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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.

1. 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 et al., 2008) to form quinones. These quinones and their derivatives are subsequently polymerized through several reactions, forming brown pigments known as melanins (Claus et al., 2014; Oliveira et al., 2011; Queiroz et al., 2008). 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 et al., 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 et al., 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 et al., 2013). Hill et al. (2019) have reported that this fungus

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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, 2012; Lleixà et al., 2018), problems of settling and filtration (Villettaz et al., 1984; Jadhav & Gupta, 2016), presence of ochratoxin A (Ponsone et al., 2012; Valero et al., 2008), mouldy odors (La Guerche et al., 2007; Steel et al., 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 et al., 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, 2020) and, more recently, to use reduced glutathione (Kritzinger et al., 2013; Zimdars, 2020) or inactivated dry yeasts rich in glutathione (Bahut et al., 2020; Gabrielli et al., 2017). 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 taken into account 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 et al., 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 et al., 2013). Thus GSH traps the orthoquinones in a colorless form and as a result the formation of brown polymers is limited (Singleton, Salgues, Zaya, & Trousdale, 1985a, 1985b; Kritzinger et al., 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 et al., 1995), L-3,4-dihydroxyphenylalanine (DOPA) (Saiya-Cork et al., 2002; Eichlerová et al., 2012), 3,30-dimethylaminobenzoic acid (DMAB) (Matsumura et al., 1987), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Prasetyo et al., 2009), pirocatecol (Perucci et al., 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 et al., 2012) and syringaldazine (Harkin & Obst, 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 taken into account 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.

2. 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 et al., 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 °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 min at 8500 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 min (including a time measurement at 0 min). 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.

2.1. Browning measurements

Volumes of 0, 50, 100, 150, 200, 250, 300, 350 and 400 μL 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 μL 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 min. The slope of the regression straight line was determined in order to express the intensity of browning. All the experiments were performed in triplicate.

2.2. 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 min.

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. The comparison between categorical variables was carried out using one-factor analysis of variance (ANOVA). The comparison between continuous variables was performed using multidimensional analysis of the Mahalanobis distance (Bedrick et al., 2000). Both statistical analyses were carried out using the XLSTAT 2017 statistical package.

3. Results and discussion

Kinetic parameters of *Botrytis cinerea* laccase browning for different substrates. Fig. 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.

It could be considered unexpected that caftaric acid, which is usually seen as one of the main substrates for laccase browning (Cheynier, Trousdale & Singleton, 1986; Singleton et al., 1985a, 1985b; 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° and −31.37° 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.

