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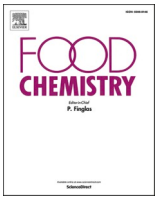

Development of a model to study browning caused by tyrosinase in grape must

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ABSTRACT

Enzymatic browning caused by polyphenol oxidases, tyrosinase and laccase, continues to be one of the main problems in winemaking. Therefore, wineries are very interested in studying the mechanisms of browning and procedures for decreasing the use sulphur dioxide. This research proposes a model to study tyrosinase activity from grape must using different substrates: one monophenol (*p*-hydroxybenzoic acid), two diphenols (caftaric acid and (−)-epicatechin) and one triphenol (gallic acid). The kinetic constants of tyrosinase, Vmax and K_M, indicate that caftaric acid is the best substrate for tyrosinase, followed in decreasing order by (−)-epicatechin, gallic acid and *p*-hydroxybenzoic acid. This last acid does not appear to be susceptible to browning by the action of grape must tyrosinase. The influence of pH, temperature and ethanol on grape must tyrosinase were also determined and the results indicate that tyrosinase Vmax increases when pH and temperature are higher and that the presence of ethanol reduces it.

1. Introduction

Enzymatic browning is still one of the major problems of current oenology (Li, Guo, & Wang, 2008), especially when the grapes are infected by grey rot (*Botrytis cinerea*) (Gancel et al., 2021). Enzymatic browning is an oxidation process that happens in many foods that increases the brown colour (Singh et al., 2018), often leading to consumer rejection. This problem is particularly damaging for the wine industry because grape must is very susceptible to enzymatic browning (Du Toit, Marais, Pretorius, & du Toit, 2006; Oliveira, Ferreira, De Freitas, & Silva, 2011).

Two polyphenol oxidases (PPOs) are responsible for enzymatic browning: tyrosinase (EC 1.14.18.1), which is naturally present in grape berries; and laccase (EC 1.10.3.2), which is only present in grapes infected by grey rot (Friedman, 1966). Both enzymes are multicopper oxidases that use oxygen to oxidize some phenolic compounds, such as hydroxycinnamic acids and other diphenols (Giménez et al., 2022). Polyphenol oxidase activity causes some diphenols to be oxidized to diquinones, which polymerize later to form melanins (Friedman, 1966). The initial formed diquinones are colourless but the melanins that are formed later by chemical polymerization are yellow-brown and therefore are responsible for the darkening of the yellow colour in white wines (browning) and also for colour deterioration in red wines (Ribéreau-Gayon, Dubourdieu, & Donèche, 2006; Singh et al., 2018). It is also quite probable that the darkening intensity of the formed melanins would be influenced by the nature of the phenolic compounds oxidized by tyrosinase.

The wine industry applies different strategies to prevent browning. The use of sulphur dioxide is the most common tool because it is a powerful inhibitor of tyrosinase and laccase (Queiroz, Lopes, Fialho, & Valente-Mesquita, 2008). Sulphur dioxide is also used in winemaking due to its antimicrobial properties (Ough & Crowell, 1987; Rose, 1993) to inhibit malolactic fermentation in white and rosé wines (Quirós, Herrero, García, & Día, 2012) or to prevent the development of spoilage microorganisms such as acetic acid bacteria (Vavrinik, Stuskova, Baron, & Sochor, 2022) or *Bretanomyces bruxellensis* (Zuehlke & Edwards, 2013).

However, the harmful effects of sulphur dioxide on the environment and human health (Stockley, 2005; Vally & Misso, 2012) have led to a current trend to reduce or even eliminate its use in winemaking. In fact,

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wineries must declare the presence of sulphites on the labels of their bottles if it exceeds 10 mg/L.

Therefore, some other additives have been proposed as alternatives to sulphur dioxide to prevent the browning of grape must, such as ascorbic acid (Barril, Rutledge, Scollary, & Clark, 2016; Bradshaw, Barril, Clark, Prenzler, & Scollary, 2011), inert gases (Cáceres-Mella et al., 2013; Martinez & Whitaker, 1995), oenological tannins (Vignault et al., 2019; Vignault et al., 2020) and, more recently, the use of reduced glutathione (Bustamante et al., 2024a; Giménez et al., 2023; Kritzinger, Bauer, & du Toit, 2013), or inactivated dried yeast extracts rich in glutathione (Bahut et al., 2020; Bustamante et al., 2024a; Gabrielli, Aleixandre-Tudo, Kilmartin, Sieczkowski, & du Toit, 2017), and bio-protection (Bustamante et al., 2024b; Chacón-Rodríguez et al., 2020; Giménez et al., 2023; Simonin et al., 2022; Windholtz, Nioi, Coulon, & Masneuf-Pomarede, 2023). However, to date there is no completely satisfactory alternative to the use of sulphur dioxide to protect grape must from enzymatic browning and thus research into alternatives continues to be necessary.

