068 C	3.00 ± 0.71 A

Results are expressed as mean ± standard deviation of three replicates. Different letters in a column indicate the existence of statistical difference ($p < 0.05$).

Considering the empirically determined values of Vmax for laccase browning (Table 6), 20 mg of glutathione/L caused an inhibition on laccase browning of 24%. This increased to 47% and 80% in the case of 50 and 100 mg/L respectively. It should be stressed that 20 mg/L is the maximum legal dose authorized by the OIV for this antioxidant (OIV, 2021). The presence of glutathione increased K_{05} values, although these differences were only significant for the high doses of this compound (50 and 100 mg/L). This suggests that the presence of glutathione reduces the affinity of laccase in respect to (-)-epicatechin. In contrast, supplementation with glutathione did not cause significant differences in the Hill number.

In summary, this paper proposes a synthetic model for measuring laccase browning in a matrix close to real grape juice that makes it possible to study how laccase browning acts in the presence of different possible substrates. It is obvious that the used matrix is much simpler than the real grape juice, but it allows a much better approach than working with the substrates usually used for the study of laccase, such as syringaldazine or ABTS. The results indicate that orthodiphenols are better substrates for laccase browning than triphenols and that monophenols, or at least 4-hydroxybenzoic acid, do not appear to be reactive. Moreover, of the orthodiphenols, (+)-catechin showed the greatest browning intensity, followed in decreasing order by (-)-epicatechin and caftaric acid.

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This model can be also used to determine the inhibitory effect toward laccase browning of the most frequently used antioxidants – sulfur dioxide, ascorbic acid and glutathione – and could certainly be tried in the future on new antioxidants. Our results confirm that sulfur dioxide, ascorbic acid and glutathione really are effective in protecting grape juice against laccase browning. The effectiveness of ascorbic acid and glutathione also confirm that both antioxidants could be useful tools when it comes to reducing the doses of sulfur dioxide in winemaking, especially when grey rot is present. However, in the case of glutathione the dose needed to effectively protect grape juice against laccase browning is higher than the current maximum dose established by the OIV (2021). Given the safety of this compound, it would be advisable to increase its maximum dosage.

Further studies are needed to verify the efficiency of the proposed model on other laccase substrates such as anthocyanins, flavonols and proantocyanidins, and also to test other possible inhibitors of laccase browning, such as oenological tannins and other possible antioxidants. In addition, the influence of other components of grape juice such as heavy metals should also be considered.

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Notes

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CHAPTER II. Study of biotechnological tools, alternative to sulfur dioxide, to preventing enzymatic browning white grape must.

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ORIGINAL PAPER

Biotechnological tools for reducing the use of sulfur dioxide in white grape must and preventing enzymatic browning: glutathione; inactivated dry yeasts rich in glutathione; and bioprotection with *Metschnikowia pulcherrima*

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One of the main problems that affects wine quality and that can provoke the consumers rejection, is enzymatic browning. This problem that is caused by the action of polyphenol oxidases is more severe when the grapes are infected by *Botrytis cinerea* since this fungi releases laccase. The main tool use to prevent browning is the use of sulfur dioxide. However, nowadays the tendence is reducing or eliminating this additive. For that reason, some alternative tools have been proposed to prevent the browning, such as glutathione, pure or in form of inactivated dry yeast, and bioprotection.

This chapter pertains to the accomplishment of the second and third objectives, which aimed to study the use of alternatives of sulfur dioxide to prevent the browning in grape juice. During the study have been working with diluted healthy grape juice samples supplemented or not with laccase, sulfur dioxide, ascorbic acid, glutathione, a specific inactivated dry yeast rich on glutathione and *Metschnikowia pulcherrima*.

The obtained results shows that glutathione, pure or in form of a specific inactivated dry yeasts, and bioprotection with *Metschnikowia pulcherrima* are really effective to prevent browning in healthy grapes in the absence of sulfur dioxide. However, their capacity to prevent browning in the presence of laccase is not enough.

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Biotechnological tools for reducing the use of sulfur dioxide in white grape must and preventing enzymatic browning: glutathione; inactivated dry yeasts rich in glutathione; and bioprotection with *Metschnikowia pulcherrima.*

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ABSTRACT

Sulfur dioxide is the most used additive today for preventing browning in grape musts and wines. However, since wine consumers are increasingly interested in healthier wines, the wine industry is keen to reduce its use. Some promising alternatives to sulfur dioxide have been proposed in recent years, including glutathione, both pure and in the form of inactivated yeasts, and *Metschnikowia pulcherrima* used as a bioprotective agent. Some information exists about the protective effect against oxidation of glutathione but there is very few about the use bioprotection for that purpose. Supplementation with glutathione, regardless of the commercial form, reduced oxygen consumption and browning intensity when laccase was not present in the grape juice. *Metschnikowia pulcherrima* also reduced browning intensity in the absence of laccase but increased the total oxygen consumption. However, in the presence of laccase, glutathione and *Metschnikowia pulcherrima* were not effective enough to adequately prevent the grape juice from browning. Glutathione, both pure and in the form of inactivated yeasts, and *Metschnikowia pulcherrima* are interesting tools for protecting grape must against browning and thus reducing the use of sulfur dioxide.

Keywords: Grape must; Browning; Laccase; Bioprotection; Glutathione; *Metschnikowia pulcherrima*

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INTRODUCTION

One of the problems that most seriously affects oenology today is enzymatic browning (Li H, *et al.,* 2008), especially when the grapes are infected by grey rot (Ky *et al.,* 2012). Enzymatic browning is an oxidation process that occurs in many foods that increases the brown colour (Friedman, 1996), which often leads consumers to reject them. This problem is especially harmful in the wine industry since grape must is highly vulnerable to browning (du Toit *et al.,* 2006; Oliveira *et al.,* 2011).

Enzymatic browning is caused by polyphenol oxidases, a broad family of oxidoreductases (EC. 1 class, according to the International Union of Biochemistry and Molecular Biology – IUBMB). However, in the case of grape juice, browning is caused by just two enzymes: tyrosinase (EC 1.14.18.1, IUBMB), which is naturally present in grapes (du Toit *et al.,* 2006; Oliveira *et al.,* 2011), and laccase (EC 1.10.3.2, IUBMB), which is present only when the grapes are infected by *Botrytis cinerea* (Oliveira *et al.,* 2011; Steel *et al.,* 2013). Both enzymes use molecular oxygen to mainly oxidize the diphenols present in grape must and wine such as caftaric and cutaric acids, catechin and other flavanols, anthocyanin, and flavanone, though laccase is able to oxidize a wider range of substrates than tyrosinase (Oliveira *et al.,* 2011; Steel *et al.,* 2013; Giménez *et al.,* 2022).

The main consequence of enzymatic browning, irrespective of whether tyrosinase and/or laccase is the enzyme responsible, is that diphenols are oxidized to quinones, which can later polymerize through various reactions to form brown pigments called melanins (Queiroz *et al.,* 2008; Claus *et al.,* 2014). These compounds are responsible for increasing the intensity of the brown colour in white wines (browning) and for precipitating the colouring matter in red wines (oxidasic haze) (Ribéreau-Gayon *et al.,* 2006).

Sulfur dioxide is the main and, until recently, only tool used by wineries to protect grape must from browning (Fazio and Warner 1990; Wedzicha *et al.,* 1991). This additive (E-220) is widely used in winemaking thanks to its well-known antioxidant, antioxidasic and antimicrobial properties (Ough and Crowell, 1987), which make it practically essential not only in winemaking but also in the manufacture of other foods (Schroeter, 1966).

However, the current trend in winemaking is to reduce and even eliminate this unfriendly additive owing to its negative effects on the environment (D'amico, Di Vita and Monaco, 2016), the increasing tendency towards minimal intervention (Massov, 2019) and health (Lester, 1995) since it could cause headaches in sensitive people (Costanigro *et al.,* 2014).

For all these reasons, the wine sector is extremely keen to find strategies for preventing oxidation and microbiological spoilage and for reducing or even eliminating sulfur dioxide.

Numerous strategies for reducing or replacing sulfur dioxide have been proposed. On one hand, inert gas (Walls *et al.,* 2022), oenological tannins (Vignault *et al.,* 2019), ascorbic acid (Peng *et al.,* 1998), glutathione (Giménez *et al.,* 2022), and inactivated dry yeasts that are rich in glutathione (Kritzinger *et al.,* 2013) or directly consume oxygen (Pons-Mercadé *et al.,* 2021) have been proposed to protect grape must against browning. On the other hand, chitosan (Petrova *et al.,* 2016), lysozyme (Gao *et al.,* 2002), bioprotection (Simonin *et al.,* 2020), ultra-high pressure homogenization (Loira *et al.,* 2018), ozone (Guzzon *et al.,* 2017) and, more recently, fumaric acid (Morata *et al.,* 2020) have been proposed for protecting wine against microbiological spoilage.

Some of the most promising alternative agents for protecting grape juice against browning are glutathione and several non-*Saccharomyces* yeasts such as *Metschnikowia pulcherrima* used as a bioprotective agent.

Glutathione (GSH) limits browning because it reacts with the orthoquinones produced by the enzymatic oxidation of orthodiphenols to form GRP (Grape Reaction product; 2-S-glutathionylcaftaric acid). GSH traps the orthoquinones in a colourless form and thus restricts the formation of brown polymers (Singleton *et al.,* 1985; Kritzinger *et al.,* 2013). The use of glutathione in winemaking was authorized by the International Organisation of Vine and Wine (OIV) in 2015 at a maximal dose of 20 mg/L (OIV-OENO 445, 2015; OIV-OENO 446, 2015). However, the high price of pure glutathione led to the use of certain inactive dry yeasts especially rich in glutathione (IDY-GSH) being proposed as more economical alternatives (Gabrielli *et al.,* 2017; Bahut *et al.,* 2019). The use of these yeasts in winemaking was authorized by the OIV in 2018 (OIV-OENO 603, 2018).

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Bioprotection is also nowadays considered a highly promising alternative tool to sulfites in winemaking (Simonin *et al.,* 2020; Windholtz *et al.,* 2021a). In fact, the OIV has authorized the use of non-*Saccharomyces* yeasts in winemaking for various purposes, one of which is bioprotection (OIV-OENO 576B, 2017; OIV-OENO 576A, 2017). Several studies on the use of certain strains of non-*Saccharomyces* yeasts for preventing the development of spoilage microorganisms have been reported in recent years (Simonin *et al.,* 2018; Rubio-Bretón *et al.,* 2018; Peña *et al.,* 2020; Morata *et al.,* 2021; Di Gianvito *et al.,* 2022). However, few of these studies have proposed their use in protecting the grape must against browning (Chacon-Rodriguez *et al.,* 2020; Windholtz *et al.,* 2022). Specifically, certain strains of *Metschnikowia pulcherrima* have shown interesting results both for preventing the development of spoilage microorganisms (Windholtz *et al.,* 2021a; Windholtz *et al.,* 2021b) and for protecting against browning (Chacon-Rodriguez *et al.,* 2020; Simonin *et al.,* 2020).

In this paper we compare the protective effect against browning in white grape musts of some of the most promising antioxidant tools – reduced glutathione, both pure and in the form of commercial inactivated dry yeasts rich in glutathione, and a commercial strain of *Metschnikowia pulcherrima* – with the protective effect of classical tools such as sulfur dioxide and ascorbic acid.

MATERIALS AND METHODS

Chemicals and equipment. All samples were handled without exposure to light. Potassium disulfite (CAS No.: 16731-55-8, purity ≥ 98%), polyvinylpolypyrrolidone (PVPP, CAS No.: 9003-39-8, purity ≥98%), syringaldazine (purity ≥ 98%), L-ascorbic acid (purity ≥ 99%), L-glutathione reduced (purity ≥ 98%) and $FeSO_4 \cdot 7H_2O$ (purity ≥ 99%) were purchased from Sigma-Aldrich (Madrid, Spain). L-(+)-tartaric acid (purity ≥ 99.5%), sodium hydroxide (purity ≥ 98%), sodium acetate (purity ≥ 99%) and $CuSO_4$ (purity ≥ 99%) were purchased from Panreac (Barcelona, Spain). Ethanol (96 % vol.) was supplied by Fisher Scientific (Madrid, Spain). Cellulose membranes of 3.5KDa (6.4mL/cm) were supplied by Spectrum Laboratories, Inc (Rancho Dominguez, USA).

