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ABSTRACT

Enzymatic parameters (K_M and V_{max}), residual activity, effect of bentonite and electrophoresis characterization of laccase in the presence of different oenological tannins (OT) were investigated in relation to *B. cinerea* negative effects in grapes and musts. Five OT were tested (gallotannin, ellagitannin, quebracho, grape-skin and grape-seed) in comparison with ascorbic acid (AA), sulfur dioxide (SO_2) and bentonite. We added OT, AA, SO_2 and bentonite to botrytized must obtained by inoculation of grapes with B. *cinerea* strain (strain 213). Laccase activity was measured by the syringaldazine method at different concentrations of substrate. Enzymatic parameters were determined using Michaelis-Menten and Lineweaver-Burk plots. The B. *cinerea* strain was also grown in a liquid medium for laccase production. Molecular weight of laccases and effect of OT upon these laccases were studied by SDS-PAGE. Results confirm that bentonite, contrary to OT, did not permit to reduce laccase activity. Regardless the tannin considered, V_{max}, K_M and laccase activity were reduced and gallotannin, grape-skin and grape-seed tannin presented the greatest ability. Efficiency of grape-seed tannin addition in order to reduce the laccase activity, was comparable to that of AA or SO_2 at the typical doses employed in oenology for each one. Oenological tannins appear to be excellent processing aids to prevent laccase effects and contribute to reduce the use of SO_2 in grapes and musts.

Keywords: oenological tannins, grapes, musts, Botrytis bunch rot, laccases, kinetics parameters, electrophoresis.

1. Introduction

Botrytis cinerea, is a ubiquitous, filamentous and necrotrophic fungus excreting metabolites (glycerol, gluconic acid, β-glucans) and enzymes (pectinases, proteases, tyrosinases and laccases) in the host cells (Steel, Blackman, & Schmidtke, 2013). Laccases (EC 1.10.3.2) are o-diphenol and p-diphenol: dioxygen oxidoreductases. These multi-copper glycoproteins use molecular oxygen to oxidize various aromatic and

36   non-aromatic compounds by a radical-catalyzed reaction mechanism (Claus, 2004). In this pathogen, two

37   genes have been found encoding laccases with molecular weights of about 60 kDa (Claus, Sabel, & König,

38   2014) and the molecular mass of the monomer ranges from about 50 to 100 kDa (Claus, 2004). An

39   important feature of laccases is to be very stable under wine conditions and they have serious impacts on the

40   phenolic composition and quality of musts and wines (More et al., 2011).

41   Under particular environmental and grape growing conditions, *B. cinerea* can affect positively the grapes by

42   causing "noble rot" leading to high-priced, natural sweet white wines, such as Tokaji Aszú, Sauternes or

43   Passito wines for example (Magyar, 2011). This particular infection pathway, associated with noble rot,

44   promotes favorable biochemical changes in grape berries, notably by the accumulation of secondary

45   metabolites enhancing the grape composition (Blanco-Ulate et al., 2015). Nevertheless, *B. cinerea*, can also

46   cause grey mould or botrytis bunch rot (BBR), responsible for huge economic losses each year for the wine

47   industry worldwide. Infection of bunch by the pathogen provokes serious biological and chemical changes

48   impacting negatively organoleptic qualities of wines (J. Ribéreau-Gayon, Ribéreau-Gayon, & Seguin,

49   1980). For a qualitative red wine, a vintage contaminated by the pathogen at harvest at the rate of 5% in

50   severity show irreversible consequences on the wine organoleptic features (Ky et al., 2012).

51   As recently reviewed and thoroughly investigated, when climatic conditions are wet under mild weather

52   conditions, *B. cinerea* can infect directly grape berries, in particular from veraison onwards (Ciliberti,

53   Fermaud, Roudet, & Rossi, 2015; Hill, Beresford, & Evans, 2018). The infection by the fungus begins in

54   the grape berry and continues in the grape juice, leading to the oxidation of polyphenols and then the

55   alteration of the color. The phenomenon of polyphenol oxidation is induced by the production of laccases

56   from *B. cinerea*. The laccases produced, oxidize the polyphenols into quinones which themselves

57   polymerized forming brown compounds. This reaction is called oxidase breakage and corresponds to a

58   color degradation and an increase in instability (Pourcel, Routaboul, Cheynier, Lepiniec, & Debeaujon,

59   2007). The oxidase breakage can take place in red grape juice as well as in white inducing brick tints or

60   brown tints, respectively. Moreover, changes in color due to the laccases produced by the pathogen are

61   accompanied by others changes in the organoleptic wine qualities which are impacted by changing their

62   equilibrium, body and/or mouthfeel (Claus et al., 2014). Several studies have shown that grey mold leads to

63   the development of organoleptic deviations in grapes and wines and depreciation of botrytized wines have

64   been attributed to off-flavors such as 'damp earth', 'vegetal/herbal like' and 'mushroom' (Ky et al., 2012).

