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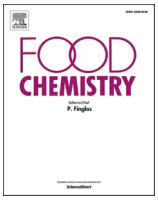

Oxygen-induced enzymatic and chemical degradation kinetics in wine model solution of selected phenolic compounds involved in browning

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ABSTRACT

Hydroxycinnamic and hydroxybenzoic acids are phenolic compounds that, upon oxidation, intensify browning, thus compromising the organoleptic quality of musts and white wines. This process is enhanced by the presence laccase, which is present in grapes after Botrytis cinerea infection. The aim of this study was to evaluate the kinetics of enzymatic and non-enzymatic degradation and browning of 15 phenolic compounds. Generally, hydroxycinnamic acids are more prone to degradation and browning reactions than hydroxybenzoic acids. The substituents present in the aromatic ring are key for determining their reaction rate. Indeed, the presence of both hydroxyl and methoxy substituents on the aromatic ring appears to facilitate enzymatic degradation in all the compounds studied. On the other hand, browning did not display a direct correlation with the degradation rate of the phenols. Finally, the chemical structure features of phenols were a reliable predictor of both browning and degradation rates.

1. Introduction

Some substances, especially phenolic compounds, of grape musts and wines may react with significant amounts of oxygen (Salmon et al., 2000). From a chemical and sensorial perspective, a controlled, slow oxidation could be beneficial for red wine by improving and stabilizing color and reducing astringency (Salmon et al., 2002; Singleton, 1987). However, the interaction between oxygen and other compounds in white grape musts and white wines can promote the degradation of phenolic structures, thus causing browning, which is one of the most harmful problems in the wine industry (Fernández-Zurbano et al., 1998; McRae et al., 2015; Patrianakou & Roussis, 2016; Vaimakis & Roussis, 1993; Vaimakis & Roussis, 1996).

The catalytic oxidation of phenolic compounds can be chemical or enzymatic. Chemical oxidation may be favored under certain physico-chemical conditions inherent to the grape musts or wine environment (Danilewicz, 2013; Li et al., 2008). The non-enzymatic oxidation process requires that transition metals ions such as iron, copper and manganese reduce the oxygen to hydrogen peroxide through the redox cycle Fe^{2+}/Fe^{3+} and Cu^{2+}/Cu^{+} (Cacho et al., 1995; Danilewicz, 2016; Vlahou et al., 2022; Voltea et al., 2022). Simultaneously, the phenols are sequentially oxidized to semiquinone radicals and benzoquinones (Danilewicz et al., 2008). Moreover, hydrogen peroxide can associate with ferrous ion in the so-called Fenton reaction, thus producing hydroxyl radicals (HO•), which are highly reactive and known to oxidize almost any organic molecule found in wine (Elias & Waterhouse, 2010; Waterhouse & Laurie, 2006). The semiquinones and quinones formed are highly unstable and, due to their strongly electrophilic character, may induce further reactions with nucleophilic compounds such as phenols, thiols and amines. The chain reactions can vary depending on the structures of the original molecules. However, the most common of these are rearrangement of the dimers' structure through an enol-like conversion reaction and polymerization through several reactions of dimers to form brown pigments called melanins (Li et al., 2008; Queiroz et al., 2008).

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In contrast, the enzymatic oxidation process occurs almost exclusively in grape must after the harvest. The enzymatic oxidation of phenolic compounds is catalyzed by oxidoreductase enzymes, a type of multicopper polyphenol oxidases. Among these are tyrosinase (E.C. 1.10.3.1) which is naturally present in grapes, and laccase (E.C. 1.10.3.2), a highly glycosylated extracellular enzyme (Claus, 2004) that is only present in grapes infected by epiphytic fungi, mainly Botrytis cinerea (Bustamante, Giménez, Just-Borràs, Solé-Clua, Gombau, Heras, Sieczkowski, Gil, Canals, & Zamora, 2024; Singleton & Cilliers, 1995; Strong & Claus, 2011). Both tyrosinase and laccase can promote oxidation by hydroxylation to the *ortho*-position adjacent to an existing hydroxyl group phenolic substrate or favor the oxidation of ortho-dihydroxybenzenes to ortho-benzoquinones thanks to their mono- and diphenol oxidase activity, respectively (Oliveira et al., 2011). Tyrosinase is produced during grape ripening and its enzymatic activity increases near the common harvest time (Rapenau et al., 2009). Laccase, on the other hand, which is only present in grapes affected by grey rot, is probably the most important cause of economic losses in the wine industry because its effects on grape must browning are much more intense than those of tyrosinase.

Phenolic compounds are the most significant substrates for polyphenol oxidases. These compounds can be classified as flavonoids or non-flavonoids depending on the presence or absence of a C6-C3-C6 carbon skeleton, respectively (Montané et al., 2020). The most significant phenolic compounds in red wines are flavonoids, a family that includes condensed tannins or proanthocyanidins, and anthocyanins, which are responsible for the wine's red color (Waterhouse, 2002; Zamora, 2003). Both groups are susceptible to enzymatic degradation (Giménez et al., 2023; Holderbaum et al., 2010). Red wines also contain flavonols and non-flavonoids, especially phenolic acids and their esters (Merkytė et al., 2020). In contrast, white wines do not contain anthocyanins and their concentration of flavanols is very low because the white winemaking process involves pressing the grapes, which removes the skins and seeds, thus reducing the concentration of these compounds (Alara et al., 2021).

Flavonols and non-flavonoid compounds, including stilbenes and phenolic acids, are therefore the main phenolic compounds present in white grape musts and white wines. It has been reported that phenolic acids are particularly significant substrates for the chemical and enzymatic oxidation processes of white wines (Cheynier et al., 1988; Fernández-Zurbano et al., 1998; Merkytė et al., 2020). Phenolic acids are present in their free form or as glycosylated derivatives or esterified with tartaric, quinic or shikimic acid (Monagas et al., 2005). These compounds are the primary cause of color deterioration in white wines through both the enzymatic and the non-enzymatic oxidation process (Fernandez-Zurbano et al., 1995).

It seems clear, therefore, that phenolic acids and their esters are key for understanding the browning process in white grape musts and white wines. Several studies on chemical and enzymatic browning have been reported (Bustamante, Giménez, Just-Borràs, Solé-Clua, Gombau, Heras, Sieczkowski, Gil, Pérez-Navarro, et al., 2024; Giménez et al., 2023; Vignault et al., 2020; Volkova et al., 2012; Zimdars et al., 2017; Zinnai et al., 2013) but, to our knowledge, no information exists on oxygen induced catalytic/enzymatic degradation kinetics of the various phenolic acids.