The equipment used was as follows: a spectrophotometer UV-Vis Helios Alpha™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); a centrifuge Heraeus™ Primo™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); and an Entris II Series Analytical Balance (Sartorius, Goettingen, Germany).

Obtaining the samples of grape must. Muscat of Alexandria grapes were handpicked in the vineyard of the Rovira i Virgili University (Mas dels Frares, Constantí, Tarragona: 41°08'44.1"N 1°11'51.0"E) during the 2022 vintage harvest. The grapes were pressed in a nitrogen-saturated hand-press and the must was collected in a bottle also saturated with N_2.

Synthetic buffer. A solution of 4 g/L of L-(+)-tartaric acid, 3 mg of iron/L, in the form of iron (III) chloride hexahydrate, and 0.3 mg of copper/L in the form of copper (II) sulfate pentahydrate adjusted to pH 3.5 with sodium hydroxide was used for all experiments.

Inactivated dry yeasts rich in glutathione and *Metschnikowia pulcherrima* strain. A commercial inactivated dry yeast (IDY-GSH) rich in glutathione (Glutastar™, Lallemand Inc, Montreal, Canada) and a strain of *Metschnikowia pulcherrima* (MP) selected for its high oxygen consumption capacity (Level2 Initia™, Lallemand Inc, Montreal, Canada) were used. Both the IDY-GSH (400 mg/L) and the MP (250 mg/L) were hydrated in ten times their weight of distilled water ten minutes before the beginning of the measurements. The water temperature was room temperature for the IDY-GSH and 30 ^{o}C for the MP.

Extracellular laccase production and enzymatic activity measurement. Active laccase extracts were obtained from the *Botrytis cinerea* isolate 213 strain following the methodology reported by Vignault *et al.* (2020). This laccase extract was treated with 0.16 g of PVPP/mL for 10 minutes and centrifuged at 7,500 rpm for 10 minutes, and the supernatant was subsequently dialysed with 3,5KDa cellulose membrane for two days in a 0,3M ammonium formate solution and for two more days in distilled water. The laccase activity of this extract was determined using an adaptation of the syringaldazine test method (Grassin and Dubourdieu, 1986).

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Reaction conditions for measuring the oxygen consumption rate. These assays were performed in 60 clear glass flasks (66mL) with an oxygen sensor spot (PreSens Precision Sensing GmbH, order code: SP-PSt3-NAU-D5-CAF; batch number: 1203- 01_PSt3-0828-01, Regensburg, Germany) for measuring the dissolved oxygen noninvasively by luminescence (Nomasense TM O2 Trace Oxygen Analyzer by Nomacorc S.A., Thimister Clermont, Belgium).

Thirteen mL of grape must and 52 mL of buffer were added to each flask, to which various antioxidant agents or combinations of them had previously been added. This dilution of the grape must was performed because the pure grape juice consumes oxygen so fast that it is nearly impossible to correctly monitor the oxygen concentration of all the samples. The antioxidants used were: sulfur dioxide (20 mg/L in the form of potassium disulfite), ascorbic acid (100 mg/L), glutathione (20 mg/L), a commercial inactivated dry yeast rich in glutathione (400 mg/L), and a selected commercial strain of *Metschnikowia pulcherrima* (200 mg/L). A control without any addition was also prepared. These assays were also performed by combining sulfur dioxide with each of the other antioxidant agents with and without the addition of 2 UA of laccase/mL. Table 1 shows all the experimental conditions and provides abbreviations for each one. All these assays were performed in triplicate.

Table 1. Experimental conditions

Without Laccase						Supplemented with 2 UA of Laccase/mL					
Experimental conditions	SO_2	AA	GSH	IDY	MP	Experimental conditions	SO_2	AA	GSH	IDY	MP
C	0	0	0	0	0	L	0	0	0	0	0
SO_2	20	0	0	0	0	L+SO_2	20	0	0	0	0
AA	0	100	0	0	0	L+AA	0	100	0	0	0
GSH	0	0	20	0	0	L+GSH	0	0	20	0	0
IDY	0	0	0	400	0	L+IDY	0	0	0	400	0
MP	0	0	0	0	200	L+MP	0	0	0	0	200
AA + SO_2	20	100	0	0	0	L+AA + SO_2	20	100	0	0	0
GSH + SO_2	20	0	20	0	0	L+GSH + SO_2	20	0	20	0	0
IDY + SO_2	20	0	0	400	0	L+IDY + SO_2	20	0	0	400	0
MP + SO_2	20	0	0	0	200	L+MP + SO_2	20	0	0	0	200

All the units are expressed as mg/L. C: Control; SO2: Sulfur dioxide; AA: Ascorbic acid; GSH: Glutathione; IDY: Inactivated dry yeast; MP: Metschnikowia pulcherrima; L: Laccase

The bottles were immediately hand-shaken for a few seconds to saturate the solution in oxygen (around 7-8 mg O_2/L), and the oxygen concentration was measured (Diéval *et al.,* 2011) periodically until reaching an asymptotic behaviour (around 5 hours) to determine the oxygen consumption kinetics. All measurements were taken in an air-conditioned laboratory at 22 ± 2 ^oC. The total oxygen consumption capacity (TOCC) was calculated using the mathematic model previously reported by Pons-Mercadé *et al.* (2021). Once the oxygen concentrations were below 1 mg/L or its consumption reached an asymptotic behaviour, the samples were supplemented with 50 mg of sulfur dioxide/L to stop colour evolution.

Colour measurements. Measurement of the yellow colour (A420nm) and the CieL*a*b* coordinates of the samples were determined according to Ayala *et al.* (1997). Data

processing was performed with MSCV software (MSCVes, 2013). The total colour difference (ΔEab*) was calculated as the Euclidian distance between two points in the CieL*a*b* space using the following formula: $\Delta Eab^* = [(L_1-L_2)^2 + (a^*_1-a^*_2)^2 + (b^*_1-b^*_2)^2]^{1/2,}$ where L* is the lightness; a* is the colour's green-red component, and b* is the colour's blue-yellow component. It is generally considered as a criterion that two samples of wine could be distinguished by the human eye through the glass when ΔEab* ≥ 3 units (García-Marino *et al.* 2013; Vignault *et al.,* 2019).

Statistics. All data are expressed as the arithmetic average ± standard deviation of three replicates. One-factor analysis of variance (ANOVA F-test) was conducted using SPSS 15.0 software (SPSS Inc., Chicago, IL). Significant differences were considered when p-value was less than 0.05.

RESULTS AND DISCUSSION

Oxygen consumption kinetics

Figures 1.A and 1.B show the oxygen consumption kinetics of the diluted grape juice under the experimental conditions with or without supplementation with laccase. Figure 1.A clearly shows that the control sample – the diluted grape juice without modification (C) – initially consumed oxygen very quickly. This oxygen consumption rate (OCR) subsequently moderated, probably due to depletion of the substrates, reaching values below 1 mg of O_2/L after five hours. This figure also shows that when the sample was supplemented with sulfur dioxide (SO_2), the OCR decreased. In order to be able to statistically compare these curves, a previously reported kinetic model (Pascual *et al.,* 2017; Pons-Mercadé *et al.,* 2021) was applied to calculate the total oxygen consumption capacity. This model involves displaying the inverse of consumed oxygen versus the inverse of time. From this mathematical model, the following equation can be established: $1/[O2] = A/t + B$. This equation, which describes the relationship between oxygen consumed and time, can be used to determine the total oxygen consumption capacity by calculating the limit when time tends towards infinity.

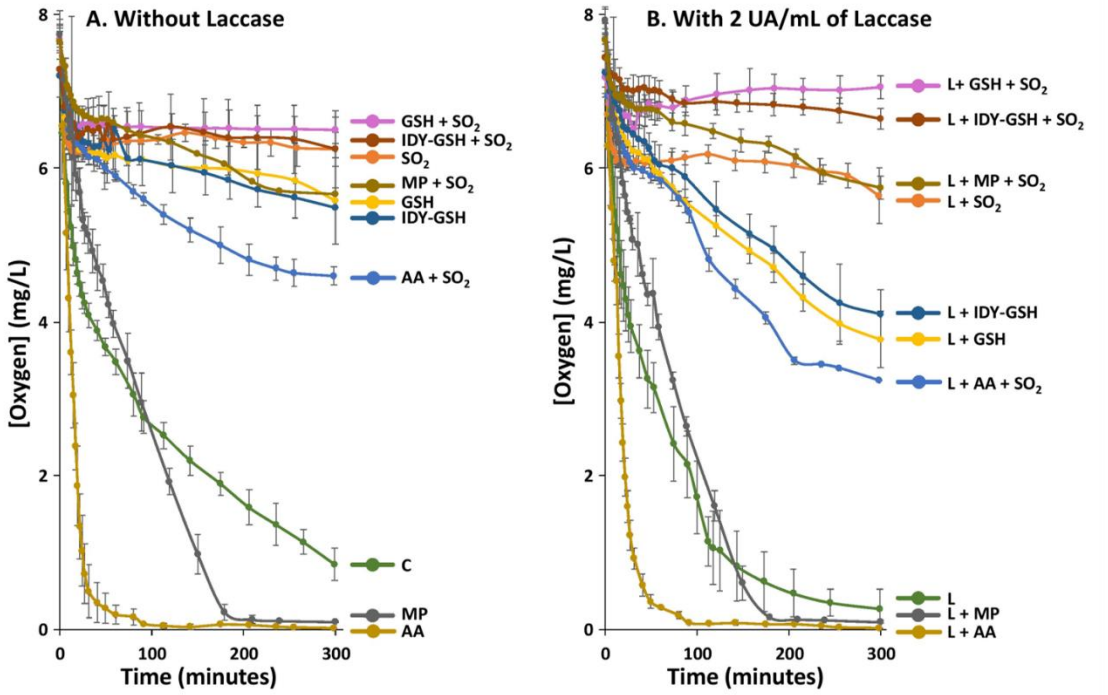

Figure 1. Oxygen consumption kinetics of grape must under different conditions

All the analysis were performed by triplicate. All the units are expressed as mg/L. C: Control; SO_2: Sulfur dioxide; AA: Ascorbic acid; GSH: Glutathione; IDY: Inactivated dry yeast; MP: *Metschnikowia pulcherrima*; L: Laccase

Total oxygen consumption capacity (TOCC)

The results for TOCC are shown in Figure 2, which confirms what was indicated in Figure 1.A, i.e., that the TOCC of the control sample (C) was significantly higher than that of the sample supplemented with sulfur dioxide (SO_2). These data confirm the well-known inhibitory effect of this additive on tyrosinase activity (Schroeter, 1966; Ough and Crowell, 1987; Fazio and Warner 1990; Wedzicha *et al.,* 1991; Giménez *et al.,* 2022). Supplementation with glutathione (GSH) and with inactivated dry yeasts rich in glutathione (IDY-GSH) also led to a significant decrease in TOCC. This inhibitory effect observed in both experimental conditions may be due to the fact that glutathione reacts with the orthodiquinones produced by the enzymatic oxidation of hydroxycinnamic acids to form the grape reaction product (GRP) (Singleton *et al.,* 1985; Kritzinger *et al.,* 2013; Nikolantonaki *et al.,* 2014; Nikolantonaki *et al.,* 2018). This process can reduce the concentration of orthodiphenols and lead to their depletion, especially in our experimental conditions where the grape must was diluted five times. The lack of substrates for polyphenol oxidases may therefore justify the TOCC reduction in our experimental conditions, though in a grape must without dilution this reduction would

probably not be so great. However, these data clearly confirm GSH's protective effect against oxidation, both pure and in the form of IDY rich in GSH.