65   Various authors have reported organoleptic defects, in musts as in wines made from rotten grapes, such as

66   mushroom, mouldy, camphoric odors (Lopez Pinar, Rauhut, Ruehl, & Buettner, 2016). Geosmin and 1-

67   octen-3-one have been also identified in musts and wines made from botrytized grapes (La Guerche,

68   Chamont, Blancard, Dubourdieu, & Darriet, 2005; La Guerche, De Senneville, Blancard, & Darriet, 2007).

69   Regarding mouthfeel, botrytized red wines were described as less astringent, according with a decrease of

70   their mDP (Ky et al., 2012).

71   Oenological tannins are usually classified in two families which are hydrolysable and condensed tannins.

72   Hydrolysable tannins include gallotannins and ellagitannins whereas condensed tannins include

73   procyanidins/prodelphinidins and profisetinidins/prorobitenidins. Each group of tannins present different

74   composition, nature, and chemical structure associated with different properties. During the winemaking

75   process, oenological tannins can be used at different doses, i.e. 5 to 50 g/hL of grape must, as a common

76 practice (Obradovic, Schulz, & Oatey, 2005). The oenological tannins are commonly used by winemakers

77 to improve and stabilize the color of red wines since they are efficient copigments and/or can form new

78 pigments (Neves, Spranger, Zhao, Leandro, & Sun, 2010; Versari, du Toit, & Parpinello, 2013).

79 Winemakers, also used them as antioxidant or antioxidasic compounds in order to protect wine against

80 oxidation (González-Centeno et al., 2012; Pascual et al., 2017; Vignault et al., 2018). They can also be

81 used in order to i) help protein fining and prevent protein haze (P. Ribéreau-Gayon, Dubourdieu, &

82 Donèche, 2006), ii) improve wine structure/mouthfeel (Preys et al., 2006) and iii) eliminate reduction odors

83 (Vivas, 2001). Nevertheless, until very recently , addition of oenological tannins was only accepted as an

84 aid practice to facilitate the fining of musts and wines, by the International Organization of Vine and Wine

85 (OIV, 2015). Very recently, OIV has also authorized its use for two new purposes. The first is to contribute

86 to the antioxidant protection of components of the must and wine, and their second is to promote the

87 expression, stabilization and preservation of color of wines (OENO-TECHNO 17-612 and OENO-

88 TECHNO 17-613).

89 Even though properties of oenological tannins have been widely described in the literature, antioxidasic

90 properties (anti-laccase) are not yet well documented. Until now, it is only possible to inactivate or inhibit

91 the laccase enzyme by thermovinification or by sulfur dioxide addition (P. Ribéreau-Gayon et al., 2006).

92 Inert gas or ascorbic acid in presence of sulfur dioxide may also be used to protect the grape juice against

93 oxidation (Li, Guo, & Wang, 2008). Nevertheless, in our previous study (Vignault et al., 2019), oenological

94 tannins have been shown as good candidates to prevent the damage induced by laccases produced by *B.*

95 *cinerea*. Oenological tannins allowed us to protect color of wines infected by *B. cinerea* and to reduce the

96 laccase activity.

97 The aim of our present research was to complete our previous study by understanding the mechanisms of

98 action of oenological tannins against laccases produced by *B. cinerea* (strain 213) in grapes and musts. Our

99 objective was also to compare the effects of oenological tannins against laccase activity in grapes/musts

100 with the two usual oenological additives used in the wine industry nowadays: ascorbic acid and sulfur

101 dioxide.