Therefore, it is necessary to study grape tyrosinase activity in order to better understand its action mechanisms and try to design new strategies for avoiding the browning that it causes. Several studies have been carried out on tyrosinase kinetics from different origins (Dolashki et al., 2012; García Molina et al., 2007; Sánchez-Ferrer, Rodríguez-López, García-Cánovas, & García-Carmona, 1995; Zaidi & Ali, 2014) but only a few of them have been performed with tyrosinase from *Vitis vinifera* (Fronk et al., 2015; Kaya & Bagci, 2021; Popescu et al., 2009) and even less with the natural substrates that this enzyme can find in the grape must (Honisch, Osto, Dupas de Matos, Vincenzi, & Ruzza, 2020; Yokotsuka, Shimizu, & Shimizu, 1991).

During the last few years, our research group has studied browning caused by laccase in grape must and some procedures to inhibit this enzyme and reduce the use of sulphur dioxide (Bustamante et al., 2024a; Bustamante et al., 2024b; Giménez et al., 2022; Giménez et al., 2023). However, all of these studies were carried out in conditions of grapes affected by the presence of laccase and not in conditions of healthy grapes. Given the need to study browning in healthy grape conditions, the aim of this research was to develop a model for studying the browning oxidation kinetics caused by tyrosinase in white grape must.

2. Materials and methods

2.1. Chemicals and equipment

All samples and standards were handled without any exposure to light. Polyvinylpolypyrrolidone (PVPP), gallic acid (purity ≥97.5 %), p-hydroxybenzoic acid (purity ≥99.9 %) and (−)-epicatechin (purity ≥98.0 %) were purchased from Sigma-Aldrich (Madrid, Spain). Caftaric acid (purity ≥99.9 %) was purchased from Biosynth S. R. O. (Bratislava, Slovakia). Activated carbon was purchased from Enartis Sepsa (Navarrete, La Rioja, 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); a centrifuge Heraeus™ Primo™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); an ultrasound bath (3,000,514 Selecta, Abrera, Barcelona, Spain); a thermostatic bath (6,000,137 Selecta, Abrera, Barcelona, Spain); and a CB Standard Balance (Cobos, Barcelona, Spain).

2.2. Preparation of grape must

The grape must used to carry out the different browning studies came from grapes of the Muscat of Alexandria variety (Variety number VIVC, n.d.: 8241) from the experimental vineyard of the Rovira i Virgili University (Mas dels Frares, Constantí, Tarragona: 41°08′44.1″N 1°11′51.0″E) during the 2023 vintage harvest. The grapes were manually harvested at the optimal maturity level (22.3 ^{o}Brix; titratable acidity 5.2 g of tartaric acid/L) and the bunches were frozen in plastic bags at −20 °C until the time of experimentation. 24 h before the experiment, the bunches were left to defrost at room temperature. Once defrosted, the berries were manually destemmed and ground with a blender (Silvercrest SSM550C1, Kompernass GMBH, Bochum, Germany). Then, 20 mg/Kg of pectolytic enzyme (Lallzyme C-Max™, Lallemand, Inc., Montreal, Canada) was added and the sample was macerated at 4 °C for 24 h in order to favour the extraction of tyrosinase enzyme from the grape skin fragments. After this time, the sample was sieved, gently manually pressed and centrifuged to separate the solid parts and obtain the clean must. The must was not supplemented with sulphur dioxide to avoid tyrosinase inhibition.

2.3. Removal of phenolic compounds from grape must

To guarantee that the tyrosinase acts only on the selected substrates, it was necessary to eliminate all the phenolic compounds present in the grape must. In this regard, clarification tests were carried out with two clarifying agents: activated carbon and Polyvinylpolypyrrolidone (PVPP). However, activated carbon was discarded because using it eliminated a very high proportion of tyrosinase activity (data not shown).