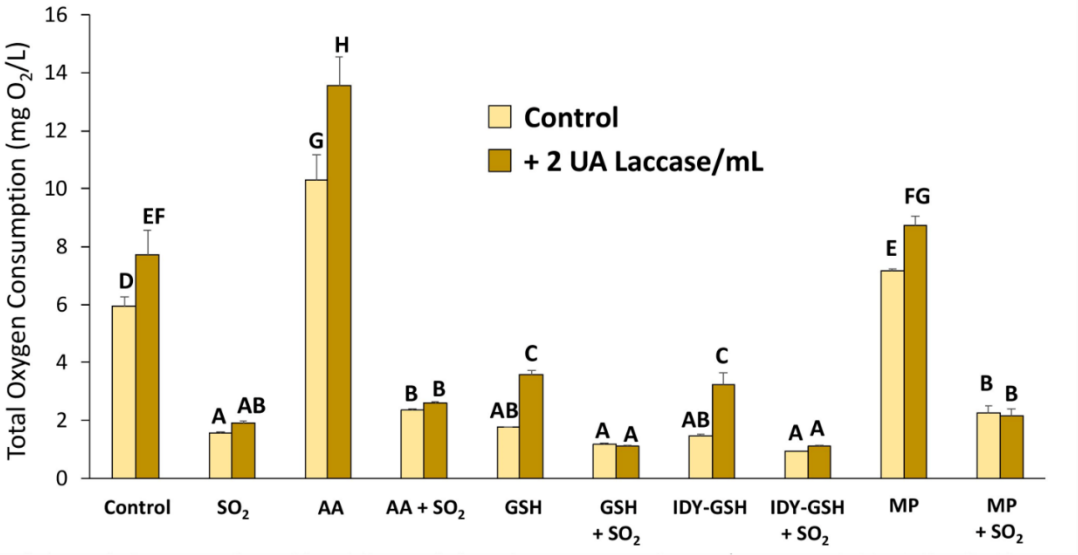

Figure 2. Influence of Laccase, SO_2, Ascorbic Acid, Glutathione, Inactivated dry yeast rich on glutathione and *M. Pulcherrima* on Total oxygen consumption (TOC)

All the analysis were performed by triplicate. All the units are expressed as mg/L. C: Control; SO_2: Sulfur dioxide; AA: Ascorbic acid; GSH: Glutathione; IDY-GSH: Inactivated dry yeast rich in glutathione; MP: *Metschnikowia pulcherrima*. Different letters indicate the existence of significant differences ($p < 0.05$)

On the other hand, supplementation with *Metschnikowia pulcherrima* (MP), and especially with ascorbic acid (AA), increased TOCC with respect to the control (C). Ascorbic acid is known to react efficiently with oxygen to produce dehydroascorbic acid and hydrogen peroxide (Oliveira *et al.,* 2011; Barril *et al.,* 2016). It can therefore compete with polyphenol oxidases (Barril *et al.,* 2016; Vignault *et al.,* 2020; Giménez *et al.,* 2022) for oxygen and prevent grape juice from browning. However, ascorbic acid must be used in combination with sulfur dioxide to eliminate the hydrogen peroxide formed and prevent subsequent oxidation (Oliveira *et al.,* 2011; Barril *et al.,* 2016). The highest TOCC observed in the presence of MP probably occurred because this non-*Saccharomyces* yeast directly consumes oxygen very effectively. In fact, use of this yeast has been recommended for that purpose (Chacon-Rodriguez *et al.,* 2020; Windholtz *et al.,* 2022). It should be noted that the used grape must was not sterilized and therefore the presence of some yeasts, *Saccharomyces* or non- *Saccharomyces* could also have contributed to the observed oxygen consumption albeit presumably to a lesser extent than the inoculated MP.

The combined supplementation of all studied antioxidants with sulfur dioxide (GSH+SO_2, IDY-GSH+SO_2, AA+SO_2 and MP+SO_2) led to a reduction in TOCC with respect to their corresponding references (GSH, IDY-GSH, AA and MP). Note that the TOCC of GSH+SO_2 and IDY-GSH+SO_2 were very similar to that of SO_2 alone. These results may be considered logical because in those conditions tyrosinase is completely inhibited and the possible inhibitory effect of glutathione cannot act because no orthodiquinones are produced. The TOCC of MP+SO_2 was also similar to that of SO_2 alone and much lower than that of MP alone. These results may indicate that the presence of sulfur dioxide inhibits this non-*Saccharomyces* yeast.

The TOCC of AA+SO_2 was higher than that of SO_2 alone and lower than that of AA alone because in those conditions oxygen consumption was only due to the direct reaction of this antioxidant with oxygen and not to polyphenol oxidase activity.

As expected, supplementation with laccase significantly increased the TOCC of the control sample. This was probably because total polyphenol oxidase activity was higher and because laccase can oxidize a wider range of substrates than tyrosinase (Oliveira *et al.,* 2011; Steel *et al.,* 2013). This trend was also observed in the samples supplemented with laccase and containing AA, GSH, IDY-GSH and MP in relation to their corresponding samples without laccase.

The samples supplemented with GSH and IDY-GSH in the presence of laccase showed significantly lower values of TOCC than the control sample supplemented with laccase but significantly higher values of TOCC than the corresponding samples without laccase. A possible explanation for these data is that laccase can oxidize more substrates than tyrosinase, especially since this polyphenol oxidase can oxidize GRP (Kritzinger *et al.,* 2013). In any case, the reduction in TOCC produced by supplementation with GSH or IDY-GSH suggests that glutathione may perform an antioxidant role even when the grape berries are affected by grey rot, though this protection is not as effective as it is in healthy grapes.

Supplementation with sulfur dioxide inhibited TOCC in all experimental groups in a similar way to the corresponding samples without laccase. These data confirm, as previously described in the literature du Toit *et al.,* 2006; Oliveira *et al.,* 2011; Verma *et*

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al., 2018; Vignault *et al.,* 2020; Giménez *et al.,* 2022), that sulfur dioxide is also a powerful inhibitor of laccase. Determining TOCC is necessary to understand which antioxidant agent is the most effective in trapping the oxygen in the grape must and preventing its consumption by polyphenol oxidases. However, since TOCC does not provide direct information about the browning intensity, the final colour of the samples was measured by spectrophotometry.

Browning intensity

Figures 3.A and 3.B show the absorbance at 420 nm (A420nm) and the CIEL*a*b* blue-yellow component (b*) of the various samples as indicators of browning intensity.

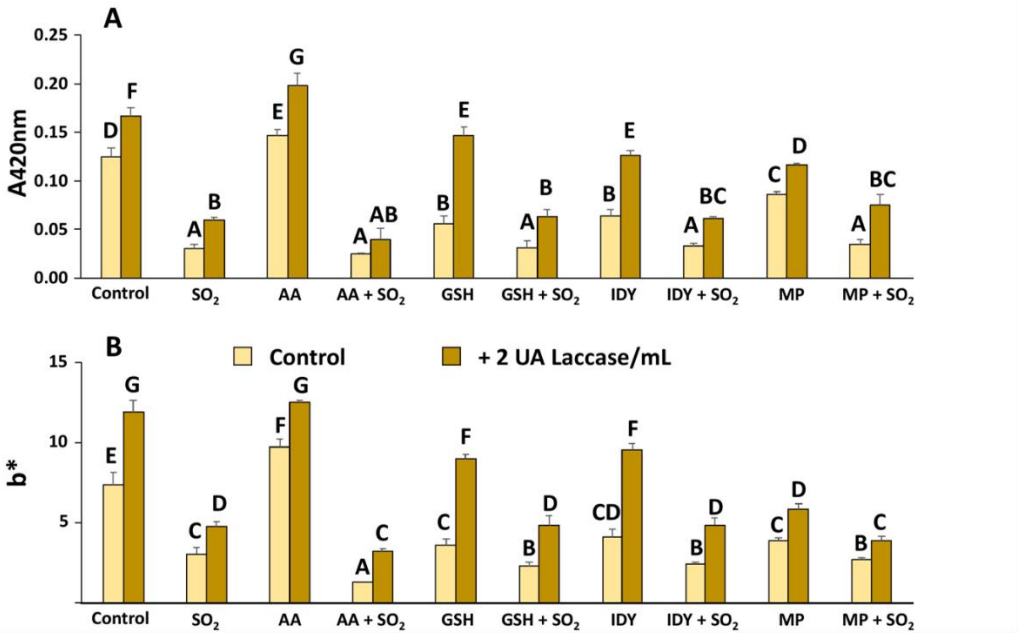

Figure 3. Influence of Laccase, SO_2, Ascorbic Acid, Glutathione, Inactivated dry yeast rich on glutathione and *M. Pulcherrima* on browning intensity

All the analysis were performed by triplicate. C: Control; SO_2: Sulfur dioxide; AA: Ascorbic acid; GSH: Glutathione; IDY-GSH: Inactivated dry yeast rich in glutathione; MP: *Metschnikowia pulcherrima*. Different letters indicate the existence of significant differences ($p < 0.05$)

As expected, supplementation with sulfur dioxide had a clear protective effect against browning since the values of A420 and b* were significantly lower than in the control sample. This effect was also observed when the samples were supplemented with laccase, which confirms that sulfur dioxide not only inhibits the polyphenol oxidase present in healthy grapes (tyrosinase) but also has a powerful inhibitory action on the

laccase present in the grapes infected with grey rot (Schroeter, 1966; Ough and Crowell, 1987; Fazio and Warner 1990; Wedzicha *et al.,* 1991; Giménez *et al.,* 2022).

In contrast, the sample supplemented with AA showed significantly higher values of A420nm and b* than the control without supplementation although it consumed oxygen faster than the control. This trend, which was also observed when the sample was supplemented with laccase, is probably due to the fact that ascorbic acid produces hydrogen peroxide when it reacts with oxygen (Oliveira *et al.,* 2011; Barril *et al.,* 2016). However, when these samples were also supplemented with sulfur dioxide, the A420nm were similar to that of the sample supplemented only with SO_2 and the CIEL*a*b* coordinate b* was even significantly lower. These data confirm that ascorbic acid must be applied together with sulfur dioxide so that it reacts with hydrogen peroxide to nullify its negative effect on browning (Peng *et al.,* 1998; Barril *et al.,* 2016).

The A420nm of the samples supplemented with GSH, IDY-GSH and MP were also significantly lower than those of the control sample. This reduction in A420nm was highly relevant, though the values were slightly but significantly higher than that of the sample treated with sulfur dioxide. The CIEL*a*b* coordinate b* of the samples supplemented with GSH, IDY-GSH and MP showed a similar trend to the A420nm but, in this case, no significant differences were found with the sample treated with sulfur dioxide. These data therefore confirm that glutathione, both pure and in the form of inactivated dry yeast, and *Metschnikowia pulcherrima* really do protect grape juice against browning. This protective effect of GSH, IDY-GSH and MP was also present in the samples supplemented with laccase, but the efficiency was much lower. However, when sulfur dioxide was also added, the values of A420 and b* were similar to those of the sample protected only with SO_2. These data indicate that these alternative tools to sulfur dioxide cannot be applied when the grapes are infected with *Botrytis cinerea*.

Note that browning intensity generally has a certain parallelism with the TOCC we observed. In nearly all samples, the higher the TOCC, the higher the browning intensity, with the only exception being the samples supplemented with MP in the presence or absence of laccase. The explanation for this different behaviour may be that since *Metschnikowia pulcherrima* consumes oxygen very effectively (Chacon-Rodriguez *et al.,*

2020; Simonin *et al.,* 2020), some of the initially dissolved oxygen is not consumed by the polyphenol oxidases.

Total colour difference (ΔEab*)

The total colour difference (ΔEab*) between the various samples and the sample supplemented with sulfur dioxide without laccase was calculated to determine whether the browning intensity of the samples can be distinguished by potential consumers.