102 **2. Materials and methods**

103 *2.1. Chemicals and equipment*

104 All samples and standards were handled without any exposure to light. L-(+)-tartaric acid, sodium

105 hydroxide, sodium acetate, polyvinylpolypyrrolidone (PVPP), Tween 80, glycerol, gallic acid, L-histidine,

106 $CuSO_4$, $NaNO_3$, NaCl, KCl, $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, KH_2PO_4, $MgSO_4.7H_2O$, Bradford reagent, bovine

107 serum albumin (BSA) and syringaldazine were purchased from Sigma-Aldrich (Madrid, Spain). D-(+)-

108 glucose, peptone, agar and yeast extract were purchased from Panreac (Barcelona, Spain). Ethanol (96 %

109 vol.) and hydrochloric acid were supplied by Fisher Scientific (Madrid, Spain). Yeast (Zymaflore® Spark),

110 nutrients (Nutristart®) and bentonite (MICROCOL® ALPHA) were provided by Laffort (Floirac, France).

111 The equipment used was as follows: a spectrophotometer UV-Vis Helios Alpha™ (Thermo Fisher

112 Scientific Inc., Waltham, MA, USA); an incubator IPP 260 (DD Biolab, Barcelona, Spain); a centrifuge

3

113 Heraeus™ Primo™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); and a CB Standard Balance
114 (Cobos, Barcelona, Spain). All the materials for the micro-vinification were provided by the cellar "Mas
115 dels Frares" of the Enology Faculty of the Rovira i Virgili University (Constanti, AOC Tarragona, Spain).

116 *2.2. Commercial tannins*

117 Five commercial tannins, representing the main botanical origins, were used including three condensed
118 tannins: one procyanidin from grape seeds, one procyanidin/prodelphinidin from grape skin and one
119 profisetinidin from quebracho. Furthermore, two hydrolysable tannins were studied: one gallotannin from
120 nut gall and one ellagitannin from oak. All these tannins were provided by Laffort (Floirac, France).

121 *2.3. Fruit sampling, inoculation with B. cinerea and botrytized-must elaboration*

122 During the 2017 vintage, healthy grapes (*V. vinifera*, cv. Muscat d'Alexandrie; Variety number VIVC 8241)
123 were collected on September 18th (around 50 kg) from the experimental vineyard, planted in 1992, in the
124 Enology Faculty of the Rovira i Virgili, University in Constanti (AOC Tarragona; 41°8′54.17″ N and
125 1°11′53.89″ E). The vineyard is at 87 m height above sea level, and groundwater is located at a depth of
126 around 4 m. The vines were trained on a vertical trellis system and arranged in rows 2.80 m apart, with 1.20
127 m spacing between vines. They were pruned using a double "Cordon de Royat" system, with 16 buds, 8 on
128 each cane.
129 The *B. cinerea* single-spore isolate 213, originally isolated from grapevine leaf in 1998, was selected from
130 the collection of UMR SAVE, Bordeaux (Martinez et al., 2003). It was selected because of its virulence on
131 grapevine leaves and berries and because it is a *transposa* type strain (Ky et al., 2012; Martinez et al., 2003;
132 Martinez, Dubos, & Fermaud, 2005). The pathogen was cultured on Yeast Peptone Dextrose Petri plates
133 (YPD: 20 g/L of peptone and glucose; 10 g/L of yeast extract and 17 g/L of agar in distilled water) and
134 incubated about 1 week at 20°C before use. The harvested grapes were placed in five plastic boxes (600 x
135 400 x 200 mm) and were inoculated by spraying a spore suspension (1.10^6 conidia/mL; 1 drop of Tween 80;
136 50 g/L of glucose; sterilized water) until the complete fruit surface was covered by the spore suspension.
137 The plastic boxes containing the grapes were then incubated, for around three weeks, at 20°C surrounded by
138 two plastic boxes containing sterile water to maintain the relative humidity, i.e. RH ranging from 90% to
139 100%.
140 The botrytized grapes were beforehand sorted visually to remove undesirable rotten berries due to other
141 fungal development, such as notably *Penicillium spp* (blue-green color) and/or other fungal species
142 (*Alternaria spp*. or *Clostridium spp*.), or acetic bacteria (red-pink color). Then, these selected botrytized
143 grapes were crushed and pressed to obtain the botrytized grape juice using a small pneumatic press
144 (Venmhidrprei-040, Invia, Vilafranca del Penedes, Spain). The juice was recovered under dry ice in order to
145 keep it protected from oxidation, but without any addition of sulfur dioxide for not inhibiting laccase
146 activity. The botrytized grape juice was centrifuged at 8,500 rpm for 5 minutes and immediately stocked in
147 glass bottles at - 4°C.