A PVPP suspension was prepared with distilled water at a concentration of 200 g/L. Four PVPP columns were prepared by depositing 10 mL of this solution in each one and the water was eliminated using a vacuum pump. One hundred mL of the grape must were then percolated through one of these columns with the help of the vacuum pump. The process was repeated 4 times using a new PVPP column each time until reaching a total polyphenol Index (TPI) lower than 2 units. The TPI was determined by measuring the 280 nm absorbance of a 1:100 dilution of wine or grape juice with a spectrophotometer after centrifugation (15,900 *g* for 20 min) using a 10-mm quartz cuvette (Ribéreau-Gayon et al., 2006).

2.4. Measurement of tyrosinase activity with different substrates by monitoring the browning of the must

To measure the must's tyrosinase activity, the kinetics of the increase in intensity of the yellow colour in the grape must were determined by measuring the absorbance at 420 nm (A420) as a function of time using different substrates. The selected substrates were: one monophenol (p-hydroxybenzoic acid), two diphenols (caftaric acid and (−)-epicatechin) and one triphenol (gallic acid). Stock solutions (20 mM) of each substrate were prepared.

Aliquots of 1.8 mL of discoloured grape must were introduced into standard spectrophotometer microcuvettes of 10 mm optical path length. Immediately afterwards, volumes of 200, 175, 150, 100, 50 and 0 μL of distilled water and 0, 25, 50, 100, 150 and 200 μL of the different substrate stock solution were added to the different microcuvettes. The final substrate concentration in the reaction media was therefore 0, 0.25, 0.50, 1.0, 1.5 and 2.0 mM respectively. Finally, the microcuvettes were vigorously shaken to homogenize and saturate the must with oxygen.

To monitor the browning reaction, periodic measurements of absorbance at 420 nm were made over time until asymptotic behaviour was observed. The duration of these measurements depended on each substrate used and/or the experimental conditions, and they took between 40 and 240 min. All experiments were performed in triplicate at 25 °C with the sole and logical exception of the study of the influence of temperature.

2.5. Determination of the kinetic constants of tyrosinase

To study the browning kinetics caused by must tyrosinase, the Michaelis-Menten diagram (Michaelis & Menten, 1913) was plotted for each substrate. The Lineweaver-Burk diagram (Lineweaver & Burk,

1934) was also plotted to determine the kinetic constants, Michaelis constant (KM) and maximum velocity (Vmax), of tyrosinase with each of the substrates. If the kinetics observed for any of the substrates did not show Michaelian behaviour, then we proceeded to represent the Hill diagram, which allowed us to determine the $K_{0.5}$ that is the equivalent of the KM for allosteric behaviour (Tsao & Madley, 1972).

2.6. Influence of pH on tyrosinase activity

To study how tyrosinase acts as a function of pH, browning kinetic measurements were performed at three different pH values, 3.87, 3.53 and 3.10, which represent the normal pH interval from most wines.

The original pH of the discoloured grape must was 3.87 and therefore aliquots of this grape must were adjusted with hydrochloric acid to pH values of 3.53 and 3.10. In this case, only caftaric acid was used because it was found to be the best substrate for tyrosinase of all those studied.

2.7. Influence of temperature on tyrosinase activity

To study the effect of temperature on tyrosinase activity, the browning kinetics of the grape must were measured at different temperatures. Specifically, the must browning measurements were carried out at temperatures of 15 °C, 25 °C and 35 °C. Only caftaric acid was used as substrate in this experiment for reasons similar to those described in the previous point.

2.8. Influence of ethanol on tyrosinase activity

To study the influence of ethanol on tyrosinase activity, the browning kinetics of the grape must were measured at different concentrations of ethanol: 0.6 % and 12.0 %. For this experiment, as for the previous ones, only caftaric acid was used.

2.9. Statistical analysis

All data are expressed as mean values ± standard deviation of three replicates. The comparison between variables was carried out using one-factor analysis of variance (ANOVA) using SPSS 15.0 software (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Kinetic parameters of grape must browning caused by tyrosinase for different substrates

Fig. 1 shows the changes in absorbance of the different substrates at 420 nm (A420) according to incubation time. 4-Hydroxybenzoic acid did not produce any browning, even at long times (240 min), and therefore it does not seem to be sensitive to the action of grape must tyrosinase. In contrast, caftaric acid and (−)-epicatechin showed a clear increase in A420 over time. Gallic acid also showed an increase in A420 but a much lower one. In addition, a clear trend was observed for the three substrates susceptible to being browned by the action of tyrosinase: the higher the substrate concentration, the higher the increase in A420. These results indicate that orthodiphenols, caftaric acid and (−)-epicatechin are better substrates for tyrosinase than triphenols, gallic acid, and especially monophenols, or at least 4-hydroxybenzoic acid, which appears to be non-reactive. Giménez et al. (2022) reported similar results in a kinetic study performed with laccase from *Botrytis cinerea*.