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

Fig. 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,

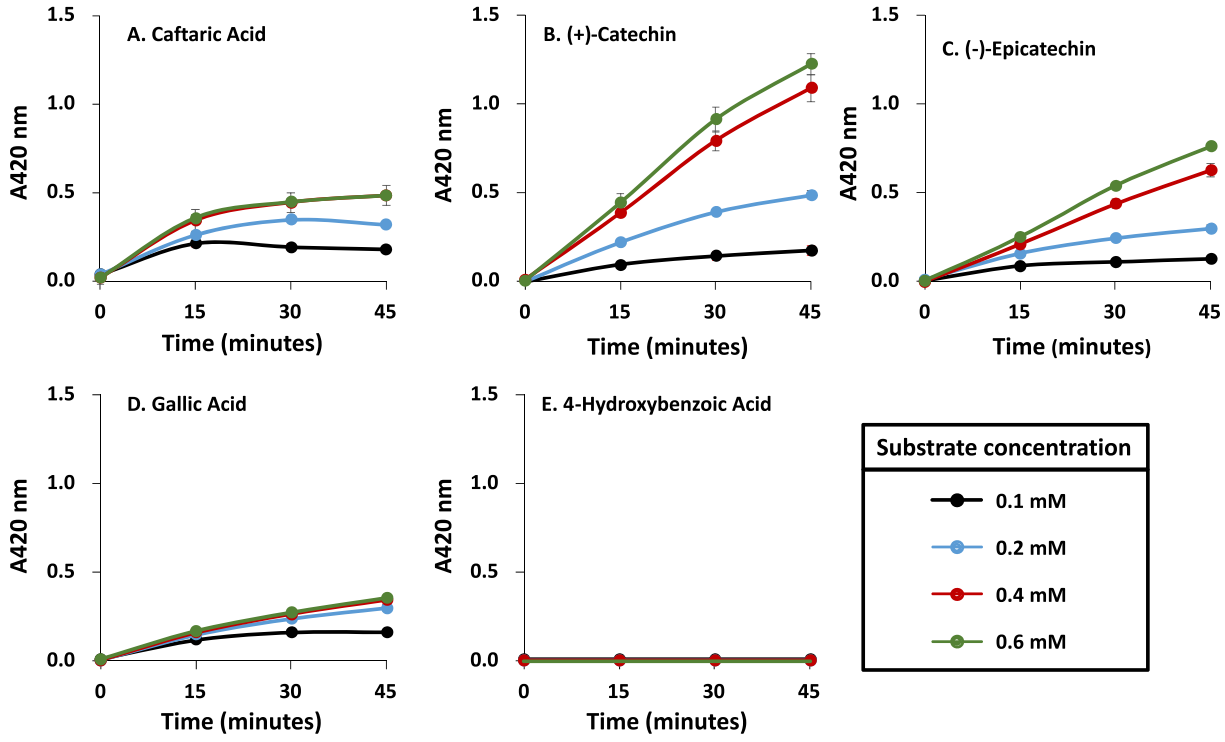

Fig. 1. Influence of the type of substrate on the laccase browning kinetics.

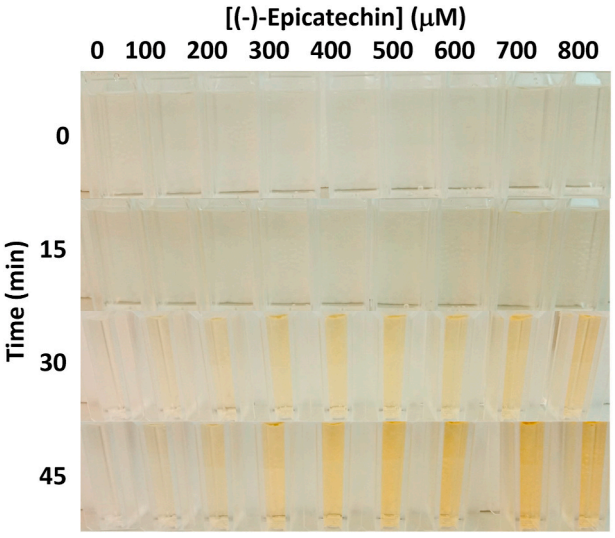

Fig. 2. Yellow color development of the synthetic grape juice model solution as a function of (−)-epicatechin concentration and reaction time. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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 taken into account 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 (Oliveira et al., 2011; Queiroz et al., 2008). 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 et al., 2002; Enaud et al., 2011; Maurya et al., 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). Fig. 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 Fig. 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