In this paper we study the kinetics of chemical and enzymatic (laccase) degradation of the phenolic compounds in a synthetic medium emulating white wine in order to better understand the relationship between their chemical structure and their degradation reactivities. Specifically, the phenolic acids studied were: three phenol derivatives (phenol, catechol and pyrogallol); five hydroxybenzoic acids (4-hydroxybenzoic acid, protocatechuic acid, vanillic acid, gallic acid and syringic acid); and seven hydroxycinnamic acids (*o*-coumaric acid, *p*-coumaric acid, *m*-coumaric acid, caffeic acid, caftaric acid, *trans*-ferulic acid and *trans*-fertaric acid). The structures and reference concentrations in grape must or wine of all these compounds are shown in Table 1. It should be noted that there are differences in the concentration of these compounds in must compared to wine, probably due to the diversity of varieties used and the different extraction methods employed for their analysis. Most of these phenolic compounds were chosen because they are natural grape constituents and because some of them are known to be involved in browning (Cosme et al., 2018; Lee & Jaworski, 1989; Macheix et al., 1991; Patel et al., 2019), and that some others were used as probes to clarify the role played by some structural features. Note that the three phenols were included in this study because, though they are not present in wine, their chemical structures can provide interesting information.

2. Materials and methods

2.1. Chemicals and equipment

Phenol (purity ≥99 %), catechol (purity ≥99 %), pyrogallol (purity ≥99 %), 4-hydroxybenzoic acid (purity ≥99 %), protocatechuic acid (purity ≥97 %), vanillic acid (purity ≥97 %), gallic acid (purity ≥97.5 %), syringic acid (purity ≥99 %), *o*-coumaric acid (purity ≥98 %), *p*-coumaric acid (purity ≥98 %), *m*-coumaric acid (purity ≥99 %), caffeic acid (purity ≥98 %), caftaric acid (purity ≥99 %), *trans*-ferulic acid (purity ≥99 %) and *trans*-fertaric acid (purity ≥98 %), ethanol absolute, L-(+)-tartaric acid (purity ≥99.5 %), sodium hydroxide (purity ≥98 %), polyvinylpolypyrrolidone (PVPP), 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 %), syringaldazine (purity ≥98 %), and sodium acetate (purity ≥99 %) were purchased from Sigma-Aldrich (Madrid, Spain). KH_2PO_4 (purity ≥99 %), $CuSO_4$ (purity ≥99 %), 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) for the browning kinetic; a HPLC-DAD (©Agilent technologies, 1200 Series, DAD G1315D, Waldbronn Germany) for phenolic compound analysis; 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).

2.2. Production, extraction and isolation of extracellular laccase

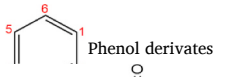
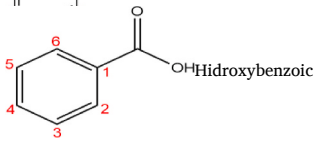
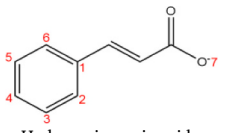
Laccase was extracted from *Botrytis cinerea,* isolate 213, obtained from grapevine leaves in 1998 from the UMR SAVE collection in Bordeaux (Martinez et al., 2003). This strain was chosen because of its high virulence on grapevine leaves and berries and because it is a transpose-type strain (Ky et al., 2012; Martinez et al., 2005). The pathogen was cultured, and the enzyme was extracted according to previous studies (Giménez et al., 2022).

2.3. Measuring activity assays

Laccase activity was measured using a modification of the syringaldazine test method (Grassin & Dubourdieu, 1986). Once the laccase was produced, 5 mL of this solution was mixed with 0.8 g of PVPP (to remove phenolic compounds that may interfere). The solution was then stirred and centrifuged at 8500 rpm for 10 min. One milliliter of the supernatant was added to a spectrophotometer cuvette to which 1.4 mL of buffer solution (8.2 g/L sodium acetate in distilled water, pH 5.5) and 0.6 mL of syringaldazine solution (60 mg/L syringaldazine in 96 % ethanol) were added. After homogenization, the absorbance was measured at 530 nm every minute for five minutes including at time zero.

Finally, the following equation was used to calculate laccase activity by using the slope of the line obtained via a calibrating linear regression

Table 1
Structure, CAS, molecular weight and reference concentration of phenolic compound in grape and wines.

Phenolic compounds group	Compound	CAS number	Molecular weight (g·mol-1)	1	2	3	4	5	6	7	Grape Concentration ($mg \cdot L^{-1}$)		Wine Concentration (mg·L-1)		References
											White varieties	Red varieties	White	Red	
 Phenol derivates	Phenol **(Ph)**	108–95-2	94.11	**OH**	H	H	H	H	H	–	N.D.	N.D.	N.D.	N.D.	
	Catechol **(Cat)**	120–80-9	110.11	**OH**	**OH**	H	H	H	H	–	N.D.	N.D.	N.D.	N.D.	
	Pyrogallol **(Pyr)**	87–66-1	126.11	**OH**	**OH**	**OH**	H	H	H	–	N.D.	N.D.	N.D.	2.44–6.93	1
 Hidroxybenzoic acids	4-Hydroxybenzoic acid **(HA)**	99–96-7	138.12	H	H	H	**OH**	H	H	–	0.02–0.09	0.35	0.01–0.18	0.13–2.35	2,3
	Protocatechuic acid **(PrA)**	99–50-3	154.12	H	H	**OH**	**OH**	H	H	–	0.03–0.31	0.14–0.72	0.05–44.54	3.76–24.52	1,2,4,5,7
	Vanillic acid **(VaA)**	121–34-6	168.14	H	H	**OCH_3**	**OH**	H	H	–	0.37–0.79	0.07–4.27	0.03–47.34	0.11–9.34	1–3,5-7,11
	Gallic acid **(GaA)**	149–91-7	170.12	H	H	**OH**	**OH**	**OH**	H	–	0.11–1.01	0.31–67.40	0.82–71.70	0.21–126.80	1–8,10–12
	Syngiric acid **(SyA)**	530–57-4	198.17	H	H	**OCH_3**	**OH**	**OCH_3**	H	–	0.01–0.02	0.04–273.22	7.03–44.72	5.28–590.76	1,2,5,6
Total, HBA											N.F.	78.27	188.17–205.70	32.52–283.30	1,5,6
 Hydroxycinnamic acids	*o*-Coumaric acid **(*o*-CA)**	614–60-8	164.16	H	**OH**	H	H	H	H	H	**	**	**	**	
	m-Coumaric acid **(*m*-CA)**	14,755–02-3	164.16	H	H	**OH**	H	H	H	H	**	**	**	**	
	p-Coumaric acid **(*p*-CA)**	501–96-4	164.05	H	H	H	**OH**	H	H	H	0.05–1.40	0.78	0.13–27.86	0.06–21.84	1,2,5,6,8,11,12
	Caffeic acid **(CaffA)**	331–39-5	180.16	H	H	**OH**	**OH**	H	H	H	0.03–1.03	0.14–8.60	0.43–3.93	0.08–28.38	1–4,6-8,10,11
	Caftaric acid **(CaftA)**	67,879–58-7	312.23	H	H	**OH**	**OH**	H	H	**$C_4H_5O_6$***	1.49–48.17	3.82–33.72	1.83–21.52	17.90–117.95	2–4,6-9,12
	trans-Ferulic acid **(FeruA)**	537–84-4	194.18	H	H	**OCH_3**	**OH**	H	H	H	0.02–0.17	0.7	0.02–0.35	0.04–9.81	3,11,12
	trans-Fertaric acid **(FertA)**	74,282–22-7	326.25	H	H	**OCH_3**	**OH**	H	H	**$C_4H_5O_6$***	0.99–3.00	N.F.	0.37–3.80	1.36–3.77	3,9,12
Total, HCA											5.00–80.00	N.F.	57.87–58.03	8.23–44.71	1,5,6,9