Fig. 2 shows the Michaelis-Menten plot (Michaelis & Menten, 1913) for the different substrates studied. As expected, the reaction rate (V) for the caftaric and gallic acids increased as their concentration augmented until asymptotic behaviour was reached, when the substrate concentration was high enough to saturate the enzyme. This confirms that tyrosinase shows an apparent Michaelian behaviour for caftaric and gallic acids. However, the plot obtained for (−)-epicatechin (Fig. 2C)

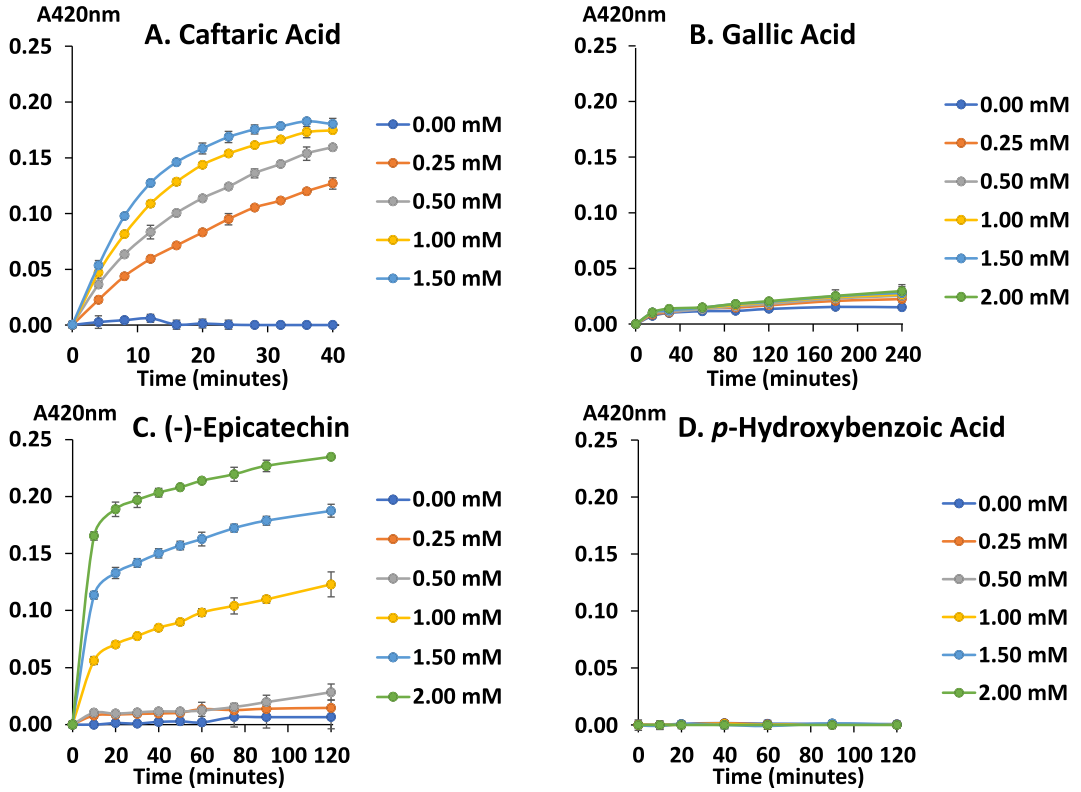


Fig. 1. Tyrosinase browning kinetics.