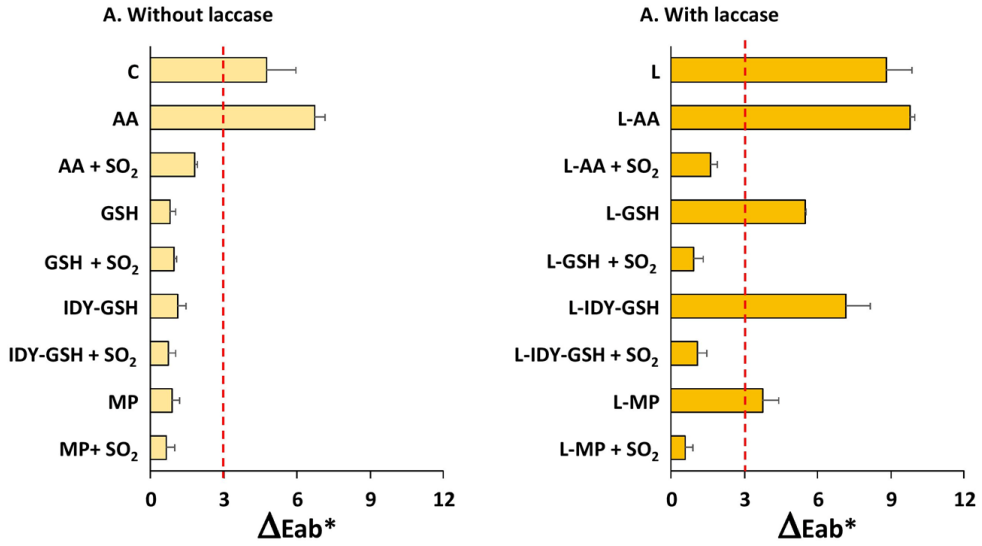

Figure 4. Influence of Laccase, SO_2, Ascorbic Acid, Glutathione, Inactivated dry yeast rich on glutathione and *M. Pulcherrima* on the total color (ΔEab*)

All the analysis were performed by triplicate. C: Control; SO_2: Sulfur dioxide; AA: Ascorbic acid; GSH: Glutathione; IDY-GSH: Inactivated dry yeast rich in glutathione; MP: *Metschnikowia pulcherrima*.

The results are clear and highly indicative of the protective effect of the various antioxidants. Figure 4.A shows the ΔEab* of all samples without supplementation with laccase and the corresponding sample with only the addition of sulfur dioxide. As expected, the control sample showed an ΔEab* above 3 units, which indicates that it was much more affected by browning than the sample protected with SO_2. The ΔEab* of the sample supplemented with only AA was even higher than the control, whereas when sulfur dioxide was also present the ΔEab* was below 3 units. These data confirm that using ascorbic acid alone leads to greater oxidation and that it must therefore be used in the presence of SO_2 (Oliveira *et al.,* 2011; Barril *et al.,* 2016).

In contrast, the samples protected with GSH, IDY-GSH, and MP with or without SO_2 showed values below this threshold. However, when the samples were also supplemented with laccase, supplementation with GSH, IDY-GSH, and MP was not effective enough to protect the samples against browning because the values of ΔEab* were above 3 units. These results agree with previously reported data (Singleton *et al.,* 1985; Kritzinger *et al.,* 2013; Gabrielli *et al.,* 2017; Bahut *et al.,* 2019; Chacon-Rodriguez *et al.,* 2020; Simonin *et al.,* 2020; Windholtz *et al.,* 2021b; Giménez *et al.,* 2022) and confirm that using glutathione, pure or in the form of inactivated dry yeasts, and *Metschnikowia pulcherrima* can really protect grape juice against browning at least in healthy grapes.

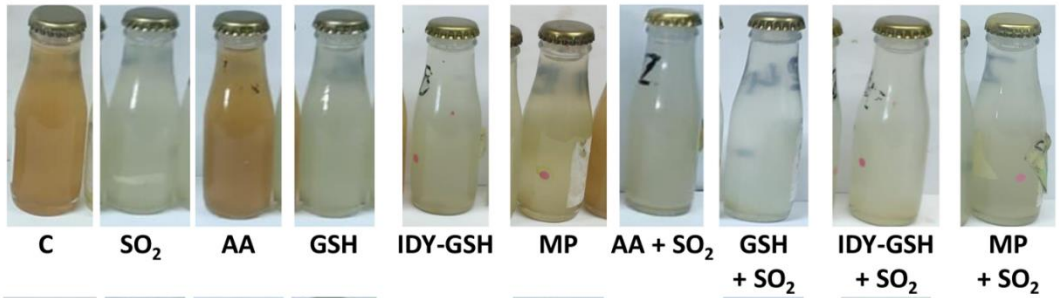

Figure 5. Influence of Laccase, SO_2, Ascorbic Acid, Glutathione, Inactivated dry yeast rich on glutathione and *M. Pulcherrima* on the final color of the samples

C: Control; SO_2: Sulfur dioxide; AA: Ascorbic acid; GSH: Glutathione; IDY-GSH: Inactivated dry yeast rich in glutathione; MP: *Metschnikowia pulcherrima*.

Since a picture is worth a thousand words, Figure 5 shows the photographs of the different samples after the experimental process. In them, it can be clearly seen the browning developed in each one of the experimental conditions and therefore the protective effect of some of the treatments, which visually corroborates all the experimental data previously shown.

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CONCLUSIONS

In this study we analyzed how various antioxidants influence oxygen consumption kinetics and browning intensity in two scenarios. The first scenario was grape must from healthy grapes while the second was grape must enriched with laccase to reproduce what occurs when the grapes are infected with *Botrytis cinerea*. Our experimental design measured oxygen consumption kinetics and browning intensity caused by polyphenol oxidases, tyrosinase and laccase. As expected, our results confirm that grape must consumes oxygen and browns very quickly and that the presence of laccase accelerates both of these processes. Our results also confirm that sulfur dioxide is highly effective in preventing browning even in grape musts with high levels of laccase activity. On the other hand, using only ascorbic acid leads to higher oxygen consumption and browning, which indicates that this antioxidant must be used in association with sulfur dioxide.

The other alternative antioxidants – glutathione, both pure and in the form of inactivated dry yeasts, and the non-*Saccharomyces* yeast, *Metschnikowia pulcherrima* used as a bioprotective agent – can be interesting tools for protecting grape juice against browning and perhaps for reducing the use of sulfur dioxide, at least in healthy grapes. Specifically, glutathione and inactivated dry yeast rich in glutathione reduced oxygen consumption and reduced the intensity of browning when no laccase was present in the medium. However, their effectivity was reduced in the presence of laccase. The mechanism by which glutathione protects against enzymatic browning and reduces oxygen consumption is probably its capacity to combine with the orthodiquinones formed by the action of the polyphenol oxidases in stopping the browning process and depleting the medium on substrates for these enzymes.

Metschnikowia pulcherrima also reduced browning intensity but its action mechanism is different from that of glutathione. This non-*Saccharomyces* yeast protects because it consumes oxygen very efficiently and therefore reduces its availability for the polyphenol oxidases.

More studies are needed to further investigate these promising alternatives to sulfur dioxide since many consumers are searching for healthier wines and the wine industry is very keen to reduce this unfriendly additive.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

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DECLARATIONS

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Compliance with Ethics requirements All authors were compliant and followed the ethical guidelines, according to the requirements of European Food Research and Technology.

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CHAPTER III. Study of enzymatic oxidation mechanisms of wine anthocyanins

Nowadays, red wines are especially appreciated by the intensity and stability of their colour. For that reason, the presence of laccase released by *Botrytis cinerea* in infected grapes is one of the main problems since it can provoke an oxidasic haze.

This chapter pertains to the accomplishment of the fourth objective, which aimed to study the enzymatic oxidation mechanisms of grape anthocyanins. With this aim, degradation kinetics catalyzed by laccase of the five grape anthocyanins has been measured in a synthetic solution using individual solutions of each one of the anthocyanins and also an equimolar solution of all five.

The results indicate that the anthocyanins with three substituents in the B-ring are more quickly degraded than anthocyanins with only two. Even, peonidin seems to be not degraded by laccase in those conditions. However, when all the anthocyanins were present in the media, the degradation kinetics of the anthocyanins were more similar among them even in the case of peonidin. This more similar degradation kinetics was probably because the less reactive anthocyanin can polymerize with the quinones formed by the action of laccase on the more reactive ones.

Supplementation with (-)-epicatechin, glutathione and especially with seed tannins has shown a protective effect on the color and on the anthocyanins analyzed by spectrophotometry suggesting that their presence could favor the formation of new red pigments.

The submission was release on 17th of May 2023, to *Oeno-One* journal for its consideration and eventual publication.

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Effects of laccase from *Botrytis cinerea* on the oxidative degradation of anthocyanins.

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ABSTRACT

The aim of this work was to study the degradation kinetics of five grape anthocyanins caused by laccase from *Botrytis cinerea*. In individual solutions, the anthocyanins with 3 substituents in the B-ring – petunidin, delphinidin and malvidin 3-*O*-glucosides – were degraded much faster than those with 2 substituents. In the latter case, cyanidin 3-*O*-glucoside did not degrade as quickly and peonidin-3-*O*-glucoside in particular was not degraded by laccase at all. In contrast, when an equimolar solution of the five anthocyanins was used, the differences in the degradation kinetics of all anthocyanins were lessened, probably because the less reactive anthocyanins were able to polymerise with the quinones formed by the laccase action on the more reactive anthocyanins. Finally, supplementation with (-)-epicatechin, glutathione and especially seed tannins seemed to protect the red colour from laccase.

Keywords: Laccase; *Botrytis cinerea*; Anthocyanins; Oxidative degradation; Oxidasic haze; Browning

III. Results

INTRODUCTION

Polyphenol oxidases are multi-copper oxidative enzymes found in plants, fungi, and bacteria that belong to the family called multi-copper oxidases (Ma *et al.,* 2009; Strong and Claus, 2011). This family of enzymes, highly important from an oenological point of view, includes tyrosinase (EC 1.14.18.1, IUBMB), which is naturally present in grape berries (du Toit *et al.,* 2006; Fronk *et al.,* 2015) and laccase (EC 1.10.3.2, IUBMB), which is only present in grapes infected by epiphytic fungi, mainly *Botrytis cinerea* (Strong and Claus, 2011; Claus *et al.,* 2014). Both tyrosinase and laccase can oxidise several substrates such as caftaric and cutaric acids, catechin, anthocyanins, flavanols and flavanone as substrates, but laccase acts on a far wider range of substrates than tyrosinase (Oliveira *et al.,* 2011; Steel *et al.,* 2013).

Botrytis cinerea, a necrotrophic pathogenic fungus, causes grey rot. This is probably the worst plague affecting vine culture since it causes huge economic losses each year for agriculture, especially in grape and wine production (Steel *et al.,* 2013). In addition to the release of laccase, which seriously affects wine colour (La Guerche *et al.,* 2007; Ky *et al.,* 2012; Vignault *et al.,* 2019; Giménez *et al.,* 2022), *Botrytis cinerea* causes several other problems such as contamination with non-desirable microorganisms (Barata *et al.,* 2008; Lleixà *et al.,* 2018), problems of settling and filtration (Villettaz *et al.,* 1984; Jadhav and Gupta, 2016), presence of ochratoxin A (Ponsone *et al.,* 2012; Valero *et al.,* 2008), and mouldy odours (Lorrain *et al.,* 2012; Meistermann *et al.,* 2021).

It is therefore obvious that the infection of grape berries with *Botrytis cinerea* is undoubtedly one of the main problems in viticulture today, since their presence seriously affects the quality of the final wine product. In the long list of problems that *Botrytis cinerea* causes, wine colour deterioration is probably the one that worries winemakers the most.

The main consequence of polyphenol oxidases, irrespective of whether tyrosinase and/or laccase is the enzyme responsible, is that diphenols are oxidized to quinones (Claus, 2004; Li *et al.,* 2008). These quinones can polymerise through several reactions, forming brown pigments called melanins (Queiroz *et al.,* 2008; Oliveira *et al.,* 2011).

These pigments, which are relatively insoluble depending on their degree of polymerisation (Moon *et al.,* 2020), are responsible for increasing the brown colour in white wines (browning) and for the precipitation of the colouring matter in red wines (oxidasic haze) (Ribéreau-Gayon *et al.,* 2006).