* Tartaric esterification; **isomers expressed as p-Coumaric acid; N.D. No Described; N.F. references not found; [1](Francesca et al., 2014); [2](Van Leeuw et al., 2014); [3](Lukić et al., 2019); [4](V. González de Peredo et al., 2021); [5](Rivero et al., 2019); [6](Pardo-García et al., 2014); [7](Cebrián-Tarancón et al., 2019); [8](Portu et al., 2016); [9](Bustamante, Giménez, Just-Borràs, Solé-Clua, Gombau, Heras, Sieczkowski, Gil, Canals, & Zamora, 2024); [10](López-Giral et al., 2015); [11](Bekar et al., 2017); [12](Cejudo-Bastante et al., 2012).

(ΔA) expressed in absorbance units/min: Laccase activity = 46.15 × ΔA μmol. $L^{-1} \cdot min^{-1}$ = 46.15 × ΔA LU. By definition, a laccase unit (LU) corresponds to the amount of enzyme that catalyzes the oxidation of a micromole of syringaldazine per minute.

2.4. Hydroalcoholic solution preparation

All experiments used a saturated solution of oxygen (roughly 7-8 mg O_2/L), 4 g/L of L-(+)-tartaric acid, 12 % ethanol, 3 mg.L^{-1} of iron, added as $FeSO_4 \cdot 7H_2O$ and 0.3 mg.L^{-1} of copper added as $CuSO_4 \cdot 5H_2O$ adjusted to pH 3.5 with sodium hydroxide.

2.5. Enzymatic and non-enzymatic browning kinetic

The browning kinetic was performed in 3 mL plastic cuvettes in triplicate for three phenol derivatives, five hydroxycinnamic acids and seven hydroxybenzoic acids. Individual solutions were prepared for each compound (2 mM) with hydroalcoholic solution. Then 200 μL of this solution, 30 μL of laccase (2 U/mL laccase activity) and 1770 μL of hydroalcoholic solution were added to the plastic cuvette where the browning kinetics were carried out (final concentration 0.2 mM). For each compound, the same experiment was performed without the addition of laccase, which was used as a control treatment. The browning kinetics were followed for five hours in a spectrophotometer by measuring the absorbance at 420 nm every 15 min, and the browning was evaluated as the slope of the regression straight line according to previous studies (Giménez et al., 2022).

2.6. Enzymatic and non-enzymatic degradation kinetic

For each compound, kinetic degradations with laccase (enzymatic consumption) and without laccase (chemical consumption) were performed in triplicate in Eppendorf tubes (1.5 mL). The same concentrations were used for the solutions as for the evaluation of the browning kinetics and the reactions were stopped with 20 mg/L of sodium bisulfite ($NaHSO_3$) at 0, 2.5 and 5 h, respectively. The phenolic concentration was measured by HPLC-DAD.

2.7. HPLC-DAD analysis of phenolic compounds

The concentrations of the different phenolic compounds were measured by HPLC-DAD according to a modification of the previously described procedure (Lago-Vanzela et al., 2011) using a calibration linear regression for each compound. The samples were filtered (0.20 μm, polyester membrane, Chromafil PET 20/25, Machery-Nagel, Düren, Germany) and then injected (20 μL) into a Zorbax Eclipse XDB C18 column (3.5 μm; 4.6 × 150 mm). The solvents used were A: [water/formic acid/acetonitrile (88.5:8.5:3, *v*/v/v)]; B: [water/formic acid/acetonitrile (41.5:8.5:50, v/v/v)]; and C: [water/formic acid/methanol (1.5:8.5:90, v/v/v)]. The flow rate was 0.19 mL/min. The gradient was: A = 96 %/B = 4 % from 0 to 37 min; A = 70 %/B = 17 %/C = 13 % from 37 to 51 min; A = 50 %/B = 30 %/C = 20 % from 51 to 57 min; A = 0 %/B = 50 %/C = 50 % from 57 to 64 min. The regimen flow rate, gradient and solvent used have been described in previous studies (Bustamante, Giménez, Just-Borràs, Solé-Clua, Gombau, Heras, Sieczkowski, Gil, Canals, & Zamora, 2024). The compounds were quantified by measuring the absorbance at 280 nm (Phenolic and Hydroxybenzoic derivates) and 320 nm (Hydroxycinnamic acids).

2.8. Color measurements

The final color of all samples was measured after five hours by means of CIE*L*a*b** coordinates according to Ayala et al., 1997. The data were processed using MSCV software (de de la Grupo Color, 2012). Total color difference (ΔEab*) was calculated using the following formula:

$$\Delta Eab^* = \left((L^*_1 - L^*_2)^2 + (a^*_1 - a^*_2)^2 + (b^*_1 - b^*_2)^2 \right)^{1/2}.$$

where L* is the lightness; a* is the color's green–red component; and b* is the color's blue–yellow component. Δ*Eab** is used to determine whether the difference between two samples can be detected by the human eye. Generally, the difference is considered to be visible to the human eye when Δ*Eab** > 3 units (García-Marino et al., 2013; Martínez et al., 2001). The color of each sample was reproduced in PowerPoint software using the RGB signals after transforming the CIE*L*a*b** coordinates (ColorMine, 2025).