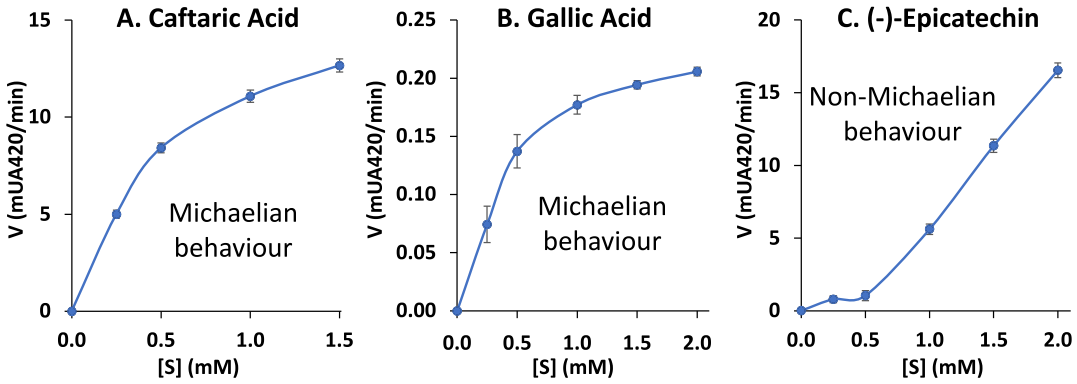
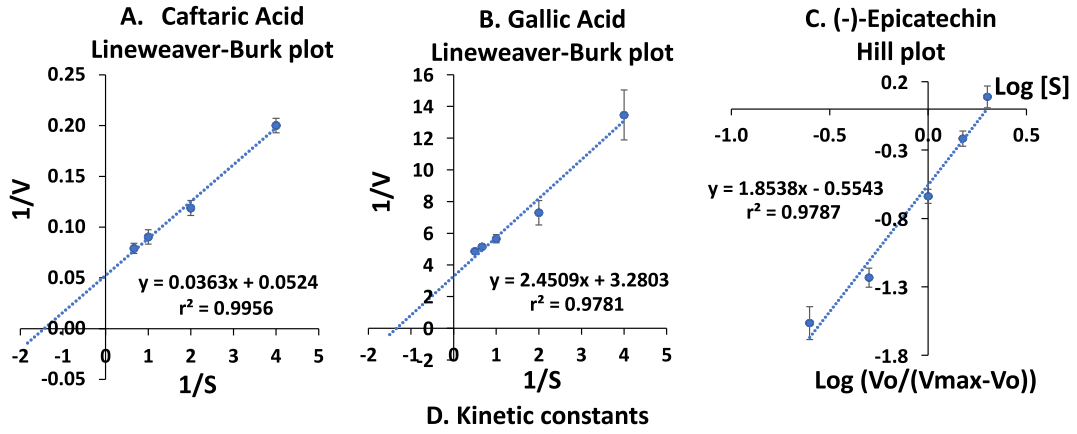


Fig. 2. Michaelis-Menten plots.

showed a sigmoidal profile that suggests an apparent allosteric behaviour.

It should be considered that this study has measured the browning kinetics and not the tyrosinase activity directly. It is obvious that tyrosinase participates in this process, but browning also involves a subsequent chain of chemical reactions that leads to the formation of melanins (Oliveira et al., 2011; Queiroz et al., 2008). Consequently, the final browning kinetics may be influenced not only by the activity of the tyrosinase itself, but also by other factors related to the subsequent melanin formation reactions, which will depend on each of the substrates. It can therefore be stated that the browning kinetics of caftaric and gallic acids apparently show a Michaelian behaviour while that of epicatechin apparently presents an allosteric behaviour. In this sense, some authors have described a Michaelian behaviour for tyrosinase (Donato, Algieri, Rizzi, & Giorno, 2014; García-Molina et al., 2007) while others have reported an allosteric behaviour (Haghbeen et al., 2010; Sánchez-Ferrer et al., 1995). It should be taken into account that this study has not directly measured tyrosinase activity but browning, a process in which the tyrosinase 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 behaviour was at least apparently Michaelian for caftaric and gallic acids and allosteric for (−)-epicatechin.

Fig. 3 shows the Lineweaver-Burk plot (Lineweaver & Burk, 1934) for caftaric acid (Fig. 3A) and gallic acid (Fig. 3B), which makes it possible to determine the kinetic parameters of grape must tyrosinase, the reaction maximal velocity (Vmax) and the Michaelis constant (K_M) for both substrates. It should be highlighted that the linear regression coefficients (r^2) obtained were reasonably good (0.9956 for caftaric acid and 0.9781 for gallic acid), which confirms the Michaelian behaviour of grape must tyrosinase for these two substrates. However, neither the Lineweaver-Burk plot nor the Eadie-Hofstee plot showed a linear behaviour for (−)-epicatechin (data not shown), which makes it impossible to determine the kinetic parameters using these procedures. Therefore, the 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. 3C shows the Hill plot for (−)-epicatechin in which it can be seen that the linear regression has a reasonably good coefficient ($r^2 = 0.9787$). This confirms the allosteric behaviour of tyrosinase for this substrate and