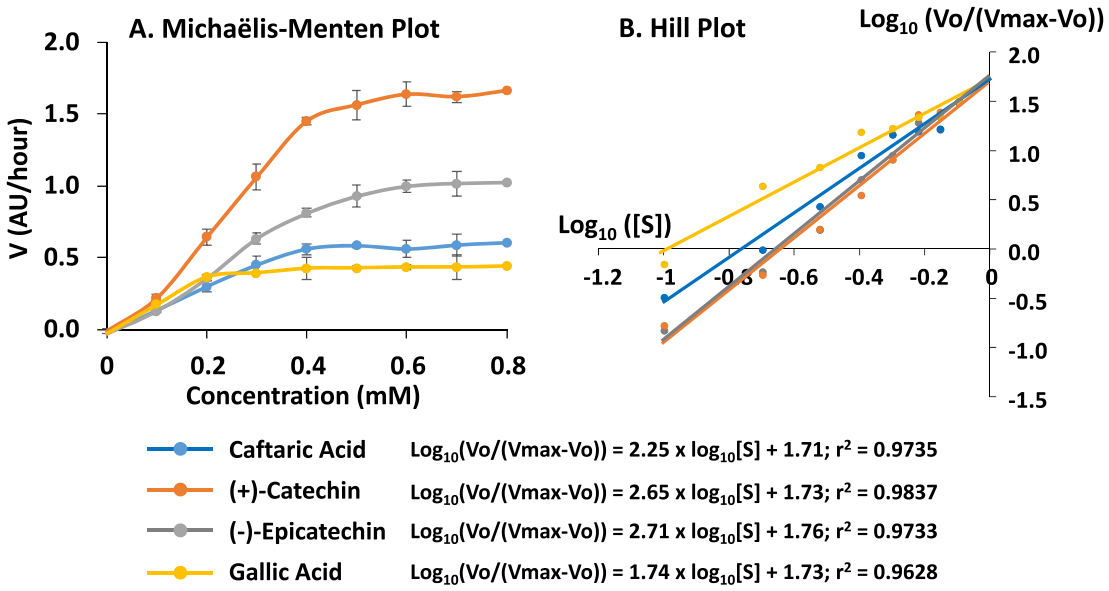

Fig. 3. Michaëlis-Menten and Hill plots of different substrate.

Table 1
Kinetic constants of laccase browning for the different substrates.

Substrate	Phenol type	Vmax (AU420/ hour)	K0.5 (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 ($p < 0.05$).

lower values. These data indicate that laccase browning shows positive cooperativity for all the substrates, since the Hill number was higher than 1 in all cases.

Influence of pH and ethanol concentration on the kinetic parameters of *Botrytis cinerea* laccase browning. Fig. 4 shows the influence of pH on the laccase browning kinetics using only (−)-epicatechin as substrate. The Michaëlis-Menten plot (Fig. 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 from 3.5 up to 5.5 (Dubernet et al., 1977; Fortina et al., 1996; Slomczynski & Nakas, 1995; 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).

Fig. 4B shows the Hill plot of laccase browning for (−)-epicatechin at

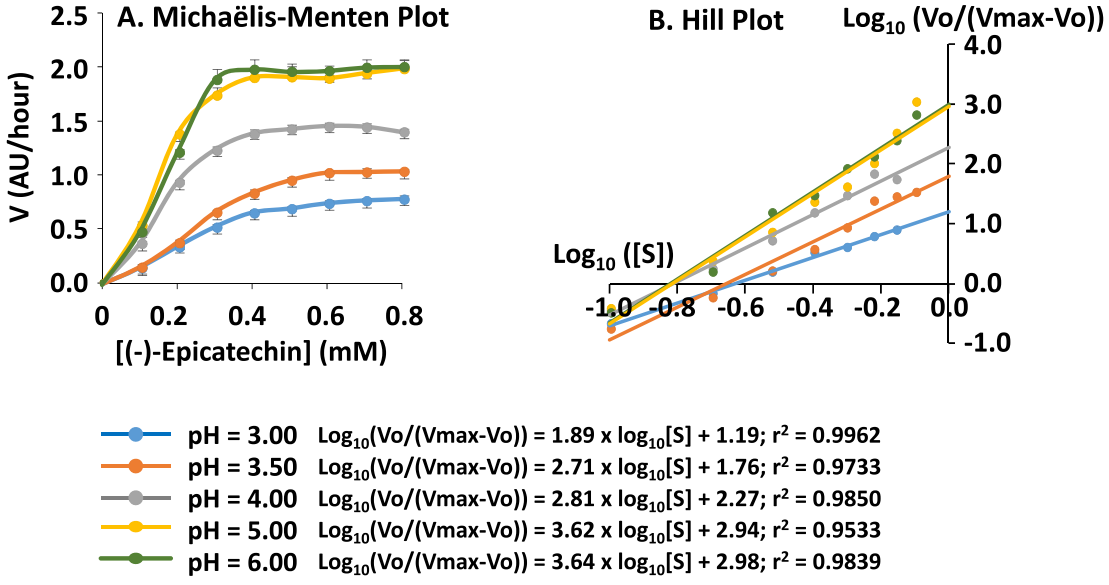

Fig. 4. Influence of pH on laccase browning kinetics of the synthetic grape juice model solution using (−)-epicatechin as substrate.

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.

Fig. 5 shows how the presence of ethanol affects the laccase browning kinetics using only (−)-epicatechin as substrate. The Michaëlis-Menten plot (Fig. 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 (Table 3). 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. Fig. 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.