2.9. Calculation of degradation and browning kinetics variables

To characterize the kinetics of the degradation of the phenolic compounds and the browning, the following five ratios were estimated from the concentrations calculated by HPLC and the absorbances at 420 nm:

a) The initial enzymatic oxygen induced degradation rate (IEOIDR) (μmol·l^{-1}·h^{-1}). This is the estimated enzymatic degradation rate of the compounds in hydroalcoholic solution. These data were calculated after 2.5 h of exposure to laccase since afterwards the degradation rate clearly decreased. IEOIDR was calculated by subtracting non-enzymatic degradation from degradation in the presence of laccase.
b) The initial non-enzymatic compound degradation rate (INECDR) (μmol·l^{-1}·h^{-1}). This is the estimated consumption rate of non-enzymatic degradation during the first 2.5 h without exposure to laccase.
c) Enzymatic/non-enzymatic consumption, which is the ratio between the enzymatic and the non-enzymatic consumption of the compounds (IEOIDR·$INECDR^{-1}$).
d) The initial enzymatic browning rate (IEBR) (A420/h). This is the estimated browning rate caused by the enzymatic degradation of the different compounds during the first 70 min of exposure to laccase since afterwards the browning rate clearly decreased. A420 without laccase was subtracted from A420 with laccase to obtain IEBR.
e) Initial browning/consumption (A420·$mmol^{-1}$), which represents the browning intensity produced by each mmol of compound degraded during the first 2.5 h of enzymatic reaction (IEBR·$IEOIDR^{-1}$).

2.10. Statistical analysis

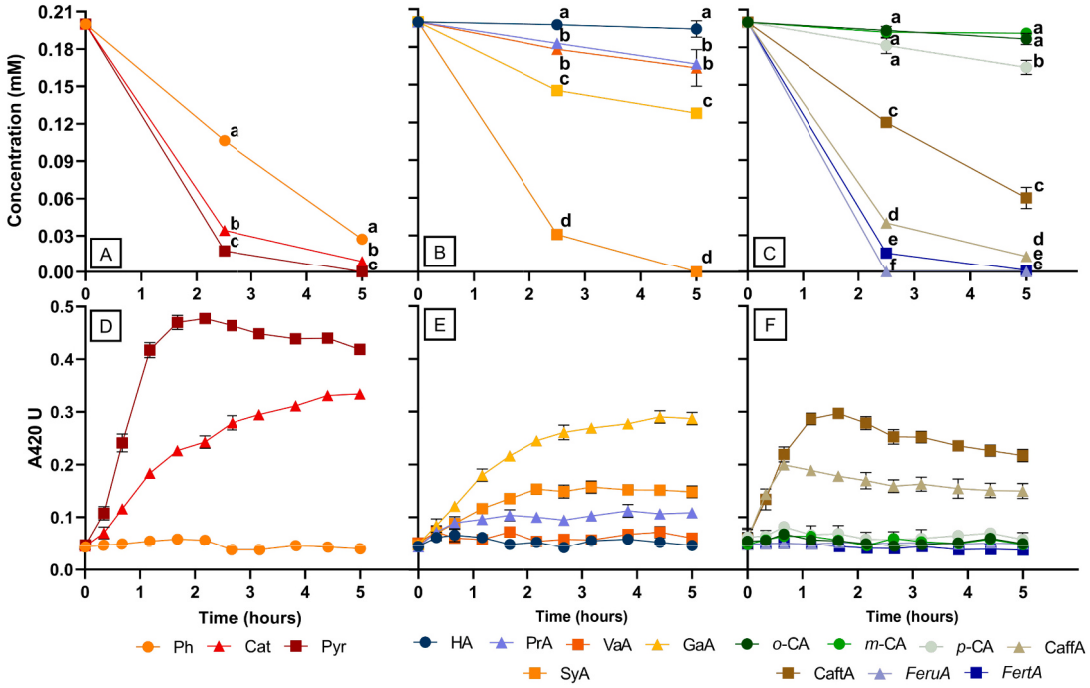
All data were expressed as the arithmetic mean ± standard deviation of three replicates. One-factor analysis of variance (ANOVA F test) was conducted using XLSTAT 2024.3 software (Addinsoft SAS; Paris, France). Different letters indicate the existence of statistical difference ($p < 0.05$). The multiple linear regression models were performed in Microsoft Excel (Microsoft®; Washington, USA).

3. Results and discussion

3.1. Degradation and browning kinetic

Fig. 1 shows the consumption and browning intensity enzymatic kinetic of the different phenolic compounds due to the action of laccase. These values were obtained by subtracting chemical degradation (without laccase) from total degradation (with laccase). In fact, chemical degradation (without laccase) of the phenolic compounds was very low in all cases (Table 2) and is not included in this figure.

Fig. 1.A shows specifically the laccase consumption of the three phenol derivatives (phenol, catechol and pyrogallol). Although these compounds have not been reported in either grape must or wine (Table 1) – except for pyrogallol (Francesca et al., 2014) – they were included in our study because they represent a simplified model for



A: Phenols derivates, **B**: Hydroxybenzoic acid and **C**: Hydroxycinnamic acid Concentration (mM) quantificated by HPLC-DAD, 0, 2.5 and 5.0 hours after laccase inoculation (2U/mL), and **C**: Phenols derivates, **D**: Hydroxybenzoic acid and **E**: Hydroxycinnamic acid browning intensity measured as absorbance at 420 nm during the same 5 hours. Results are expressed as mean ± standard deviation of three replicates. Different letters in a row indicate the existence of statistical difference ($p < 0.05$). Ph: Phenol; Cat: Catechol; Pyr: Pyrogallol; HA: Hydroxybenzoic acid; PrA: Protocatechuic acid; VaA: Vanillic acid; GaA: Gallic acid; SyA: Syringic acid; *o*-CA: *o*-Coumaric acid; *m*-CA: *m*-Coumaric acid; *p*-CA: *p*-Coumaric acid; CaffA: Caffeic acid; CaftA: Caftaric acid; FeruA: Ferulic acid; FertA: Fertaric acid.

Fig. 1. Phenolic compound degradation and browning intensity kinetics after laccase inoculation (Enzymatic oxidation).

Table 2
Compound parameters of enzymatic and non enzymatic phenolics oxidation.