D. Kinetic constants

Substrate	Apparent behaviour	$K_M/K_{0.5}$ (mM)		Vmax (mUA420/min)	
Caftaric Acid	Michaelian	0.693 ± 0.021	A	19.09 ± 0.57	B
Gallic Acid	Michaelian	0.747 ± 0.043	B	0.30 ± 0.02	A
(-)-Epicatechin	Allosteric	1.991 ± 0.112	C	30.03 ± 1.69	C

Fig. 3. Kinetic constant determination.

allows $K_{0.5}$ to be calculated. Fig. 3D shows the tyrosinase browning kinetic parameters for the different substrates. The highest value for Vmax was that of (−)-epicatechin (30.03 mUA420/min), followed in decreasing order by caftaric acid (19.09 mUA420/min) and gallic acid (0.30 mUA420/min), which more strictly confirms the results showed in Fig. 1.

Fig. 3D also shows the KM values (or $K_{0.5}$ in the case of (−)-epicatechin) for the different substrates. These results indicate that grape must tyrosinase has the lowest $K_M/K_{0.5}$ value for caftaric acid (0.693 mM) followed in increasing order by gallic acid (0.747 mM) and (−)-epicatechin (1.991 mM), which would indicate that this enzyme has a higher affinity for caftaric acid followed by gallic acid and finally by epicatechin, which has the lowest affinity. It is somewhat surprising that epicatechin is the substrate with the lowest affinity when it is the one that generates the highest Vmax. This behaviour is probably related to the allosteric kinetics that this enzyme presents towards (−)-epicatechin. It is not easy to compare the KM values obtained with data previously reported in the literature because, as mentioned in the introduction, there are few kinetic studies on grape tyrosinase. In addition, it is difficult to compare kinetic parameters due to the use of different grape cultivars, different measurement conditions and in particular the use of different substrates. Even so, Fronk and Jaeckels (2017) reported for tyrosinase extracted from Riesling grapes, an apparent K_M of 5.5 mM for dopamine and 34.4 mM for catechol, and Kaya and Bagci (2021) reported a K_M of 4.8 mM for 4-methylcatechol for Kirmizi Kismis grape. These values are higher than those obtained in this work. However, Honisch et al. (2020) reported a K_M of 0.31 mM for tyrosine for Riesling grapes, a value that is closer to those obtained in this research.

It can also be noted that Giménez et al. (2022) determined the $K_{0.5}$ values of laccase from *Botrytis cinerea* with the same substrates (caftaric acid, gallic acid and (−)-epicatechin) and the values that they found were lower than those of grape must tyrosinase (0.173, 0.222 and 0.101 mM respectively). These data indicate therefore that laccase shows a higher affinity than tyrosinase with these substrates.

In any case, the high affinity of grape must tyrosinase versus caftaric acid agrees with previous works in which this hydroxycinnamic acid has been considered as one of the main substrates for browning (Cheynier, Rigaud, Souquet, Duprat, & Moutounet, 1990; Cheynier, Trousdale, Singleton, Salgues, & Wylde, 1986; Macheix, Sapis, Fleuriet, & Lee, 1991; Singleton, Salgues, Zaya, & Trousdale, 1985). In addition, caftaric acid is one of the phenolic compounds present at higher concentrations in the grape must (Honisch et al., 2020; Singleton et al., 1985). Therefore, caftaric acid was selected for the following assays precisely because it was the substrate for which grape tyrosinase showed the highest affinity.

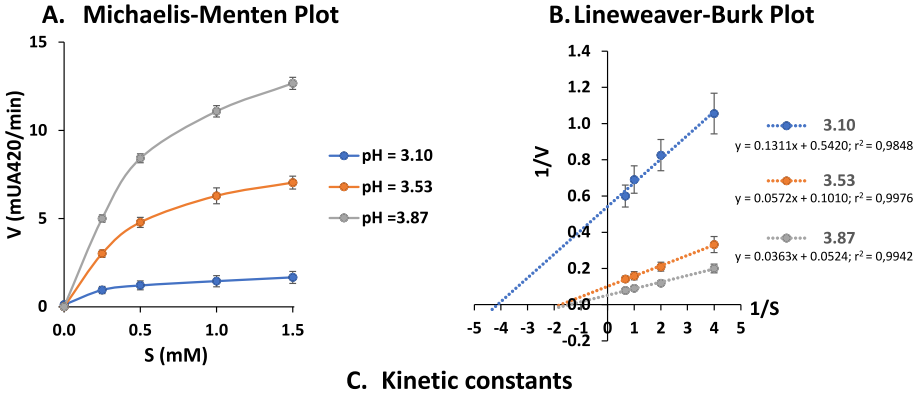
It should be noted that these results demonstrate that the proposed method for measuring browning caused by tyrosinase in grape must works adequately and can therefore be used for future studies on how we can reduce the doses of sulphur dioxide added to wine and, at the same time, prevent deterioration due to enzymatic oxidation.