148 *2.4. Laccase activity assays*

149 Tannins solutions were prepared in model wine solution (12% of ethanol, 4 g/L of tartaric acid and pH 3.5)
150 at different doses: 10, 20, 30 and 40 g/hL. Then, five different solutions were prepared by adding 4 ml of
151 botrytized must (obtained in 2.3) to 1 ml of each tannins solutions (samples) or 1 ml of model wine solution
152 (control). The mix was left four minutes in contact before being added by 0.8 g of PVPP, stirred and
153 centrifuged for 10 minutes at 8,500 rpm. Laccase activity was determined using the syringaldazine test
154 method as previously described (Vignault et al., 2019). All analyses were performed in triplicate. A laccase
155 unit (UL) corresponds to the amount of enzyme catalyzing the oxidation of a micromole of syringaldazine
156 per minute. The following equation was used for the calculation of laccase activity by using the slope from
157 a calibrating linear regression (ΔA) expressed in absorbance units/minute:

158
$$Laccase\ activity = 46.15\ x\ \Delta A\ \mu mol.\,L^{-1}.\,min^{-1} = 46.15\ x\ \Delta A\ UL$$

159 Moreover, the residual laccase activity was determined for each sample using the following equation:

160
$$\%\ of\ residual\ activity = (laccase\ activity\ sample/laccase\ activity\ control)\ x\ 100$$

161 *2.5. Determination of kinetics parameters (Km and Vmax)*

162 Solutions were prepared (see previous paragraph 2.4) and the enzyme kinetic of the *B. cinerea* laccase was
163 studied using different concentrations of syringaldazine as the substrate. The Michaelis-Menten plot was
164 represented for each sample allowing us to represent the Lineweaver-Burk curve (double reciprocal plots).
165 Then, the values of K_M and V_{max} were determined from the linear regression of double reciprocal plots
166 obtained by varying the final concentration of syringaldazine in the reaction medium from 0 to 33 μM.

167 *2.6. Influence of bentonite treatment*

168 Increasing volumes of a 200 g/hL bentonite suspension were added to 3 mL of botrytized must (as obtained
169 in 2.3) to reach final bentonite concentration between 0 and 100 g/hL. The samples were left 20 minutes in
170 contact before being added by 0.5 g of PVPP, stirred and centrifuged for 5 min at 8,500 rpm. Laccase
171 activity was then determined as previously described (paragraph 2.4).

172 *2.7. Extracellular laccase production*

173 Extracellular laccases were produced from the strain 213 of *B. cinerea* as previously described (paragraph
174 2.3). The strain was kept as a stock suspension of spores in 20% glycerol and stored at -80°C. *B. cinerea*
175 laccases were produced as previously described by (Quijada-Morin et al., 2018) with some modifications.
176 Briefly, cultures on YPD medium were kept for 1 week at 20°C in incubator to induce growth and
177 sporulation. The spores were then gently scraped from one 55 mm diameter Petri dish to inoculate 125 mL
178 of sterilized culture medium in an Erlenmeyer flask of 500 mL. Sterilized culture medium was composed as
179 followed (in g/L): glucose, 40; glycerol, 7; L-histidine, 0.5; $CuSO_4$, 0.1; $NaNO_3$, 1.8; NaCl, 1.8; KCl, 0.5;
180 $CaCl_2.2H_2O$, 0.5; $FeSO_4.7H_2O$, 0.05; KH_2PO_4, 1.0; and $MgSO_4.7H_2O$, 0.5. After 3 days of incubation (20°C,
181 dark, 140 rpm), 100 mL of these pre-cultures were transferred into an Erlenmeyer flask of 5 L containing

182   1.4 L of the culture medium. After 2 days of growth (20°C, dark, 140 rpm), gallic acid was added at 2 g/L

183   and cultures were maintained 5 more days under the same conditions. Then, the liquid medium was filtered

184   through a filter paper and kept until use at -80°C. Fresh fungal biomass was lyophilized and dried biomass

185   weighed. Protein concentration was determined by the Bradford method using bovine serum albumin (BSA)

186   as standard (Okutucu, Dınçer, Habib, & Zıhnıoglu, 2007). Initial laccase activity of the liquid medium was

187   also determined.