Phenolic compounds group	Compound	Initial non-enzymatic compound degradation rate (INECDR) (µmol·l-1·h-1)	Initial enzymatic oxygen induced degradation rate (IEOIDR) (µmol·l-1·h-1)	Enzymatic/non-enzymatic consumption rate	Initial browning rate (IEBR) (A420/h)	Initial browning/consumption rate (A420/mmol)
Phenol derivates	Phenol	4.12 ± 0.18 **f**	37.41 ± 1.20 **f**	9.07 ± 0.29 **e**	0.009 ± 0.005 **b**	0.23 ± 0.11 **b**
	Catechol	0.62 ± 0.15 **c**	66.28 ± 0.57 **i**	106.90 ± 0.92 **h**	0.107 ± 0.011 **g**	1.61 ± 0.15 **d**
	Pyrogallol	N.D.	72.91 ± 0.91 **j**	–	0.292 ± 0.018 **j**	4.00 ± 0.23 **f**
Hidroxibenzoic acids	4-Hydroxybenzoic acid	0.37 ± 0.06 **a**	1.29 ± 0.02 **a**	3.51 ± 0.54 **b**	N.D.	–
	Protocatehuic acid	0.77 ± 0.07 **c**	6.81 ± 0.43 **d**	8.86 ± 0.56 **e**	0.045 ± 0.005 **e**	6.57 ± 0.90 **g**
	Vanillic acid	1.43 ± 0.45 **d**	8.74 ± 1.64 **d**	6.07 ± 1.14 **c**	0.014 ± 0.003 **b**	1.55 ± 0.23 **d**
	Gallic acid	0.52 ± 0.12 **b**	21.83 ± 1.78 **e**	41.69 ± 5.44 **g**	0.110 ± 0.018 **g**	5.01 ± 0.83 **g**
	Syngiric acid	N.D.	67.72 ± 1.08 **i**	–	0.062 ± 0.001 **f**	0.91 ± 0.03 **c**
Hydroxycinnamic acids	*o*-Coumaric acid	0.57 ± 0.07 **b**	2.63 ± 0.80 **b**	4.65 ± 1.42 **b**	0.021 ± 0.013 **bc**	8.31 ± 0.44 **i**
	m-Coumaric acid	1.23 ± 0.02 **d**	4.29 ± 0.26 **c**	2.62 ± 1.50 **ab**	0.018 ± 0.002 **b**	4.23 ± 0.85 **f**
	p-Coumaric acid	4.75 ± 0.14 **g**	7.39 ± 2.64 **d**	1.55 ± 0.56 **a**	0.029 ± 0.012 **c**	3.91 ± 0.85 **f**
	Caffeic acid	8.44 ± 1.14 **h**	64.06 ± 0.43 **h**	7.59 ± 0.05 **d**	0.212 ± 0.026 **h**	3.00 ± 0.39 **e**
	Caftaric acid	N.D.	31.92 ± 0.54 **g**	–	0.240 ± 0.009 **i**	7.50 ± 0.21 **h**
	trans-Ferulic acid	3.04 ± 0.13 **e**	80.00 ± 0.00 **k**	26.27 ± 0.23 **f**	0.004 ± 0.002 **a**	0.05 ± 0.00 **a**
	trans-Fertaric acid	0.50 ± 0.09 **b**	73.74 ± 0.11 **j**	146.40 ± 0.21 **i**	0.005 ± 0.003 **a**	0.06 ± 0.00 **a**

Results are expressed as mean ± standard deviation of three replicates. Different letters in a row indicate the existence of statistical difference ($p < 0.05$). N.D. No detected.

understanding the effect of the hydroxyl ions (OH-) on affinity with the laccase enzyme. These three phenol derivatives were effectively degraded by laccase. Pyrogallol exhibited the highest Initial enzymatic compound consumption rate (IEOIDR) (72.91 $\mu mol \cdot l^{-1} \cdot h^{-1}$), followed in descending order by catechol (66.28 $\mu mol \cdot l^{-1} \cdot h^{-1}$) and phenol (37.41 $\mu mol \cdot l^{-1} \cdot h^{-1}$) (Table 2). It appears, therefore, that the higher the number of hydroxyl groups in the phenolic structure, the higher the enzymatic consumption rate. With regard to the Initial Non-Enzymatic Compound Consumption Rate (INECDR), the ranking of these compounds was inverted with respect to the IEOIDR. In fact, pyrogallol was not degraded, catechol only slightly while phenol was the most effectively degraded. In any case, IEOIDR and INECDR showed a ratio of at

least 9:1, which indicates that the enzyme had an affinity for all these compounds (Table 2).

Fig. 1.B shows the corresponding data for the hydroxybenzoic acids (4-hydroxybenzoic acid, protocatechuic acid, vanillic acid, gallic acid and syringic acid). Syringic acid showed the highest IEOIDR (67.72 $\mu mol \cdot l^{-1} \cdot h^{-1}$) followed in descending order by gallic acid (21.83 $\mu mol \cdot l^{-1} \cdot h^{-1}$), vanillic acid (8.74 $\mu mol \cdot l^{-1} \cdot h^{-1}$), protocatechuic acid (6.81 $\mu mol \cdot l^{-1} \cdot h^{-1}$) and 4-hydroxybenzoic acid, which was barely degraded by laccase (1.29 $\mu mol \cdot l^{-1} \cdot h^{-1}$). Although syringic acid showed the highest IEOIDR in this group of compounds, it was not possible to identify any non-enzymatic consumption (INECDR $\approx$ 0), while the IEOIDR values for 4-hydroxybenzoic acid suggest a low affinity of laccase for this compound (Table 2).

With regard to hydroxycinnamic acids (Fig. 1.C and Table 2), *trans*-ferulic acid (80.00 $\mu mol \cdot l^{-1} \cdot h^{-1}$) degraded the most rapidly followed in descending order by *trans*-fertaric acid (73.74 $\mu mol \cdot l^{-1} \cdot h^{-1}$), caffeic acid (64.06 $\mu mol \cdot l^{-1} \cdot h^{-1}$), caftaric acid (31.92 $\mu mol \cdot l^{-1} \cdot h^{-1}$), *p*-coumaric acid (7.39 $\mu mol \cdot l^{-1} \cdot h^{-1}$), *m*-coumaric acid (4.29 $\mu mol \cdot l^{-1} \cdot h^{-1}$) and *o*-coumaric acid (2.63 $\mu mol \cdot l^{-1} \cdot h^{-1}$). Takahama (1995) previously reported that laccase has a high affinity for ferulic acid and a very low affinity for *p*-coumaric acid, which agrees with our results.