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.

Fig. 6 shows the influence of different doses of SO_2 on laccase browning. The Michaëlis-Menten plot (Fig. 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).

Fig. 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.

Data from Fig. 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.

Fig. 7 shows how the presence of ascorbic acid affects laccase browning. The Michaëlis-Menten plot (Fig. 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 (Fig. 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%. 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 et al., 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.

Fig. 8 shows the influence of different doses of glutathione on laccase browning. The Michaëlis-Menten plot (Fig. 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 et al., 2013; Zimdars, 2020; Bahut et al., 2020).

The corresponding Hill plot (Fig. 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).

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

Table 2
Influence of pH on the Kinetic constants of laccase browning.

pH	Vmax (AU420/hour)	K0.5 (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
p-value	<0,0001	<0,0001	<0,0001

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

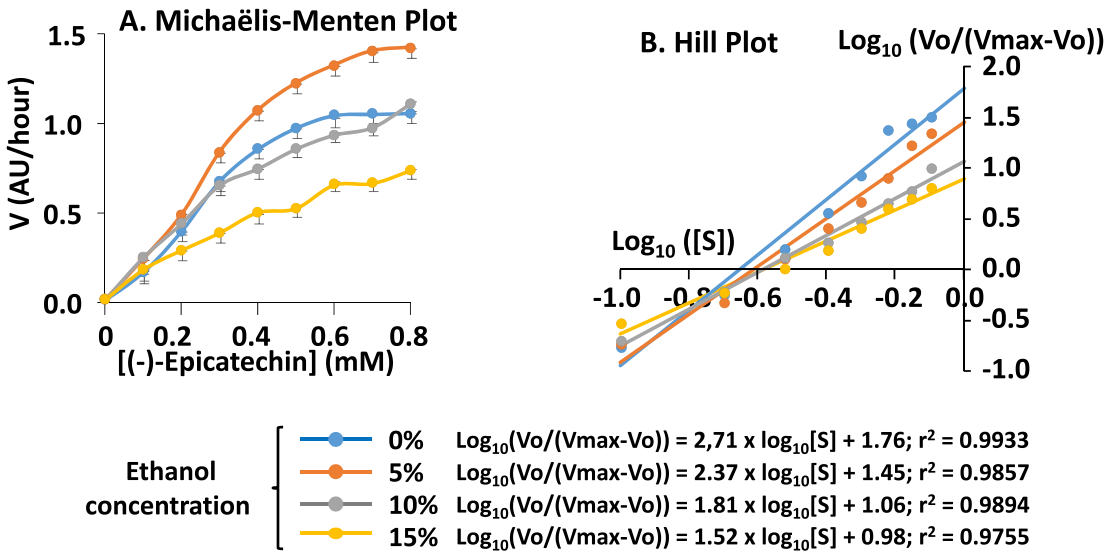

Fig. 5. Influence of ethanol on laccase browning kinetics of the synthetic grape juice model solution using (−)-epicatechin as substrate.

Table 3
Influence of ethanol on the Kinetic constants of laccase browning.

Ethanol (% v/v)	Vmax (AU420/hour)	K0.5 (mM)	Hill's number
0	1.08 ± 0.06 B	0.223 ± 0.015 A	2.71 ± 0.09 D
5	1.45 ± 0.05 C	0.244 ± 0.011 AB	2.37 ± 0.07 C
10	1.13 ± 0.08 B	0.258 ± 0.012 B	1.81 ± 0.10 B
15	0.75 ± 0.05 A	0.260 ± 0.009 B	1.52 ± 0.11 A
p-value	<0,0001	0.0189	<0,0001

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

differences were only significant for the highest dose of this compound (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

Table 4
Effects of SO2 on the kinetic constants of laccase browning.