188

189   *2.8. Enzyme electrophoresis*

190   *B. cinerea* laccase was analyzed by SDS-PAGE as previously described (Cilindre, Castro, Clément, Jeandet,

191   & Marchal, 2007) with some modifications. Briefly, 10 µL of the enzyme solution was mixed with

192   Laemmli buffer: 62.5 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol and 0.01% bromophenol blue. The gel

193   was a 10% Mini-PROTEAN® TGX™ Gel (Bio-Rad Laboratories, Hercules, CA, USA) and the

194   electrophoresis was carried out at 120 V, using the Mini-PROTEAN® Tetra Vertical Electrophoresis Cell

195   (Bio-Rad Laboratories, Hercules, CA, USA). A Spectra™ Multicolor Broad Range Protein Ladder (10-260

196   kDa, Thermo Fisher Scientific Inc., Waltham, MA, USA) was included in each electrophoresis run as

197   standards for molecular weight estimation. The gel was stained with a Pierce™ Silver Stain Kit (Thermo

198   Fisher Scientific Inc., Waltham, MA, USA). A digitized image of the gel was obtained by photography. The

199   molecular weight, intensity and relative quantity in proteins of the bands were determined with GeneTools

200   analysis software (Syngene, Cambridge, UK).

201   *2.9. Statistical analysis*

202   All the chemical and physical data were expressed as mean values ± standard deviation. The statistical

203   analyses were carried out using the XLSTAT 2017 statistical package. The normality and homoscedasticity

204   of the data were tested for all parameters by using the Shapiro-Wilk test and Levene's test, respectively.

205   When populations were distributed normally and presented homogeneity in variance, parametric tests were

206   used, i.e. ANOVA and Tukey. In contrast, when data were not distributed normally and/or presented

207   heterogeneity in variance, non-parametric tests were used (Kruskal-Wallis and Pairwise-Wilcox).

208   Differences were statistically significant at *p*-value < 0.05.

209   **3. Results and discussion**

210   *Enzyme kinetics*

211   As expected, laccase showed a classical Michaelis-Menten kinetics since the reaction rate shows a first-

212   order kinetics behavior when the substrate concentration is low and becomes a zero-order kinetics when

213   the substrate concentration is saturating. Supplementation with increasing concentration of oenological

214   tannins, sulfur dioxide and ascorbic acid in botrytized must induced a diminution of the maximal reaction

215   rate (V_{max}) (Figure 1) which confirm that all these additives exert an inhibitory effect on laccase activity.

216   However, this inhibitory effect does not seem to be similar for all these products. Oenological tannins and

217   sulfur dioxide seems to exert uncompetitive inhibition since the Lineweaver-Burk plots of laccase kinetics

218   in the presence of these additives were parallel with that obtained without any addition (Figure 2). An

219   uncompetitive inhibition takes place when an enzyme inhibitor (I) binds only to the complex "ES" formed

220   by the enzyme (E) and the substrate (S). This phenomenon induced the reduction of the concentration of

221   ES complex which can be explained by the fact that ES complex may be essentially converted into ESI

222   complex ("enzyme-substrate-inhibitor"), thus considered a separate complex altogether. This reduction in

223   ES complex concentration decreased V_{max}, since it takes longer for the substrate or product to leave the

224   active site. The reduction of the binding affinity (K_M) can also be linked back to the decrease in ES

225   complex concentration.

226   In contrast, ascorbic acid seems to exert a noncompetive inhibition since Lineweaver-Burk plots of

227   laccase kinetics in the presence of this additive the x intercept stays constant in comparison with the

228   control plot. In non-competitive inhibition, the inhibitor will bind to an enzyme at its allosteric site;

229   therefore, the K_M of the substrate with the enzyme will remain the same. On the other hand, the V_{max} will

230   decrease relative to an uninhibited enzyme.

231   In this way, Michaelis-Menten (Figure 1) and Lineweaver-Burk plots (Figure 2) allowed to qualify

232   oenological tannins and sulfur dioxide as uncompetitive inhibitors, whereas ascorbic acid can be rather

233   qualified as non-competitive inhibitor. To confirm this graphical statement, kinetics parameters were

234   calculated as follows.

235   *Kinetic parameters (Determination of K_M and V_{max})*

236   The values of V_{max} and K_M were calculated by the extrapolation of the curve obtained from the Lineweaver-

237   Burk plot. The enzyme obtained for the *B. cinerea* strain 213 had a V_{max} of 0.364 ± 0.026 µmol/L · min and

238   a K_M of 0.697 ± 0.133 µmol/L.