Red wines are particularly appreciated for the intensity and stability of their colour. For this reason, the presence of laccase from *Botrytis cinerea* in red grapes poses a serious problem since it typically makes those wines have a less intense and less stable red colour. Several studies about the effect of laccase on the browning of white grape must and wines (Gómez *et al.,* 1995; La Guerche *et al.,* 2007; Li *et al.,* 2008; El Hosry *et al.,* 2009; Oliveira *et al.,* 2011; Ky *et al.,* 2012; Zimdars *et al.,* 2017; Vignault *et al.,* 2019; Giménez *et al.,* 2022) have been reported but only very little information exists about the effects of laccase on red wine colour (Ky *et al.,* 2012; Steel *et al.,* 2013; Vignault *et al.,* 2019; Kelly *et al.,* 2022) and to our knowledge even less about the effect of laccase on grape anthocyanins (Ky *et al.,* 2012; Fang *et al.,* 2015; Detering *et al.,* 2018) . Moreover, the differences that exist in the B ring of the various grape anthocyanins (Figure 1) make the study of the relationship between the anthocyanin structure and the laccase degradation kinetics a matter of great interest.

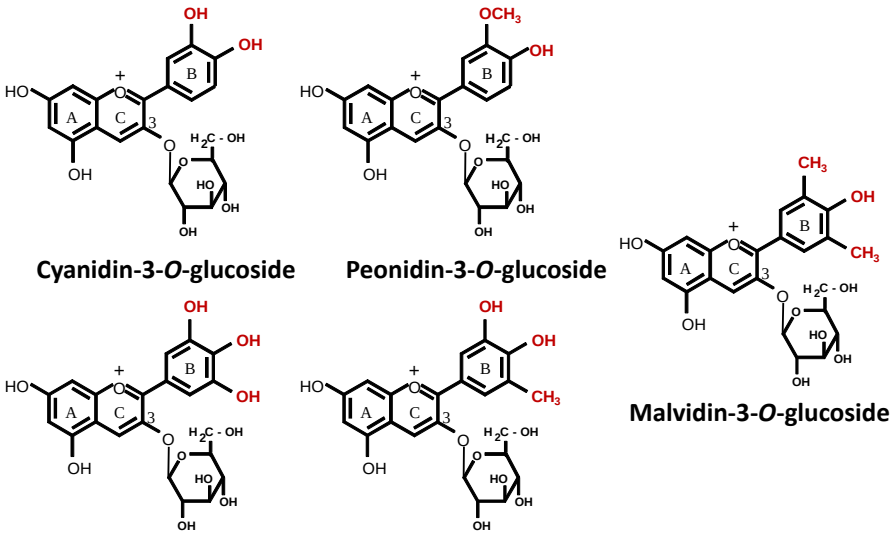

Figure 1. The five natural grape anthocyanins.

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Otherwise, the most common solutions that winemakers use to protect grape juice from the browning generated by polyphenol oxidases are basically to increase the dose of sulphur dioxide (Ribéreau-Gayon *et al.,* 2006). However, the current trend in oenology is trying to reduce or even eliminate the use of this unfriendly additive (Lester, 1995; Costanigro *et al.,* 2014; D'Amico *et al.,* 2016; Massov, 2019). To this end, the use of oenological tannins (Vignault *et al.,* 2019; Vignault *et al.,* 2020) and glutathione (El Hosry *et al.,* 2009; Giménez *et al.,* 2022; Giménez *et al.,* 2023) have been suggested as alternatives to protect wine colour from laccase action.

The aim of this work was to study the effects of laccase activity on the degradation of different anthocyanins in a synthetic media similar to grape juice and also to determine the possible protective effects of oenological tannins and glutathione.

MATERIALS AND METHODS

Chemicals and equipment. Polyvinylpolypyrrolidone (PVPP, CAS No.: 9003-39-8, purity ≥ 98%), syringaldazine (purity ≥ 98%), L-ascorbic acid (purity ≥ 99%), L-glutathione reduced (purity ≥ 98%), (-)-epicatechin (purity ≥ 98%) and $FeSO_4 \cdot 7H_2O$ (purity ≥ 99%) were purchased from Sigma-Aldrich (Madrid, Spain). L-(+)-tartaric acid (purity ≥ 99.5%), sodium hydroxide (purity ≥ 98%), methanol (purity minimum 99,9%) and formic acid (purity ≥ 99,9%) were high-performance liquid chromatography (HPLC) grade, sodium acetate (purity ≥ 99%) and CuSO4 (purity ≥ 99%) were purchased from Panreac (Barcelona, Spain). Ethanol (96% vol.) was supplied by Fisher Scientific (Madrid, Spain). Delphinidin-3-glucoside-chloride (purity = 97.38%), peonidin- 3- glucoside – chloride (purity = 98%), petunidin – 3- glucoside – chloride (purity = 96.71%) were purchased from Phytolab. D-glucose and D-fructose were purchased by VWR International (Leuven, Belgium). Cyanidin -3- glucoside -chloride (purity ≥ 98%) was supplied from TargetMol (Wellesley Hills, USA). Tannins from seeds (purity ≥ 85%) were from Alvinesa Natural Ingredients S.A. (Daimel, Spain). Malvidin-3-glucoside (purity ≥ 95%) was supplied from Extrasynthese (Genay Cedex, France).

The equipment used was: High performance liquid chromatography (HPLC), an Agilent 1200 series liquid chromatograph equipped with a G1362A refractive index detector

(RID), a G1315D diode array detector (DAD), a G1311A quaternary pump, a G1316A column oven, and a G1329A autosampler (Agilent Technologies, Santa Clara, CA, USA); a UV-Vis Helios Alpha™ spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA); a Heraeus™ Primo™ centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA); and an Entris II Series Analytical Weighing Balance (Sartorius, Goettingen, Germany).

Synthetic grape must solution buffer. A solution of 4 g/L of L-(+)-tartaric acid, a solution containing 100 g/L of D-glucose, 100 g/L of D-fructose adjusted to pH 3.5 with sodium hydroxide was used for all experiments.

Extracellular laccase production and enzymatic activity measurement. Active laccase extracts were obtained from the *Botrytis cinerea* isolate-213 strain following the methodology reported by Vignault *et al.* (2020). This laccase extract was treated with 0.16 g of PVPP/mL for 10 min and then centrifuged at 7500 rpm for 10 min. The supernatant was subsequently dialysed with 3.5 KDa cellulose membrane for 2 days in a 0.3 M ammonium formate solution and for 2 more days in distilled water. The laccase activity of this extract was determined using an adaptation of the syringaldazine test method (Grassin and Dubourdieu, 1986). The used purified laccase solution had exactly 100 UA of laccase activity/mL.

Colour measurements. Degradation of red colour (A520nm) and increasing of yellow colour (browning) (A420nm) of the samples were determined by spectrophotometry.

Anthocyanins quantification by HPLC. Delphinidin-3-*O*-monoglucoside, cyanidin-3-*O*-monoglucoside, petunidin-3-*O*-monoglucoside, peonidin-3-*O*-monoglucoside and malvidin-3-*O*-monoglucoside concentration were determined by reverse-phase HPLC analyses with an Agilent 1200 series liquid chromatograph (HPLC–diode array detection) using an Agilent Zorbax Eclipse XDB-C18, 4.6 × 250 mm 5-µm column (Agilent Technologies, Santa Clara, CA, USA), in accordance with the method described by Gil *et al.* (2012). The aim of this quantification was to estimate the possible losses of each anthocyanin due to enzymatic oxidation.

Anthocyanins quantification by spectrophotometry. Anthocyanins were determined using the adaptation of a method reported by Ribérau-Gayon and Stonestreet, 1965. A

sample of 70 µL was extracted from the original thermostated cuvette. A sample of 70 µL was extracted from the original thermostated cuvette. This sample was homogenised in an eppendorf with 70 µL of pure ethanol and 280 µL of 2.8% hydrochloric acid. Subsequently, 210 µL of this eppendorf was mixed with 83.7 µL of distilled water (eppendorf 1), while the other 210 µL was mixed with 83.7 µL of a 15% potassium metabisulfite solution (eppendorf 2), stirred and left to react for 10 min. Once the reaction time had passed, the absorbance of the two samples (eppendorf 1 and 2) was measured at 520 nm. Anthocyanin concentration was obtained from the difference between eppendorf 1 and 2 by multiplying the factor corresponding to the molar absorptivity coefficient of malvidin (Ribéreau-Gayon and Stonestreet, 1965) and the corrections corresponding to the applied dilution factor. In our experimental conditions the absorbance difference multiplied by 238.82 gives the total quantity of anthocyanins (mg/L).

Laccase degradation kinetics of individual anthocyanins. Solutions of the five anthocyanins were prepared at a concentration of 300 µM. The reaction mixture was prepared in a 1 mL spectrophotometer cuvette (ref: 7592 20, UV cuvette micro, BRAND®) mixing 600 µL of a stock solution of each of the anthocyanins (500 µM), 380 µL of synthetic grape must solution buffer and 20 µL of laccase solution. This reaction mixture therefore had an anthocyanin concentration of 300 µM and 2 UA of laccase activity/mL.

After mixing, the cuvettes were kept at 28oC throughout all the experiment. Absorbance at 520 nm was measured at 0, 1 and 2 hours. At exactly the same frequency (0, 1 and 2 hours), aliquots of 40 µL were extracted, and the reaction was stopped by adding 5 µL of sodium azide (10 mM). These aliquots were immediately used for an HPLC anthocyanin analysis.

Laccase degradation kinetics of a mixture of the 5 grape anthocyanins. A similar procedure to that reported in point 7 was performed using a mixture of the five anthocyanins at an individual concentration of 60 µM, representing a total anthocyanin concentration of 300 µM. In this case, samples were used for colour measurements and

HPLC analysis at 0, 1, 2, 6, 10 and 24 hours as in the previous experiment, in order to extend the laccase action time.

Study of the possible protective effect of seed tannins, (-)-epicatechin and glutathione on the laccase degradation kinetics of a mixture of the 5 grape anthocyanins. A similar procedure to that reported in point 8 was performed by adding or not adding seed tannin (200 mg/L), (-)-epicatechin (200 mg/L) or glutathione (20 mg/L). In addition to the measurement of the red colour (A520) and anthocyanin by HPLC, the absorbance at 420 nm (A420), indicative of browning, and an anthocyanin analysis by spectrophotometry were also carried out.

Statistical analysis. The data shown are the arithmetic means of triplicates with the standard deviation for each parameter. Two-way ANOVA Tukey comparison tests were carried out using the XLSTAT software (Addinsoft, Paris, France).

RESULTS AND DISCUSION

Laccase degradation kinetics of the five different grape anthocyanins. Figure 2 shows the degradation kinetics of the 5 different pure grape anthocyanins by the action of laccase from *Botrytis cinerea*.

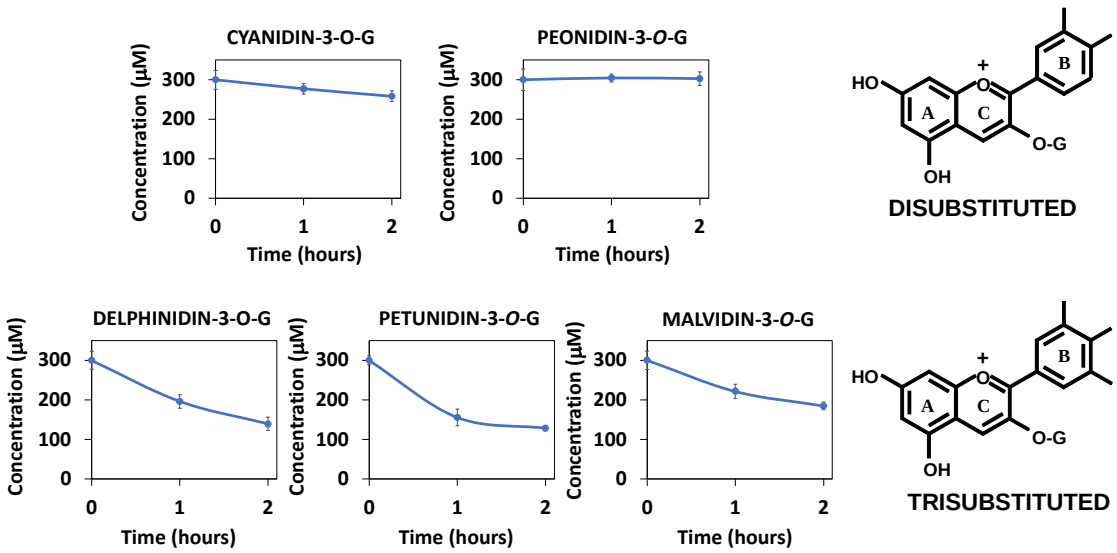

Figure 2. Degradation kinetics of the five different pure grape anthocyanins by the action of laccase determined by HPLC

Results are expressed as mean ± standard deviation of three replicates.