[SO2] (mg/L)	Vmax (AU420/hour)	K0.5 (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 A	2.74 ± 0.06 A
20	0.73 ± 0.21 B	0.376 ± 0.110 AB	3.65 ± 1.05 AB
30	0.04 ± 0.02 A	0.490 ± 0.250 B	4.49 ± 2.29 B
p-value	<0,0001	0.0412	0.0496

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

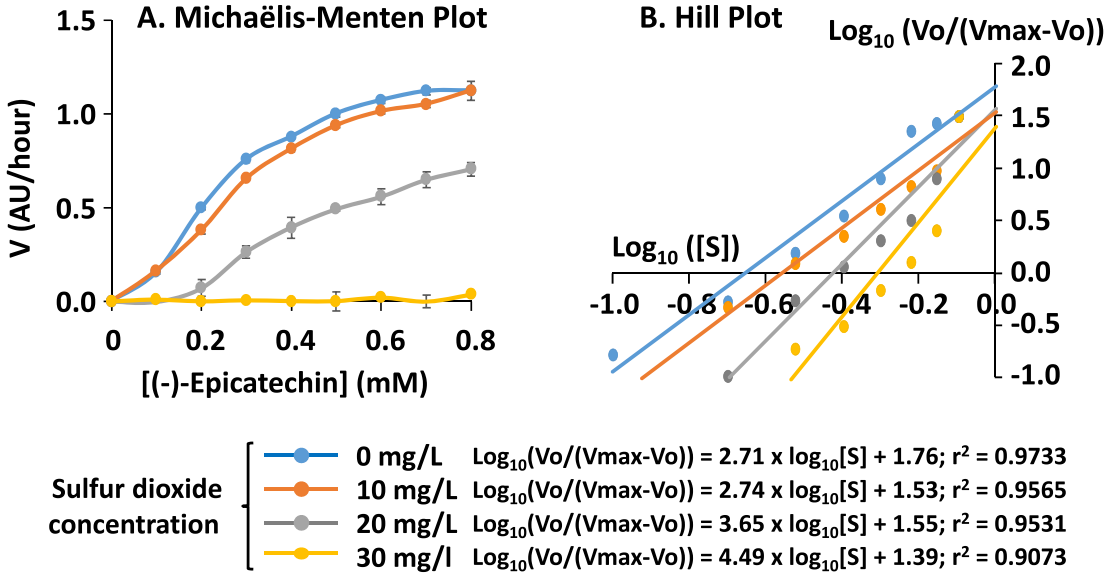

Fig. 6. Influence of SO2 concentration on laccase browning inhibition of the synthetic grape juice model solution using (−)-epicatechin as substrate.

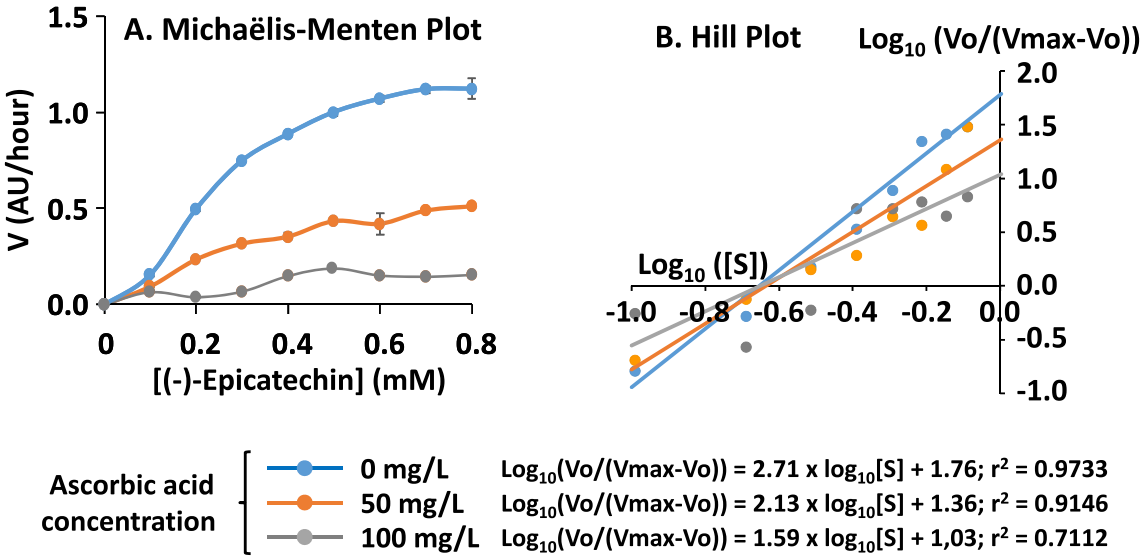

Fig. 7. Influence of ascorbic acid concentration on laccase browning inhibition of the synthetic grape juice model solution using (−)-epicatechin as substrate.