239   According to the previous statement, oenological tannins and sulfur dioxide presented, as excepted, a

240   significant decrease in their V_{max} and K_M compared to the control (without any inhibitor) (**Table 1**).

241   Likewise, except for quebracho tannin, the significant differences of V_{max} were already shown at the lowest

242   dose of inhibitor compared to the control (**Table 1**). Nonetheless, at the highest dose, sulfur dioxide

243   presented the greatest ability to reduce the V_{max} (0.003 ± 0.002 µmol/L · min), while ellagitannin (0.274 ±

244   0.002 µmol/L · min) had the lowest ability. The other oenological tannins presented similar effects (0.201 ±

245   0.007 µmol/L · min to 0.220 ± 0.006 µmol/L · min), since no significant differences were observed between

246   them (**Table 1**). All the inhibitors tested, i.e. oenological tannins and sulfur dioxide, at the highest dose

247   showed the same ability to decrease K_M without any significant difference. The initial K_M was diminished

248   from 0.697 ± 0.133 µmol/L (control) at 0.417 ± 0.048 µmol/L (gallotannin) to 0.506 ± 0.019 µmol/L

249   (ellagitannin). Nevertheless, for both ellagitannin and quebracho tannin, the lowest dose was not enough to

250   diminish significantly the enzyme's K_M compared to the control. Indeed, 40 g/hL and 20 g/hL were

251   necessary, respectively. However, 10 mg/L and 25 g/hL were enough for sulfur dioxide and the other

252   oenological tannins, respectively.

253   In the case of ascorbic acid, as it was a non-competitive inhibitor, the enzyme's V_{max} decreased significantly

254   with addition of the lowest dose (50 mg/L) compared to the control (0 mg/L). Moreover, at the highest dose

255   (200 mg/L), V_{max} reached values three fold time lower than control, ranking from 0.364 ± 0.026 µmol/L ·

256   min to 0.156 ± 0.004 µmol/L · min. However, K_M values remained constant, since no significant differences

257 were observed with control, even if values were ranked between 0.667 ± 0.121 µmol/L (100 mg/L) and

258 0.996 ± 0.137 µmol/L (200 mg/L).

259 Based only on the determination of V_{max} and K_M parameters, it was then not possible to compare the effect

260 of enological tannins with ascorbic acid, since they are different type of inhibitors. It must also be

261 considered that laccase enzyme, in our reaction conditions, has two subtracts, syringaldazine and oxygen.

262 Oenological tannins, sulfur dioxide and ascorbic acid can react with oxygen, decreasing therefore its

263 concentration in the medium (Pascual et al., 2017; Vignault et al., 2018). Consequently, this reduced the

264 V_{max} of the reaction. However, considering the oxygen consumption rate of oenological tannins and sulfur

265 dioxide, as reported by (Pascual et al., 2017), the oxygen consumption by these compounds can be

266 considered as negligible. Indeed, the oxygen consumption of oenological tannins and sulfur dioxide at

267 normal concentrations (400 mg/L for oenological tannins and 50 mg/L for sulfur dioxide) represent less

268 than 0.2 % compared with the consumption capacity of laccase in our botrytized grape juice, for 30 UL

269 (Figure 3). In contrast, ascorbic acid consumes oxygen much faster and can really compete with laccase

270 (Pascual et al., 2017). Specifically, the oxygen consumption rate of 100 mg/L of ascorbic acid is around the

271 50 % of the oxygen consumption rate of the botrytized must use with a laccase activity of around 30 UL.

272 Thus, the inhibitory effect of ascorbic acid on laccase activity seems to result from a competition with

273 laccase for the oxygen present in the medium.

274 In this way, the residual activity was additionally determined, for each sample, to make possible the

275 comparison between the different oenological tannins.

Residual laccase activity

277 Initial activity of the control was 27.3 ± 0.6 UL and was considered as the 100 ± 6.40% of residual activity

278 (**Table 1**). All the samples tested, including oenological tannin, sulfur dioxide and ascorbic acid, diminished

279 significantly the residual activity at the lowest dose tested. Sulfur dioxide clearly appeared as the most

280 efficient to reduce laccase activity, since with 100 mg/L, no residual laccase activity was detected (0.9 ±

281 0.4%). Ascorbic acid and grape-seed tannin appeared also as great candidates to reduce laccase activity,

282 since at the highest dose (200 mg/L and 40 g/hL respectively), only 11.5 ± 3.2% and 12.8 ± 6.4% of the