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As can be seen in the graphs, petunidin-3-O-glucoside and delphinidin-3-O-glucoside showed the maximal degradation rate, followed in descending order by malvidin-3-O-glucoside and cyanidin-3-O-glucoside. Surprisingly, peonidin-3-O-glucoside was not degraded by the action of laccase.

It is worth highlighting that the three anthocyanins with 3 substituents in the B-ring were degraded much faster than the two anthocyanins with 2 substituents, as the degradation rate of cyanidin-3-O-glucoside was much slower than that of petunidin, delphinidin and malvidin 3-O-glucosides, with penodin-3-O-glucoside appearing to be resistant to the laccase action. It seems therefore that the presence of the third substituent can favour the oxidation reactivity catalysed by laccase. This activation may be related to the fact that the third substituent, of the hydroxy or methoxy groups, could act as an electron donor which would induce the appearance of a delocalized negative charge in the B-ring.

Figure 3 shows the degradation of the red colour (A520nm) in the different anthocyanin solutions by the laccase action.

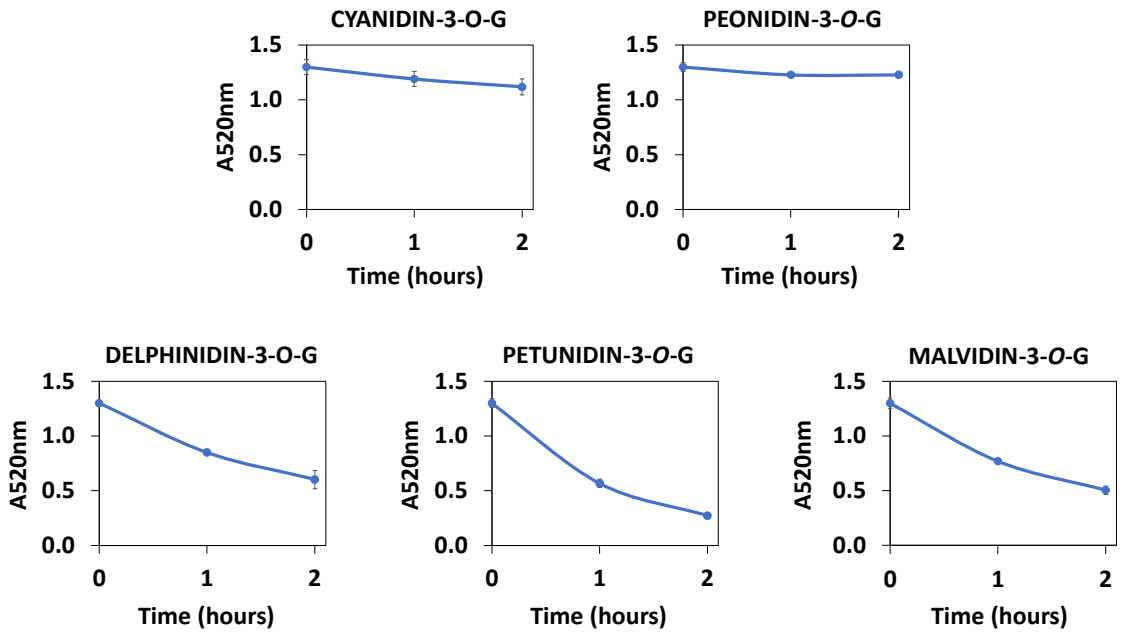

Figure 3. Degradation kinetics of red colour (A520nm) of the five different grape anthocyanins by the action of laccase

Results are expressed as mean ± standard deviation of three replicates.

In general, the red colour (A520) behaved similarly to that observed for the anthocyanin concentration, with the intensity of the red colour decreasing faster in the case of the petunidin-3-O-glucoside solutions, and followed in descending order by delphinidin-3-O-glucoside, malvidin-3-O-glucoside and cyanidin-3-O-glucoside. As was the case for the concentration of anthocyanins, the colour of the peonidin-3-O-glucoside solution seems to be resistant to the laccase action. The variations in colour of these solutions confirms that the presence of the third substituent makes the anthocyanin more sensitive to the degradation catalysed by laccase.

As the following images clearly illustrate, Figure 4 shows what happens to the final colour of the different anthocyanin solutions after 24 hours of laccase action. These photographs were taken at the beginning of the experiment and after 24 hours to emphasise the colour differences and make them visible to the human eye.

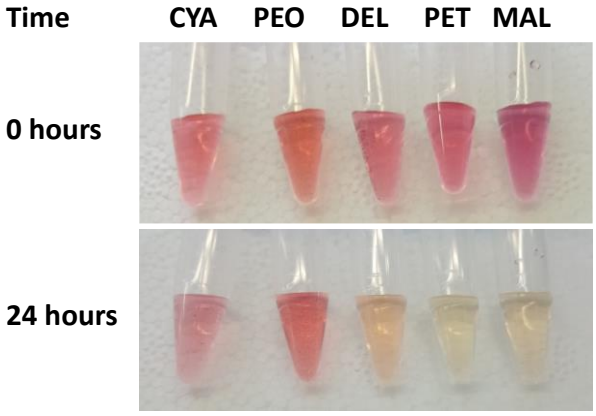

Figure 4. Visual aspect of the five different grape anthocyanins (300 mM) at the beginning of the experiment and after 24 hours of laccase action.

CYA: Cyanidin-3-O-G; **PEO**: Peonidin-3-O-G; **DEL**: Delphinidin-3-O-G; **PET**: Petundin-3-O-G; **MAL**: Malvidin-3-O-G.

It is clear that the solutions of the three anthocyanins with three substituents, petunidin, delphinidin and malvidin 3-O-glucosides have almost completely lost their red colour whereas the cyanidin-3-O-glucoside still retains some. However, the colour of the peonidin-3-O-glucoside solution is virtually the same as it was at the beginning. This image visually confirms what was observed by HPLC and by spectrophotometry.

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Laccase degradation kinetics of an equimolar mixture of the five grape anthocyanins.

Figure 5 shows the degradation kinetics of an equimolar mixture of cyanidin, peonidin, delphinidin, petunidin and malvidin 3-O-glucosides.

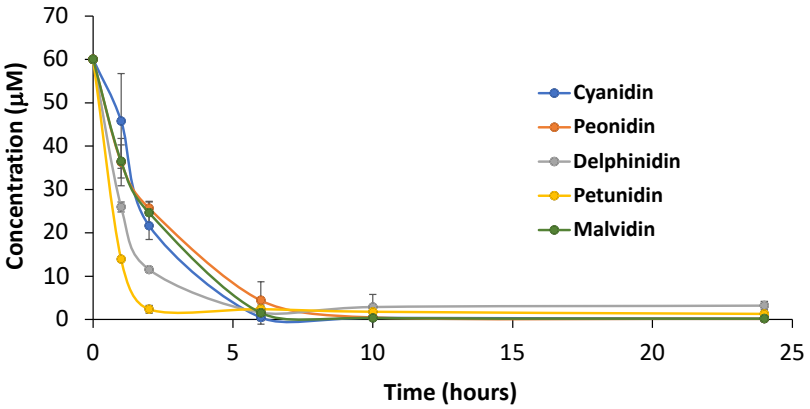

Figure 5. Laccase degradation kinetics of an equimolar mixture of the five grape anthocyanins

Results are expressed as mean ± standard deviation of three replicates.

This figure shows that all anthocyanins, even peonidin, are degraded by the laccase action. Under these conditions, petunidin-3-O-glucoside degrades the fastest, followed by delphinidin-3-O-glucoside, while the other three anthocyanins – malvidin, cyanidin and peonidin 3-O-glucosides – are degraded more gradually. It must be highlighted that the differences observed in the degradation rate between the different anthocyanins when they are in a mixture are smaller than when they are not mixed, although petunidin and delphinidin are still degraded the most quickly. This may be due to the fact that after the primary quinones' initial formation, chemical polymerisation occurs with other phenols without the need for the laccase action (Queiroz *et al.,* 2008; Oliveira *et al.,* 2011). Consequently, the less reactive anthocyanins such as peonidin and cyanidin 3-O-glucosides can be used to form polymers without the laccase action. This is probably the reason why peonidin-3-O-glucoside cannot be degraded by laccase when it is alone, whereas it can be degraded in the presence of other more reactive anthocyanins. This effect would also probably occur in the presence of other phenols which could generate insoluble polymers that, when precipitated, would cause oxidasic haze.

Influence of the supplementation with seed tannin, (-)-epicatechin or glutathione on the degradation kinetics of an equimolar mixture of the five grape anthocyanins

Figure 6 shows the effect of the supplementation with seed tannin, (-)-epicatechin or glutathione on the degradation kinetics of an equimolar mixture of the five grape anthocyanins.

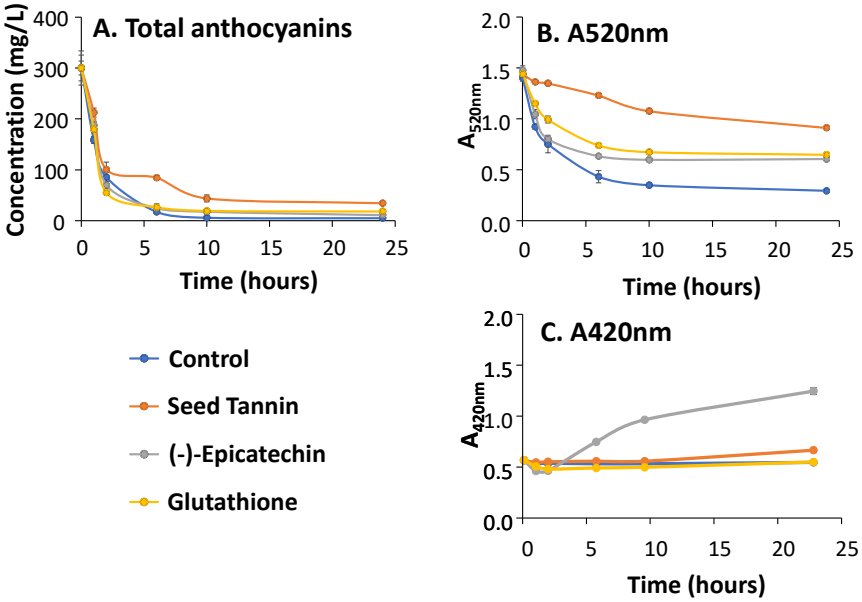

Figure 6. Influence of the supplementation with seed tannins, (-)-Epicatechin or glutathione on the degradation kinetics of a mixture of the five different grape anthocyanins by the action of laccase

Results are expressed as mean ± standard deviation of three replicates.

As shown in Figure 6.A, no great differences in the degradation rate of total anthocyanins were observed between the control conditions and when the solution was supplemented with (-)-epicatechin and glutathione. In contrast, the supplementation with seed tannin seems to slow down the degradation rate of total anthocyanins to some extent. This apparent reduction in the degradation rate can be attributed to the proven laccase inhibitory effect of oenological tannins (Vignault *et al.,* 2019; Vignault *et al.,* 2020).

Figure 6.B shows the changes in the red colour (A520nm) of the various anthocyanin solutions which were either supplemented or not with the different substances. In this

case, the degradation of the red colour was clearly slower when the solution was supplemented with (-)-epicatechin and glutathione, and especially with seed tannins. These data indicate that the concentration of total anthocyanins determined by HPLC does not match the changes in the red colour since the differences in colour in this case are much more evident.