Table 5
Effects of ascorbic acid on the kinetic constants of laccase browning.

[Ascorbic acid] (mg/L)	Vmax (AU420/hour)	K0.5 (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
p-value	<0,0001	0.9010	<0,0001

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

caftaric acid.

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

Table 6
Effects of glutathione on the kinetic constants of laccase browning.

[Glutathione] (mg/L)	Vmax (AU420/hour)	K0.5 (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 A	2.57 ± 0.17 A
100	0.22 ± 0.05 A	0.451 ± 0.068 B	3.00 ± 0.71 A
p-value	<0,0001	<0,0001	0.5590

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

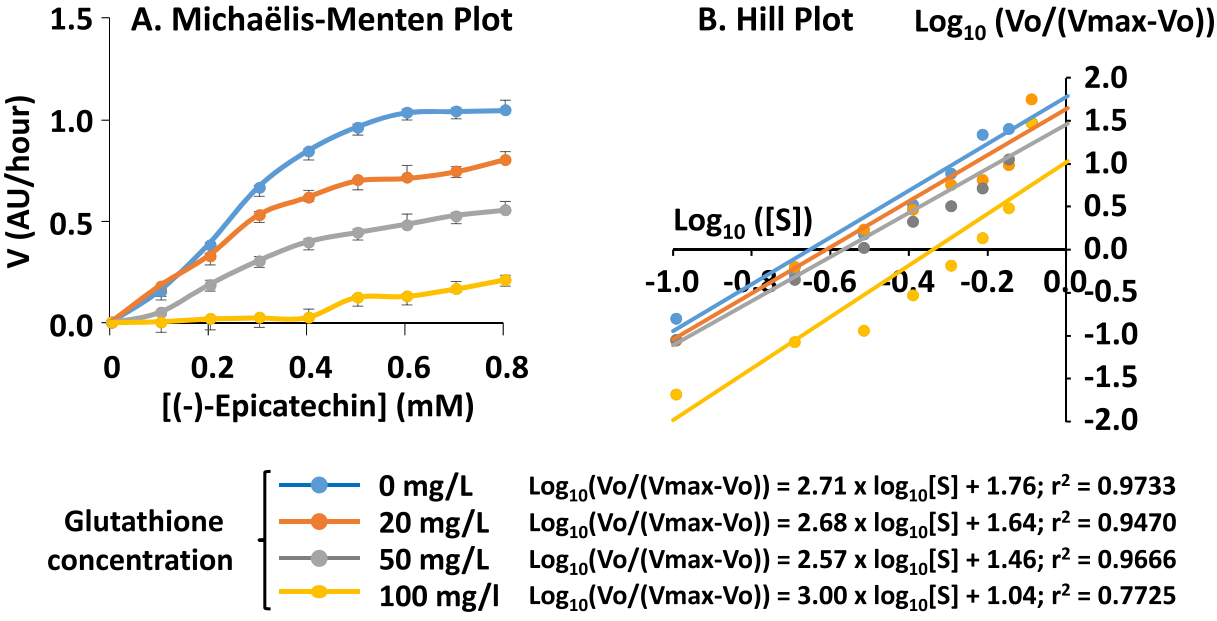

Fig. 8. Influence of glutathione concentration on laccase browning inhibition of the synthetic grape juice model solution using (−)-epicatechin as substrate.

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

The authors declare no competing financial interest.

CRediT authorship contribution statement

Pol Giménez: Methodology, Investigation, Writing – original draft, Data curation. **Sergi Anguela:** Methodology, Investigation. **Arnau Just-Borras:** Methodology, Investigation. **Pere Pons-Mercadé:** Methodology, Investigation. **Adeline Vignault:** Supervision. **Joan Miquel Canals:** Supervision, Methodology, Investigation. **Pierre-Louis Teissedre:** Supervision. **Fernando Zamora:** Supervision, Methodology, Investigation, Writing – original draft, Data curation.

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