283 laccase activity remained present, respectively (**Table 1**). Similarly, gallotannin and grape-skin tannin at the

284 highest dose presented an equivalent effect, since they reduced by half the laccase activity: 44.6 ± 7.8%

285 and 50.7 ± 5.5%, respectively (**Table 1**). Nevertheless, gallotannins reached this value at the lowest dose

286 used (10 g/hL), while grape-skin tannin only reduced around by 35% the laccase activity at the lowest dose

287 (65.6 ± 3.2%). Concerning ellagitannin and quebracho tannin, their ability to reduce laccase activity was

288 clearly lower than in the case of the other oenological tannin. Indeed, including at the highest dose (40

289 g/hL), they were able to reduce the activity only between 20 and 30%, respectively (**Table 1**).

Influence of bentonite treatment

291 Bentonite is an inorganic clay finning agent, negatively charged, which is responsible for the binding of

292 proteins who have a net positive charge at wine pH, resulting in their removal from the wine (Vincenzi,

293 Panighel, Gazzola, Flamini, & Curioni, 2015). Bentonite is universally used in the wine industry to remove

wine proteins by electrostatic adsorption, because it is efficiency , low cost, and because its use needs a simple batch process that does not require any specialized equipment or knowledge (Lira et al., 2015). Laccases produced by *B. cinerea* are proteins which have a isoelectric point lower than usual wine pH (Baldrian, 2006) and, consequently, they are charged negatively at wine pH. Theoretically they cannot be removed with bentonite. The influence of a bentonite treatment on botrytized white must is showed in **Figure 3**. No significant differences were observed regarding laccase activity, by comparing the botrytized white must not treated and the botrytized must treated at different doses of bentonite. The initial laccase activity was around 12 µmol.L^{-1}.min^{-1} and remained stable when the botrytized must was previously treated with bentonite. According to these results, it was decided to use bentonite treatment for the enzyme electrophoresis when it was necessary to remove other proteins without affecting laccase activity .

Enzyme production

The strain 213 produced extracellular enzymes when cultured under the conditions described in material and methods (section 2.7.). The amount of the dry fungal biomass was 0.0429 g/mL and the content of protein found in the culture medium was 0.15 mg/mL. The yield of protein per gram of dry fungal biomass was 3.49 mg/g for the strain 213. These results were in accordance with previous published ones (Quijada-Morin et al., 2018) in similar conditions for three other different *B. cinerea* strains: yields values ranging between 2 and 5 mg/g.

Enzyme electrophoresis

The SDS-PAGE of laccase sample showed the presence of only one band corresponding to the laccase proteins (**Figure 4** – well 1). The strain produced laccase enzymes with a molecular weight of 97 kDa, which is in accordance with previous results published (Quijada-Morin et al., 2018; Slomczynski, Nakas, & Tanenbaum, 1995). Nevertheless, values ranging from 36 kDa to 97 kDa have been also reported, suggesting a great variability between the enzymes with laccase activity produced by different strains of the pathogen. When bentonite (100 g/hL) was added to laccase sample (**Figure 4** – well 2), a part of proteins was removed, since the band at 97 kDa appeared weaker suggesting that bentonite has eliminated other proteins of similar molecular weight but not laccase since its enzymatic activity remains stable. The SDS-PAGE of the white wine (**Figure 4** – well 3) showed the presence of two bands with a molecular weight of 57 kDa and 20 kDa, as previously reported (Esteruelas et al., 2009). When bentonite was added to the wine (**Figure 4** – well 4), these two proteins bands were not detectable. It has been previously shown (Sauvage, Bach, Moutounet, & Vernhet, 2010) that more than 50% of the whole protein content was removed with only 50 g/hL of bentonite, but around 15% of this protein content remained non-adsorbed even for a bentonite concentration as high as 150 g/hL. This means that the dose of 100 g/hL could be the most appropriate in order to remove all the protein content, nevertheless such a high addition of bentonite can decrease the quality of the wines (Sauvage et al., 2010). After bentonite treatment of laccase samples and white wine, 70% and 100% of the proteins were removed respectively (**Table 2**). Therefore, at this dose, bentonite was able to remove all the proteins from wine. However, bentonite was not able to remove all proteins of laccase sample since 30% of them remained after the treatment. These results are not in