The explanation for why there is more colour in the case of the samples supplemented with seed tannins and (-)-epicatechin than that which would justify the remaining concentration of anthocyanins may be associated with the formation of new pigments that retain the red colour but are not detected by HPLC. In the case of glutathione, the results are more difficult to interpret. However, it has been shown that glutathione can react with the orthodiquinones formed by the action of laccase, and that these reconstitute the original orthodiphenols, thereby avoiding the formation of brown pigments (Cheynier *et al.* 1995; Robards *et al.* 1999). Consequently, it might be possible for glutathione to react with the initial anthocyanin degradation products of laccase action to reconstitute adducts between anthocyanins and glutathione. While these cannot be detected by HPLC, they do contribute to the red colour.

Figure 6.C shows the changes in yellow colour (A420nm), indicative of browning, of the various anthocyanin solutions which were supplemented or not with the different substances. No great differences were observed between the control samples and those supplemented with seed tannins or glutathione. In contrast, the sample supplemented with (-)-epicatechin showed a high increase in the absorbance at 420 nm. (-)-Epicatechin has been identified as a very good substrate for laccase (Giménez *et al.,* 2022). Consequently, this data seems to indicate that laccase, in addition to catalysing the degradation of anthocyanins, also oxidises epicatechin, which leads to the well-known browning process (Rigaud *et al.* 1991; Singleton *et al.* 1995).

Figure 7 illustrates the changes after 24 hours in the colour of the various anthocyanin solutions supplemented or not with the different substances in the presence or not of 2 UA laccase/mL.

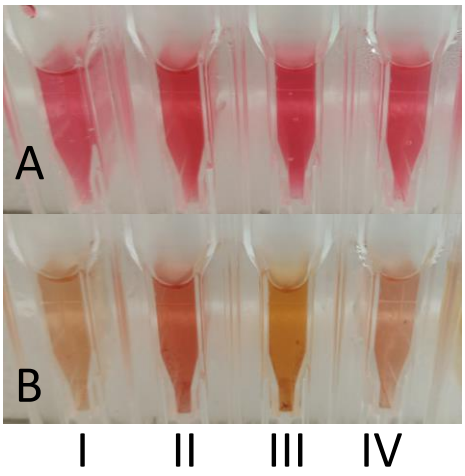

Figure 7. Visual aspect of anthocyanin samples after 24 hours in the absence or presence of 2 UA of laccase activity/mL

A: Without Laccase; **B**: With laccase; **I**: Control; **II**: seed tannins, **III**: (-)-epicatechin; **IV**: glutathione

This picture visually demonstrates what has been previously explained and confirms that glutathione, and especially seed tannins, exert a protective effect on the red colour against laccase action. The (-)-epicatechin also protects the red colour slightly but the browning induced by its presence predominates.

With the aim of verifying if new pigments were formed in the presence of seed tannins, (-)-epicatechin or glutathione, a spectrophotometric analysis of anthocyanins in the different samples was performed. Figure 8 shows the results.

III. Results

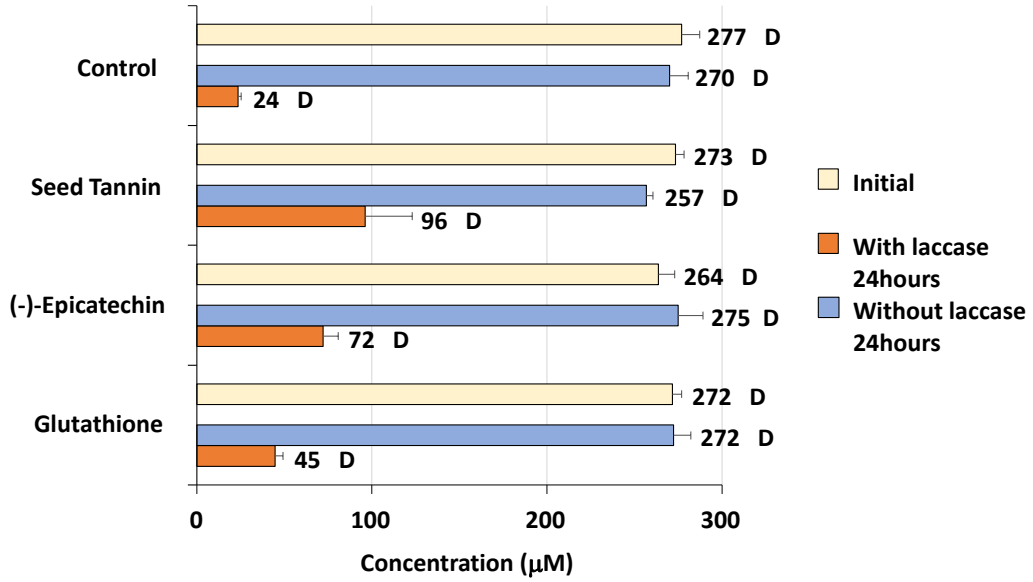

Figure 8. Influence of the supplementation with seed tannins, (-)-epicatechin or glutathione on initial and final anthocyanin concentration analysed by spectrophotometry.

All data are expressed as the arithmetic mean of 3 replicates ± standard deviation. Different letters indicate statistically significant differences ($p < 0.05$) between the samples.

The results indicate that in the absence of laccase there are no significant differences between the initial concentration of total anthocyanins and that obtained after 24 hours.

As expected, the presence of laccase caused a drastic reduction in the total anthocyanin concentration in all the experimental groups. However, the total values of anthocyanins remaining after 24 hours of laccase reaction determined by spectrophotometry show that the supplementation with 200 mg of seed tannin/L caused a 30% inhibition of anthocyanidin degradation. This inhibitory effect was 24% when the solution was supplemented with 200 mg/L of (-)-epicatechin and 10% when it was supplemented with 20 mg/L of glutathione. It should be stressed that 20 mg/L is the maximum legal dose authorised by the OIV for this antioxidant (OIV, 2021). These differences, which were not so clearly evident when anthocyanins were analysed by HPLC, seems to indicate that new red pigments were formed in the presence of these substances.

CONCLUSIONS

In this study, we analysed the degradation kinetics of anthocyanins by laccase from the Botrytis cinerea grape in three scenarios. The first scenario was a synthetic grape must using each one of the five grape anthocyanins separately, while the second was in a similar matrix but containing an equimolar of the five grape anthocyanins with the aim of getting closer to the real grape must conditions. Finally, the third scenario was a replication of the second scenario but with the supplementation of three possible protectors: seed tannin, (-)-epicatechin and glutathione.

Our results show that the three anthocyanins with three substituents on the B-ring are more sensitive to the laccase action than those with only two when they are alone in the matrix. Even so, peonidin seems to be non-reactive to the laccase action in these conditions. These data could perhaps indicate that the varieties rich in peonidin-3-O-glucoside such as Nebbiolo (Rolle *et al.,* 2012) are more resistant to the colour degradation caused by laccase. However, when all the anthocyanins were present in the matrix, peonidin was also degraded probably because peonidin-3-O-glucoside can polymerise with the quinones formed by the oxidation of other anthocyanins or phenols (Queiroz *et al.,* 2008; Oliveira *et al.,* 2011). Consequently, the advantage of using varieties rich in peonidin-3-O-glucoside would not be as significant under real grape must conditions where many other phenols are present.

Our results also confirm that supplementation with seed tannin is effective to prevent oxidasic haze, even at high levels of laccase activity, since the red colour is clearly protected and the total anthocyanin concentration, as determined by spectrophotometry, is significantly higher than in the control conditions. These data suggest the formation of new red pigments is not detectable by HPLC.

On the other hand, the supplementation with (-)-epicatechin also seems to protect the red colour and anthocyanin concentration, as determined by spectrophotometry, but to a lesser extent than seed tannins. It appears therefore that its presence also favours the formation of new red pigments, however despite being a very good substrate for laccase, it also causes very intense browning, a point which must be taken into consideration.

Finally, supplementation with glutathione also protected the red colour and anthocyanins determined by spectrophotometry but in this case the mechanism is expected to be different to that of seed tannins and (-)-epicatechin since it is not a flavanol that can react with anthocyanidins to form new red pigments.

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IV. GENERAL DISCUSSION

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The main research line of this work was to study of the mechanisms of the enzymatic oxidation of the grape must. When the enzymatic oxidation occurs the color in the grape must is affected. This investigation is especially focused on the main effects of grape must enzymatic oxidation. This is therefore a study about the mechanisms of laccase browning and oxidasic haze.

With the aim of performing this study, a model using a synthetic grape must is proposed. This synthetic grape must was used in order to study the mechanism of action of *Botrytis cinerea* laccase for evaluating the main compounds implied on the browning process.

The used synthetic grape must was so simple as possible to minimize the possible interferences of other grape must compounds that would modify the enzymatic browning results. This synthetic grape must was a buffer containing tartaric acid (4 g/L) and 100 g/L of D-glucose and 100 g/L of D-Fructose. This media was supplemented with 2 UA of laccase/mL and with different concentrations of five substrates for laccase oxidation: one monophenol (4-hydroxybenzoic acid), three diphenols (caftaric acid, (+)-catechin and (-)-epicatechin) and one triphenol (gallic acid).

This experiment was performed using different substrate concentrations in order to determine the kinetic constants (Vmax and $K_{0.5}$) using the classical Michaelis-Menten kinetic model. The Vmax was maximal for (+)-catechin, followed in decreasing order by (-)-epicatechin, caftaric acid, gallic acid, and 4-hydroxybenzoic acid which was almost not oxidized. This data indicates that diphenols are better substrate for laccase oxidation followed by triphenols and by monophenols that are nearly unaffected. Considering that (+)-catechin, (-)-epicatechin and caftaric acid, all of them are orthodiphenols but with different structure, seems that the greater reactivity in front of laccase could be related with the molecular resonance and the capability to go to their own semiorthoquinone and then to orthoquinone. Another subject to be considered is that the laccase kinetics was apparently allosteric. However, it must be considered that all the measurements were performed using the absorbance at 420 nm and consequently this allosteric behavior not necessarily must be related with the action of the enzyme since it must be

also due to the subsequent chemical reactions of polymerization that occurs once the orthodiquinones are formed.

The results also indicated that small concentrations of ethanol (5%) activated the enzyme. This data indicates that in real conditions of grapes infected by *Botrytis cinerea* the oxidative action of laccase is favored since rotten grapes have small amounts of ethanol because fermentation can be started in infected grapes. On the other hand, the optimal pH of laccase seems to be around 5.0-6.0 although the enzyme remains very active at grape must pH (3.0-4.0).

This synthetic grape must model for studying browning was also used to determine de influence of sulfur dioxide, ascorbic acid and glutathione. The results indicate that as expected sulfur dioxide is a powerful inhibitor of laccase since the Vmax decrease in its presence whereas the $k_{0.5}$ increased which indicates that this additive really decrease the rate of phenol oxidation by the action of laccase and also decrease the affinity of the enzyme versus the substrate.

Supplementation with ascorbic acid also inhibit the intensity of browning since Vmax is reduced by its presence. It should be noted that in the Hill's Plot, all the regression lines cut the abscissa axis at the same intersection point but show different slopes. This behavior would indicate, in a Lineweaver-Burk plot, that inhibition is of non-competitive type. Since the enzyme showed an allosteric comportment, the representation to obtain kinetic parameters was the Hill's plot but given the similarities it could be considered that the inhibition is of non-competitive type. In that case, the value of $K_{0.5}$ remained constant probably because ascorbic acid acts reducing the availability of one of the laccase substrates (oxygen) and does not directly inhibit the enzyme. Moreover, it must be considered that ascorbic acid cannot be used without the presence of sulfur dioxide since it produces hydrogen peroxide that can oxidize even more than oxygen. Consequently, ascorbic acid can be considered as a complement but not an alternative to sulfur dioxide.

Supplementation with glutathione also inhibits the intensity of browning since its presence reduces the Vmax. In that case the Hill's Plot show that all the regression lines

are parallel. Considering the similarities between Lineweaver-Burk and Hill's plots, this behavior would indicate that the inhibition is of uncompetitive type.