3.2. Influence of pH on grape must tyrosinase activity

Fig. 4 shows the influence of pH on grape must tyrosinase activity. More specifically Fig. 4A shows the Michaelis-Menten plot. In this graph it can be seen that pH plays a very important role in the tyrosinase browning kinetics. In synthesis, the higher the pH the higher the grape tyrosinase browning rate. Similar results have been found by other authors who have established that the optimal pH of grape tyrosinase is between 5 and 6 (Gilly, Mara, Oded, & Zohar, 2001; Sánchez-Ferrer, Bru, Valero, & Garcia-Carmona, 1989). The Lineweaver-Burk plot (Fig. 4B) resulted in relatively good regression coefficients for the three studied pH values ($r^2 = 0.9848$ for pH = 3.10; $r^2 = 0.9976$ for pH = 3.53 and $r^2 = 0.9942$ for pH = 3.87), which confirms the Michaelian behaviour for the three pH values. Moreover, this plot makes it possible to determine how the kinetic constants, Vmax and K_M, change as a function of pH (Fig. 4C). The results are very clear and show that both Vmax and K_M increase as the pH is higher, indicating that not only the maximal rate of the reaction increases but also that the affinity of tyrosinase for caftaric acid decreases.

3.3. Influence of temperature on grape must tyrosinase activity

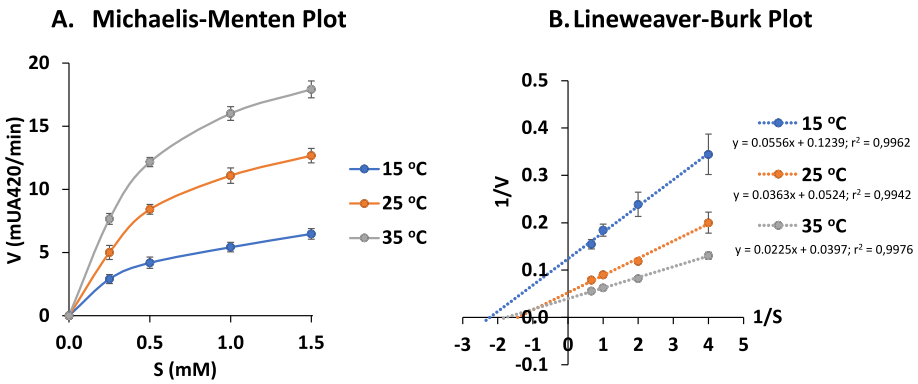
Fig. 5 shows the influence of temperature on grape must tyrosinase activity. As expected, the Michaelis-Menten plot (Fig. 5A) shows that the higher the temperature, the higher the reaction rate. The Lineweaver-Burk plot (Fig. 5B) also originated relatively good regression coefficients for all temperatures ($r^2 = 0.9962$ for 15C; $r^2 = 0.9942$ for 25 °C; and $r^2 = 0.9976$ for 35 °C). These results indicate that grape must



C. Kinetic constants

pH	K_M (mM)		Vmax (mUA420/min)	
3.10	0.242 ± 0.051	A	1.85 ± 0.39	A
3.53	0.566 ± 0.035	B	9.90 ± 0.61	B
3.87	0.693 ± 0.021	C	19.08 ± 0.57	C

Fig. 4. Influence of pH.