Hydroxycinnamic acids were more susceptible to to enzymatic and non-enzymatic degradation than hydroxybenzoic acids. The results for IEOIDR and INECDR between homologous pairs between the two groups according to the type and number of substituents were considered. These pairs are: *p*-hydroxycinnamic acid >4-hydroxybenzoic acid; caffeic acid > protocatechuic acid; and *trans*-ferulic acid > vanillic acid. Also, the compounds esterified with tartaric acid (caftaric and *trans*-ferulic acids) showed lower enzymatic and non-enzymatic degradation than their non-esterified homologous pairs (caffeic and *trans*-ferulic acids) (Table 2). It appears, therefore, that the chemical structure of various phenolic compounds significantly influences their degradation rate by laccase.

Fig. 1 also shows the browning intensity of the various phenolic compounds over time. These values were obtained by subtracting the A420 without laccase from the A420 with laccase. Note that the increase in A420 without laccase was very small in all cases and is therefore not included in this figure. Table 2 also shows the Initial Enzymatic Browning Rate (IEBR) of the various phenolic compounds. Specifically, Fig. 1.D and Table 2 show the enzymatic browning intensity kinetics of the three phenols. Pyrogallol showed the maximum browning intensity (0.292 A420/h), followed in decreasing order by catechol (0.107 A420/h) and phenol (0.009 A420/h), which hardly produced any increase in A420. The browning behavior of these three phenols is consistent with their observed degradation kinetics. However, it is surprising that phenol hardly causes any browning.

Among the five benzoic acids studied (Fig. 1.E and Table 2), gallic acid showed the greatest browning intensity (0.110 A420/h), followed in decreasing order by syringic acid (0.062 A420/h), protocatechuic acid (0.045 A420/h), vanillic acid (0.014 A420/h) and 4-hydroxybenzoic acid, which hardly produced any increase in A420. Here, this order of browning intensity of the various benzoic acids does not coincide with the order of degradation kinetics. These data suggest that browning intensity depends not only on laccase activity in relation to a given phenolic compound but also on the ability of the primary quinone formed to undergo subsequent chemical polymerization to form melanins without the need for laccase action (Oliveira et al., 2011; Queiroz et al., 2008). Consequently, a less reactive benzoic acid to laccase, such as gallic acid, can produce greater browning intensity than the more reactive syringic acid. Note also that, like phenol, 4-hydroxybenzoic acid hardly caused any browning, which suggests that monophenols are not good substrates for browning.

Among the hydroxycinnamic acids (Fig. 1.F and Table 2), caftaric acid showed the greatest browning intensity (0.240 A420/h), followed by caffeic acid (0.212 A420/h). All other hydroxycinnamic acids produced very little browning: p-coumaric acid (0.029 A420/h), o-coumaric acid (0.021 A420/h), m-coumaric acid (0.018 A420/h), trans-ferulic acid (0.005 A420/h) and trans-fertaric acid (0.004 A420/h) although this compound was completely degraded.

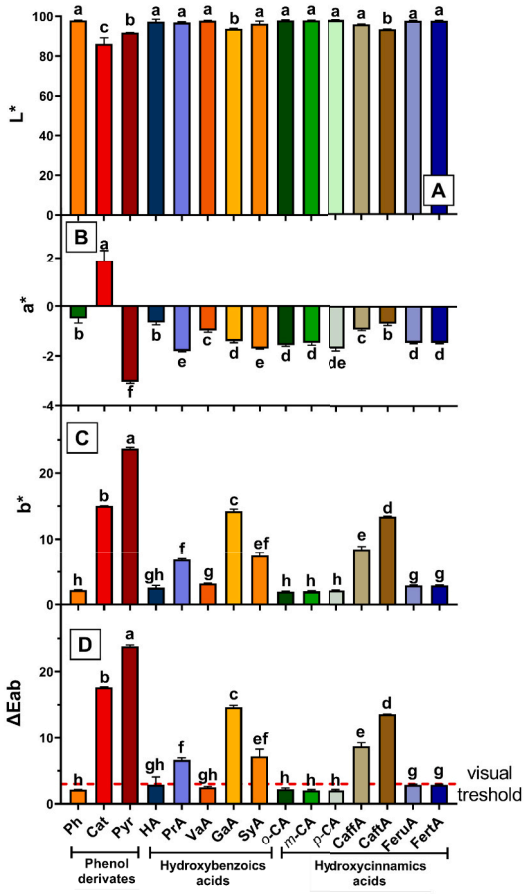
As with hydroxybenzoic acids, there was no correlation between the order of the degradation kinetics of the various hydroxycinnamic acids and the order of the browning intensity. The most surprising case was that of trans-ferulic acid, which degraded the fastest but produced little browning intensity. However, *trans*-ferulic acid produced very high turbidity, with centrifugation being required before absorbance could be measured. These data suggest that the action of laccase on ferulic acid causes a very rapid polymerization of the quinones produced, which become insoluble. The precipitation of these melanins may be why the browning intensity was lower than expected from their degradation rate. However, the degradation rate of the other hydroxycinnamic acids did not match the intensity of browning. This was the case of *trans*-ferulic acid, which was degraded very effectively but, in contrast, produced relatively little browning. These data confirm that browning intensity does not depend only on laccase activity in relation to a certain phenolic compound. It seems, therefore, that the chemical structure of the quinones formed by the action of laccase plays an important role in the subsequent polymerization that produces the melanins responsible for browning. In this context, Table 2 shows the relationship between the initial browning intensity and the initial degradation rate for all phenolic compounds studied. This parameter gives very scattered values that range from 0.05 for trans-ferulic acid to 8.31 for o-coumaric acid, thus confirming the absence of a relationship between degradation rate and browning intensity.

Finally, Table 2 also shows the enzymatic/non-enzymatic degradation ratio. As expected, these data confirm that non-enzymatic degradation was significantly lower than enzymatic degradation in all cases.