In any case, the presence of glutathione increased the values of $k_{0.5}$ which indicated that the affinity of laccase for its substrate decrease in the presence of this additive.

In synthesis, all this data indicates that as expected sulfur dioxide and ascorbic acid are powerful inhibitors of laccase, and that glutathione is a very interesting alternative tool to sulfur dioxide to prevent browning. It should be noted that actually OIV authorize only a dose of 20 mg of glutathione/L and that the European Union has not yet authorized its use. Our results suggest that glutathione is effective enough that it should be completely authorized and even the maximal doses could be augmented.

With the aim of clarifying the influence of all compounds of real grape must, a new approach was proposed using a grape must diluted at 20% with a buffer. The reason of this dilution was to uniformize a bit the possible differences between the grape musts used in the different days of work and also to make it possible the measurement of the oxygen consumption rate that in undiluted grape must was really too high.

In that case, the diluted grape must was supplemented or not with laccase, sulfur dioxide, ascorbic acid, glutathione, a specific inactivated dry yeast rich on glutathione and with *Metschnikowia pulcherrima*. The combinations of sulfur dioxide with all the other conditions were also studied.

The results concerning the oxygen consumption kinetics showed that the supplementation with laccase increased the oxygen consumption rate (OCR) and as expected supplementation with sulfur dioxide, alone or in combination with other agents, reduced it drastically. In contrast, ascorbic acid and *Metschnikowia pulcherrima* increased the OCR whereas glutathione reduced the OCR but in a lesser extent than sulfur dioxide.

In relation to browning intensity, the presence of laccase significantly increased the absorbance at 420 nm (A420nm) and the blue-yellow CieL*a*b* coordinate (b*) which indicates, as it was expected, a higher browning caused by the presence of this enzyme.

IV. General discussion

Sulfur dioxide, alone or combined with other agents, showed a powerful effect on the laccase inhibition since the values of A420nm and b* remained very low in its presence. Supplementation with glutathione, a specific inactivated dry yeast rich in glutathione and *Metschnikowia pulcherrima* also significantly reduced the intensity of browning (A420nm and b*) at levels somewhat higher than those of sulfur dioxide but low enough to cannot be distinguished by the human eye (ΔEa*b*) at least in the absence of laccase. Unfortunately, in the presence of laccase these antioxidant agents are not effective enough to prevent completely browning although significantly reduced the values of A420nm and b*.

The mechanism by which glutathione, pure or in form of a specific inactivated dry yeast, protects against enzymatic browning and reduces oxygen consumption is probably its capacity to combine with the orthodiquinones formed by the action of the polyphenol oxidases which impedes therefore the browning process and depletes the medium on substrates for these enzymes. In contrast, *Metschnikowia pulcherrima* also reduced browning intensity but its action mechanism is different from that of glutathione. This non-*Saccharomyces* yeast protects because it consumes oxygen very efficiently, and therefore reduces its availability for the polyphenol oxidases.

Consequently, this data confirms that glutathione, both pure and in the form of inactivated yeasts, and *Metschnikowia pulcherrima* really protect against browning. Consequently, both treatments can be considered as interesting tools for protecting grape must against browning, and thus reducing the use of sulfur dioxide, at least in conditions of healthy grapes.

The kinetics of oxidative laccase degradation of the five natural grape anthocyanins, cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside and malvidin-3-*O*-glucoside was measured in a synthetic medium in order to determine the affinity of this enzyme for each one of them. This study has the interest of verifying how the presence of different substituents in the B-ring of anthocyanins affects the affinity of laccase.

The results indicate that the three anthocyanins with three substituents in the B-ring are faster degraded than the two anthocyanins with only two substituents. In fact, the

decreasing order in the degradation kinetics was as follows: petunidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside and finally peonidin-3-*O*-glucoside that was really insensible to the action of laccase when it was the only phenolic substance in the media. The degradation of the red colour of the solutions with the different anthocyanins showed a similar behavior than that observed in the HPLC analyses of them.

When an equimolar solution of the five anthocyanins was used in a similar experiment, the differences in the degradation kinetics of each one of the anthocyanins seems to be damped although petunidin and delphinidin continue to be the faster degraded. In fact, all anthocyanins were nearly completely degraded at the end of the experience, even peonidin-3-*O*-glucoside that was not reactive when it was alone. This data should be probably due to the fact that, after the initial formation of the primary quinones, chemical polymerization occurs with other phenols without the need for the action of laccase. Consequently, the less reactive anthocyanins, such as peonidin and cyanidin 3-*O*-glucosides, can be used to form polymers without the action of laccase. That is probably the reason because peonidin-3-*O*-glucosie cannot be degraded by laccase when it is alone whereas can be degraded in the presence of other more reactive anthocyanins. This effect would probably also occur in the presence of other phenols, which could generate insoluble polymers that, when precipitated, would cause oxidasic haze.

The hypothetic protective effect of seed tannins, (-)-epicatechin and glutathione was analyzed by supplementing the equimolar anthocyanin solution with these substances. The results indicate that seed tannins seem to protect the degradation of the anthocyanins considering the HPLC analyses of them. In contrast, neither (-)-epicatechin not glutathione affected the degradation kinetics when comparing with the control. However, the analysis of the red colour of these samples did not match with the observed degradation of anthocyanins using HPLC since the color of the samples supplemented with (-)-epicatechin, glutathione and especially with seed tannins showed higher colour intensity than the control. The analysis of the anthocyanins of this solution by spectrophotometry indicates that the sample supplemented with glutathione has 10 %, the sample supplemented with (-)-epicatechin has 24 % and finally

seed tannins has 30 % more anthocyanins than the control. This data seems to indicate that new red pigments, non-detectable in our HPLC analysis conditions, were formed which would justify the reason because these samples have higher red colour intensity than the control.

These results indicate therefore that supplementation with seed tannin is effective to prevent oxidasic haze, even at high levels of laccase activity, since the red colour is clearly protected and the total anthocyanin concentration, determined by spectrophotometry, is significantly higher than in the control conditions. This data confirms therefore that oenological tannins, at least seed tannins, can be used in winemaking to prevent the damage caused by *Botrytis cinerea* infection as it has been proposed by other authors.

The supplementation with (-)-epicatechin seems also protect the red colour and anthocyanin concentration, determined by spectrophotometry, but in a lesser extent than seed tannins. It seems therefore that its presence also favors the formation of new red pigments. Nevertheless, it must be considered that the presence of (-)-epicatechin also caused a very intense browning because this substance is a very good substrate for laccase. Consequently, it seems that flavanol monomers are more susceptible to browning than to form new red pigments in the presence of laccase.

Finally, supplementation with glutathione also protected red colour and anthocyanins determined by spectrophotometry but in that case the mechanism should be different than that of seed tannins and (-)-epicatechin since it is not a flavanol that can react with anthocyanidins to form new red pigments. These results are therefore more difficult to interpret. However, it has been described that glutathione can react with the orthodiquinones, formed by the action of laccase, to reconstitute the original orthodiphenols, and by this way, avoid the formation of brown pigments. Consequently, it would be possible to hypothesize that glutathione could react with the initial anthocyanin degradation products of laccase action to reconstitute adducts between anthocyanins and glutathione that cannot be detected by HPLC but that contribute to red colour.

V. CONCLUSIONS

V. Conclusions

CONCLUSIONS

1. A synthetic model to measure browning caused by laccase has been proposed. Using this model, it has been shown that diphenols are more sensible to the oxidation caused by laccase than triphenols, and that monophenols are practically not oxidized.

2. The Michaelis-Menten kinetic model and the application of the Hill's Plot indicated that the obtained maximal laccase degradation Vmax was for (+)-catechin followed in decreasing order by (-)-epicatechin, caftaric acid, gallic acid and finally 4-hydroxybenzoic acid that was practically not affected by the action of laccase.

3. The presence of ethanol at low concentration seems to increase the laccase activity.

4. The optimal pH of laccase from *Botrytis cinerea* is between 5.0 and 6.0, but it remains quite active at wine pH (3.0-4.0)

5. The supplementation with sulfur dioxide reduced significantly the Vmax and increased the $K_{0.5}$ of laccase in front of (-)-epicatechin which confirms that sulfur dioxide is a powerful inhibitor of laccase and that its presence not only prevent browning but it reduces the affinity of the enzyme versus its substrate as well.

6. Supplementation with ascorbic acid also reduces Vmax but not affect the $K_{0.5}$ of laccase in front of (-)-epicatechin. It seems therefore that its inhibitory action is related to the competence for oxygen and not by a direct enzyme inhibition.

7. Glutathione seems to be a very effective browning inhibitor since significantly protects against (-)-epicatechin laccase oxidation.

V. Conclusions

8. The use of 20 % diluted grape must seems to be a more real model since it consumes oxygen and develops browning in our experimental conditions.

9. The presence of laccase in the diluted grape must increase the oxygen consumption rate and the browning intensity.

10. Sulfur dioxide exerts a powerful laccase inhibitory effect in the diluted grape must since reduces drastically the oxygen consumption rate and also the browning intensity.

11. Supplementation with ascorbic acid increases the oxygen consumption rate but also the intensity of browning probably by the production of hydrogen peroxide. Consequently, it can be considered a complement to sulfur dioxide but never as an alternative.

12. Glutathione, both pure and in form of a specific inactivated dry yeast also prevents effectively browning in the absence of laccase. Unfortunately, at the authorized doses is not effective enough in the presence of laccase, although can helps to diminish browning.

13. The use of *Metschnikowia pulcherrima* seems to be also effective to prevent browning in the absence of laccase. It seems that it consumes oxygen quickly avoiding its availability for laccase. Unfortunately, is not effective enough in the presence of laccase, although can helps to diminish browning.

14. The analysis of the degradation kinetics caused by laccase of the five natural grape anthocyanins shows that the three anthocyanins with 3 substituents in the B-ring are faster degraded than the 2 anthocyanins with only 2 substituents. More specifically, petunidin-3-*O*-glucoside the one that is most rapidly degraded followed in decreasing order by delphinidin, malvidin, cyanidin 3-*O*-glucosides and finally peonidin-3-*O*-glucoside was not affected by the action of the enzyme.

15. When the degradation kinetics was studied with an equimolar mixture of the five anthocyanins all of them, even peonidin were degraded by the laccase action. In these conditions the faster degradation is also that of petunidin-3-*O*-glucoside followed by delphinidin-3-*O*-glucoside whereas the other three anthocyanins, malvidin, cyanidin and peonidin 3-*O*-glucosides are degraded more gradually.

16. This different kinetic behavior of the five anthocyanins when they are alone or in a equimolar mixture may be probably due to the fact that, after the initial formation of the primary quinones, chemical polymerization occurs with other phenols without the need for the action of laccase. Consequently, the less reactive anthocyanins, such as peonidin and cyanidin 3-*O*-glucosides, can be used to form polymers without the action of laccase. This effect would probably also occur in the presence of other phenols, which could generate insoluble polymers that, when precipitated, would cause oxidasic haze.

17. The results obtained by HPLC indicate that the supplementation with (-)-epicatechin or glutathione did not affect the degradation kinetics of anthocyanins. In contrast, the supplementation with seed tannins seems to slow down the anthocyanin degradation confirming its previously described protective effect.

18. The degradation kinetics of the red colour (A520nm) indicate that supplementation with (-)-epicatechin, glutathione and especially with seed tannins really protect the red colour of the anthocyanin solutions. This data does not match with the observed HPLC analytical concentration of the anthocyanins which suggest that some of these anthocyanins have been transformed in new red pigments that cannot be determined under our HPLC analytical conditions.

19. The analysis of anthocyanins by spectrophotometry seems to confirm that the supplementation with (-)-epicatechin, glutathione and especially with seed tannins really protects the red colour of the anthocyanins solution since in the

presence of these additives the anthocyanin concentration is significantly higher than in control conditions.

20. The protective effect of (-)-epicatechin and especially of seed tannins seems to be related with the formation of new red pigments that are not detectable under our HPLC analytical conditions. In contrast, the mechanism of the protective effect of glutathione should be different than that of seed tannins and (-)-epicatechin since it is not a flavanol that can react with anthocyanidins to form new red pigments.

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