C. Kinetic constants

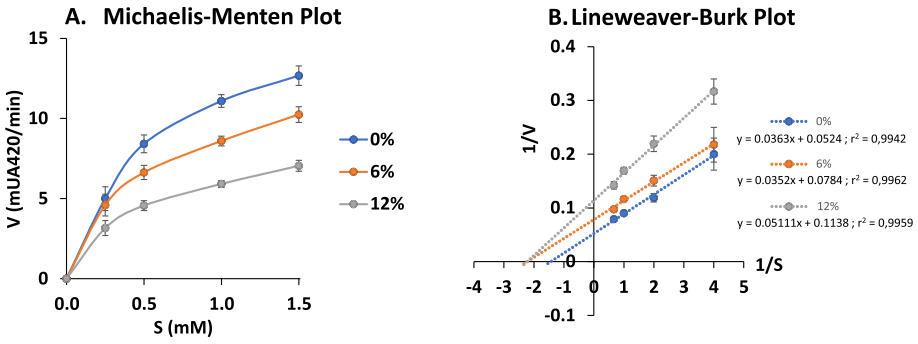
Temperature	K_M (mM)		Vmax (mUA420/min)	
15 °C	0.449 ± 0.038	A	8.07 ± 0.68	A
25 °C	0.693 ± 0.040	C	19.08 ± 1.10	B
35 °C	0.567 ± 0.021	B	25.19 ± 0.93	C

Fig. 5. Influence of temperature.

tyrosinase maintains the Michaelian behaviour at the three studied temperatures. The slopes and y-intercepts of the three regression lines were used to calculate the kinetic constants, Vmax and K_M (Fig. 5C). The results corresponding to Vmax were very clear because it increased as the temperature increased. However, the K_M behaviour, as a function of temperature, was not clear since it increased between 15 °C and 25 °C, and subsequently decreased at 35 °C. In any case, it is clear that at higher temperatures the browning caused by grape tyrosinase is greater.

3.4. Influence of ethanol on grape must tyrosinase activity

Fig. 6 shows the influence of ethanol on grape must tyrosinase activity. The results shown in the Michaelis-Menten (Fig. 6A) plot clearly indicate that ethanol is an inhibitor of tyrosinase activity since the higher the ethanol content the lower the reaction rate. This inhibitory effect of ethanol on tyrosinase from other vegetal origins has been described previously (Chen et al., 2021; Husni, Jeon, Um, Han, & Chung, 2011). This indicates that enzymatic browning caused by grape tyrosinase would be less harmful in wine than in grape must. The Lineweaver-Burk plot (Fig. 6B) also gave rise to relatively good regression coefficients ($r^2 = 0.9942$ for 0 % of ethanol; $r^2 = 0.9962$ for 6 % of ethanol and $r^2 = 0.9959$ for 12 % of ethanol), indicating that grape must tyrosinase also exhibits Michaelian behaviour in the presence of ethanol. The Lineweaver-Burk plot regression line equations were used to calculate the kinetic constants, Vmax and K_M, which are shown in Fig. (6C). These results confirm the inhibitory effect of ethanol since its presence reduces the Vmax values. Surprisingly, K_M decreased in the presence of ethanol, which indicates that the affinity of tyrosinase towards caftaric acid increased in its presence. In any case, it is clear that the presence of ethanol inhibits tyrosinase given its clear effects on



C. Kinetic constants

Ethanol	K_M (mM)		Vmax (mUA420/min)	
0%	0.693 ± 0.043	A	19.08 ± 1.18	C
6%	0.450 ± 0.029	B	12.77 ± 0.82	A
12%	0.449 ± 0.029	C	8.79 ± 0.56	B

Fig. 6. Influence of ethanol.

Vmax.

4. Conclusions

A model is proposed to determine the grape must tyrosinase activity by monitoring browning with different substrates, one monophenol (p-hydroxybenzoic acid), two diphenols (caftaric acid and (−)-epicatechin) and one triphenol (gallic acid). This model makes it possible to determine the kinetic constants of grape must tyrosinase, Vmax and KM with the different substrates. It was found that caftaric acid is the best substrate for tyrosinase of all those studied. This research has also determined the influence of pH, temperature and ethanol on the grape must tyrosinase, demonstrating that tyrosinase Vmax increases with pH, when the temperature increases and that it is reduced by ethanol. This model can be useful in further research for studying the mechanisms by which tyrosinase can be inhibited to avoid grape must browning and thus determine how to decrease the doses of sulphur dioxide added to wine while still preventing spoilage due to enzymatic oxidation.

CRediT authorship contribution statement

Aitor García-Roldán: Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Antoni Canalda-Sabaté:** Methodology, Investigation. **Jordi Gombau-Roigé:** Methodology, Investigation. **Marco Bustamante-Quiñones:** Methodology, Investigation. **Arnau Just-Borras:** Methodology, Investigation. **José M. Heras:** Supervision, Conceptualization. **Nathalie Sieczkowski:** Supervision, Conceptualization. **Joan Miquel Canals:** Project administration, Investigation, Conceptualization. **Fernando Zamora:** 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.

Data availability

Data will be made available on request.

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