3.2. Color contribution

Fig. 2 shows the CIEL*a*b* coordinates of the various samples after 5 h of reaction time. For lightness (L*), no statistically significant differences were found between most of the phenolic compounds, with significantly lower levels observed only in the case of catechol, pyrogallol, gallic acid and caftaric acid, i.e. the compounds that caused the most browning (Fig. 1 and Table 2). With regard to the color's red-green component (a*), all samples except catechol showed negative values. These data suggest that, through enzymatic degradation, catechol generates products that tend to be reddish, while the other compounds tend to be greenish. With regard to the color's yellow-blue component (b*), all phenolic compounds showed positive values, thus confirming that the intensity of the yellow color was increased by the action of laccase. These data confirm that catechol, pyrogallol, gallic acid and caftaric acid are the phenolic compounds responsible for the greater browning intensity. The total color difference (ΔEab^*) between all samples before and after enzymatic degradation showed a similar pattern to b*. However, this parameter enables us to determine whether the human eye can discriminate between two samples. The difference is generally considered to be visible to the human eye when $\Delta Eab^* > 3$ units (García-Marino et al., 2013; Martínez et al., 2001). Given all these values, the phenolic compound that causes the greatest browning intensity is pyrogallol, followed in decreasing order by catechol, gallic acid, caftaric acid, caffeic acid, syringic acid and protocatechuic acid. The remaining phenolic compounds also cause browning but the intensity is so low that the human eye cannot distinguish them. Note that trans-ferulic acid did not cause a high level of browning intensity because the products generated by its enzymatic degradation become insoluble and precipitate.

Fig. 3 shows the reference color so as to better illustrate the changes caused by the action of laccase on the various phenolic compound samples. This figure visually confirms that the browning of pyrogallol, catechol, gallic acid, caftaric acid, caffeic acid, syringic acid and protocatechuic acid can be clearly distinguished whereas the browning



A,B,C: L*,a*,b* of de different phenolic compound; **D:** Eab at 5 hours after laccase inoculation in relation to initial colour. Results are expressed as mean ± standard deviation of three replicates. Different letters in a row indicate the existence of statistical difference ($p < 0.05$). Ph: Phenol; Cat: Catechol; Pyr: Pyrogallol; HA: Hydroxybenzoic acid; PrA: Protocatehuic acid; VaA: Vanillic acid; GaA: Gallic acid; SyA: Syngiric acid; *o*-CA: *o*-Coumaric acid; *m*-CA: *m*-Coumaric acid; *p*-CA: *p*-Coumaric acid; CaffA: Caffeic acid; CaftA: Caftaric acid; FeruA: Ferulic acid; FertA: Fertaric acid.

Fig. 2. CIE*L*a*b** Coordinates of the phenolics compound 5 h after laccase inoculation.

Phenol		Catechol		Pyrogallol		4-Hydroxybenzoic acid		Protocatehuic acid	
0 h	5 h	0 h	5 h	0 h	5 h	0 h	5 h	0 h	5 h
R: 255 G: 254 B: 252	R: 250 G: 249 B: 245	R: 251 G: 251 B: 248	R: 255 G: 254 B 252	R: 254 G: 254 B 251	R: 255 G: 254 B 252	R: 255 G: 255 B 253	R: 248 G: 248 B 243	R: 254 G: 255 B 253	R: 248 G: 247 B 233
Vanillic acid		Gallic acid		Syngiric acid		*o*-Coumaric acid		*m*-Coumaric acid	
0 h	5 h	0 h	5 h	0 h	5 h	0 h	5 h	0 h	5 h
R: 254 G: 253 B 250	R: 249 G: 249 B 242	R: 254 G: 255 B 253	R: 246 G: 237 B 210	R: 253 G: 254 B 251	R: 247 G: 244 B 230	R: 248 G: 250 B 246	R: 248 G: 250 B 245	R: 252 G: 255 B 252	R: 248 G: 250 B 245
p-Coumaric acid		Caffeic acid		Caftaric acid		trans-Ferulic acid		*trans*-Fertaric acid	
0 h	5 h	0 h	5 h	0 h	5 h	0 h	5 h	0 h	5 h
R: 251 G: 255 B 252	R: 248 G: 250 B 245	R: 252 G: 255 B 253	R: 250 G: 243 B 227	R: 251 G: 254 B 251	R: 246 G: 236 B 211	R: 250 G: 255 B 253	R: 247 G: 249 B 244	R: 251 G: 255 B 252	R: 248 G: 249 B 243

Results are expressed as mean of three replicates. The reference colors are descripted in RGB composition colour system.

Fig. 3. Reference color of the phenolics compound at 0 and 5 h after laccase inoculation.

intensity of the other phenolic compounds is barely perceptible.

3.3. *Influence of structure and functional groups*

A statistical model was developed to better understand the relationship between the chemical structure of the various phenolic

compounds as well as their degradation kinetics and browning intensity rate. For this, a multiple correlation analysis was carried out by taking into account the following structural variables: x1, the number of hydroxyl groups; x2, the number of methoxy groups; x3, the presence or absence of an organic acid group; and x4, the presence or absence of a double bond in the carbon chain. Table 3 shows the multiple correlation coefficients obtained for the initial enzymatic compound consumption rate (IEOIDR) and the initial enzymatic browning rate (IEBR). These multiple correlation coefficients – 0.9051 for IEOIDR and 0.8547 for IEBR – can be considered acceptable. In both cases the ANOVA regression indicates that the correlations are statistically significant since the calculated F values (45.29 and 27.09, respectively) are much higher than the critical F value (2.4×10^{-14} and 6.4×10^{-11}, respectively). Note also that the values obtained for the y-intercept and the slope coefficients (ki) were statistically significant ($p < 0.05$) for all structural variables considered. This multiple correlation analysis can be used to estimate the experimental IEOIDR and IEBR as a function of the chemical structure of the various phenolic compounds.

Fig. 4 shows the linear regression analysis between the estimated values of IEOIDR and IEBR obtained with this model and the experimental values. This regression was performed to evaluate the effectiveness of the statistical model for both parameters. The linear regression coefficients obtained for IEOIDR (0.8191) and IEBR (0.7304) were statistically significant in both cases ($p < 0.05$). In addition, the slopes obtained for the regression lines were very close to one in both cases. These data as a whole indicate that this statistical model works acceptably and can therefore be used to estimate the relationship between the degradation kinetics and the browning rate of the various phenolic compounds as a function of their chemical structure.

According to this statistical model, the coefficients of the multiple correlation analysis (Table 3) indicate the influence of the corresponding chemical structure variable on the IEOIDR and IEBR. More specifically, a positive value suggests that the presence of this chemical structure variable favors the degradation or browning rates, whereas a negative value suggests that it hinders it. Moreover, the higher the value of the coefficient, the greater the contribution of each chemical structure variable.

In the model obtained for the IEOIDR, the presence of methoxy groups, followed in decreasing order by the presence of a double bond in the carbon chain and the presence of hydroxyl groups, appears to be the structural feature that most favors the degradation rate. In contrast, the presence of an acid group seems to slow down the degradation rate.

All these hypotheses are attributed to the reactivity properties conferred by the various structural characteristics present in the phenolic compounds studied in this paper. First, our investigations confirmed that the electron-donating nature of methoxy groups ($-OCH_3$) significantly increases the degradation rate, as the aromatic ring becomes more reactive towards degradation by laccase. The presence of methoxy groups can also help to stabilize degradation reaction intermediates by resonance and hyperconjugation (such as quinones), thus promoting polymerization and, therefore, the browning of the product (Chen et al., 2020; Farhoosh et al., 2016). Similarly, the presence of a double bond in the carbon chain attached to the aromatic ring (and which presents conjugation with the ring as in cinnamic acid derivatives) also accelerates degradation by increasing the electron density of the aromatic ring (which is the same as making the ring a better nucleophile). In the intermediate conjugated systems formed, the electrons can be easily transferred to molecular oxygen, which accelerates the reaction with laccase (Joglekar et al., 1991). The third structural characteristic that promotes the degradation of these compounds is the presence of hydroxyl groups. Like methoxy groups, hydroxyl groups are electron-donors. However, their donor effect is weaker than that of methoxy groups, which makes the increase in the degradation rate less significant. On the other hand, carboxyl groups are electron-withdrawing, which reduces the charge density of the aromatic rings to which they are attached. Phenolic compounds that contain carboxyl groups are therefore less prone to degradation by laccase since the presence of the carboxyl group interferes with the stability of the quinones that are formed.

The presence of methoxy and hydroxy groups as well as double bonds in the carbon chain also seemed to favor the browning rate of the phenolic compounds, whereas the presence of an acid group seemed to disfavor it. However, in that case the structural characteristic that most favored the browning rate was the presence of hydroxy groups, followed in decreasing order by the presence of double bonds and the presence of methoxy groups. These data suggest, as mentioned earlier, that browning intensity depends not only on enzymatic degradation activity in relation to a certain phenolic compound caused by laccase activity but also on the ability of the primary quinone formed to subsequently chemically polymerize to form melanins (Oliveira et al., 2011; Queiroz et al., 2008). It appears, therefore, that the presence of hydroxy groups favors the formation of melanin more than the presence of methoxy groups does, even though the presence of methoxy groups favors the degradation more.

Table 3
Statistical model of Initial enzymatic oxygen induced degradation rate (IEOIDR) and Initial browning rate (IEBR) of phenols.

Regression model	IEOIDR ($\mu mol \cdot l^{-1} \cdot h^{-1}$) ($y_i$) $= k_0 + k_1x_1 + k_2x_2 + k_3x_3 + k_4x_4$	IEBR ($A420 \cdot h^{-1}$) (y_i) $= k_6 + k_7x_1 + k_8x_2 + k_9x_3 + k_{10}x_4$
Multiple correlation coefficient	0.9052	0.8541
y-axis intercept	k_0: 13.3763*	k_6: −0.099506*
x_1: Hydroxyl groups number (-OH) in aromatic ring	k_1: 22.7434*	k_7: 0.117504*
x_2: Methoxy groups number (-OCH3) in aromatic ring	k_2:45.8058*	k_8: 0.029350*
x_3: Organic acid groups (-COOH) presence	k_3: −55.9700*	k_9: −0.060295*
x_4: Double bond in carbon chain presence	k_4: 38.2670*	k_{10}: 0.075768*

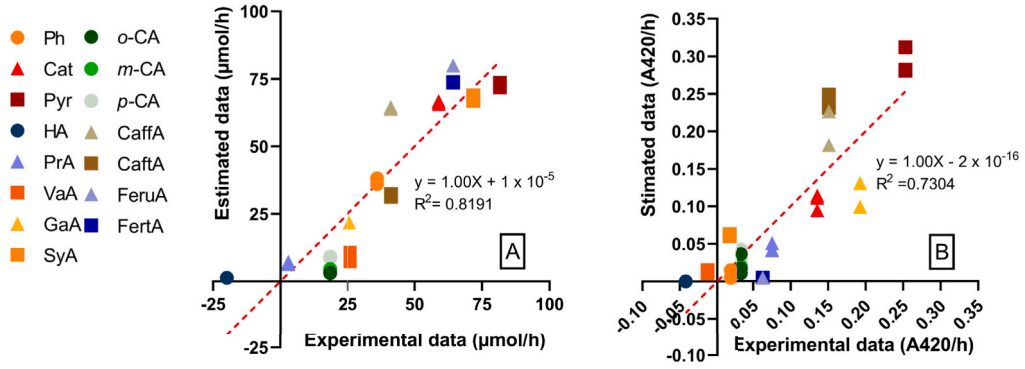
* Significance of variable probability (p-value <0.05).

4. Conclusions

Hydroxycinnamic and hydroxybenzoic acids contribute differently to the color deterioration of white wines after exposure to the action of enzyme laccase: hydroxycinnamic acids are more susceptible to degradation and browning processes than hydroxybenzoic acids. The substituents present in the aromatic ring of these compounds are key for determining their reaction rate. Indeed, the presence of both hydroxyl and methoxy substituents on the aromatic ring appears to facilitate enzymatic degradation in all the compounds studied. With regard to the relationship between the consumption of these compounds and the browning intensity, no direct correlation could be established. Further studies are needed to determine the individual action mechanism behind the degradation of phenolic compounds after the formation of quinones and the subsequent polymerization to melanins.

CRediT authorship contribution statement

Marco Bustamante: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Mariona Gil-Cortiella:** Supervision. **Álvaro Peña-Neira:** Supervision. **Jordi Gombau:** Methodology, Investigation. **Aitor García-Roldán:** Methodology, Investigation. **Matias Cisterna:** Methodology, Investigation. **Xavier Montané:** Writing – original draft, Supervision. **Francesca Fort:** Supervision. **Nicolas Rozès:** Supervision. **Joan Miquel Canals:** Project administration, Investigation, Conceptualization. **Fernando**



Ph: Phenol; Cat: Catechol; Pyr: Pyrogallol; HA: Hydroxybenzoic acid; PrA: Protocatehuic acid; VaA: Vanillic acid; GaA: Gallic acid; SyA: Syngiric acid; *o*-CA: *o*-Coumaric acid; *m*-CA: *m*-Coumaric acid; *p*-CA: *p*-Coumaric acid; CaffA: Caffeic acid; CaftA: Caftaric acid; FeruA: Ferulic acid; FertA: Fertaric acid.

Fig. 4. Regression model between experimental data and estimated data by statical model of the IEOIDR (A) and IEBR (B).

Zamora: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing 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.

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Data availability

Data will be made available on request.

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