331 accordance with those from (Riebel et al., 2017) in which laccase was not removed by bentonite fining..
332 Nevertheless, as it has been commented above, laccase activity is not impacted by bentonite treatment
333 (**Figure 3**), meaning that, probably, bentonite have removed some other proteins of similar molecular
334 weight but not laccase. Additionally, the SDS-PAGE of white wine supplemented by laccase sample
335 (**Figure 4** – well 5) presented three band at 97 kDa, 57 kDa and 20 kDa corresponding to the bands
336 obtained for laccase sample and white wine proteins in wells 1 and 3, respectively. When bentonite was
337 added to the mixture made of the white wine and laccase (**Figure 4** – well 6), only the band corresponding
338 to laccases remained visible. This result confirms once again the ability of bentonite to precipitate white
339 wines proteins, but not laccase.
340 Regarding the ability of oenological tannins to precipitate laccase proteins in white wine treated with
341 bentonite, grape tannins (grape-seed and grape-skin) followed by gallotannin were the most efficient ones
342 (**Figure 4** – well 7, 10 and 11). Grape-seed and grape-skin led to precipitate 77.8% and 74.5% of laccase
343 proteins, respectively (**Table 2**). Gallotannin allowed to precipitate almost 50% of laccase proteins (**Table
344 2**). In contrast, ellagitannin and quebracho tannin did not present any ability to precipitate laccase proteins
345 (**Figure 4** – well 9 and 12), since 100% of laccase proteins remained present (**Table 2**). These results are in
346 accordance with the previous ones obtained in **Table 1**, in which grape tannins and gallotannins were the
347 most efficient products tested to reduce laccase activity. Nevertheless, gallotannin showed a lower ability to
348 precipitate laccase proteins, but presented similar kinetics parameters and ability to reduce laccase activity
349 than grape-skin tannin. This could be explained by the fact that, on one hand, oenological tannins can
350 precipitate laccase proteins, thus reducing laccase activity, or on the other hand, by inhibition of laccase
351 activity without protein precipitation. Indeed, the binding of an inhibitor and its effect on the enzymatic
352 activity are two distinct and quite different mechanisms. According to this hypothesis, grape tannins and
353 gallotannin presented the ability to inhibit laccase activity and to precipitate (bounds) laccase proteins.
354 Specifically, gallotannin was the most efficient to inhibit laccases produced by *B. cinerea*, whereas grape-
355 skin tannin was most efficient regarding laccase proteins precipitation. Additionally, grape-seed tannin was
356 the most efficient in the oenological tannins to inhibit laccase activity and precipitate laccase proteins.
357 Concerning quebracho tannin, it should be noted its ability to inhibit laccase activity, but its ability to
358 precipitate laccase proteins was almost inexistent. Ellagitannin presented the lowest ability to inhibit and to
359 precipitate laccase from *B. cinerea*. Nevertheless, even though ellagitannin presented low ability to inhibit
360 laccase, it can reduce the activity by 20%.

361 **4. Conclusions**

362 As expected, bentonite did not permit to reduce laccase activity in white must, even if it was responsible
363 for precipitation of other proteins similar in molecular weight to laccase from *B. cinerea* strain 213. Oe-
364 nological tannins were characterized by different abilities regarding prevention of *B. cinerea* damage,
365 since they showed different ability to precipitate laccases produced and/or reduce their activity. They
366 were all uncompetitive inhibitors, but gallotannin, grape-skin and even more so grape-seed tannin pre-
367 sented the greatest ability to reduce V_{max}, K_M and residual laccase activity. Addition of grape-seed tannin
368 was efficient to reduce laccase activity with a similar level effect than that of ascorbic acid and that of
369 sulfur dioxide at traditional doses employed in oenology during winemaking for each one. Our results

370 confirmed our previous study (Vignault et al., 2019), and demonstrated that oenological tannins and
371 more specifically, grape tannins and gallotannin, are excellent processing aids to prevent *B. cinerea* dam-
372 age in grape musts. Oenological tannins used on botrytized grapes should permit also to decrease sulfur
373 dioxide use during winemaking. Further studies are required to further study the laccase inhibitory mech-
374 anisms of oenological tannins, but the actual findings justify why the OIV has included this functionality
375 in the OIV International Oenological Codex.

376

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503

Figure 1. Michaëlis-Menten plots to visualize the kinetics of laccase and the inhibitory effect of oenological tannins (A, B, C, D, E), SO$_2$ (F) or ascorbic acid (G)
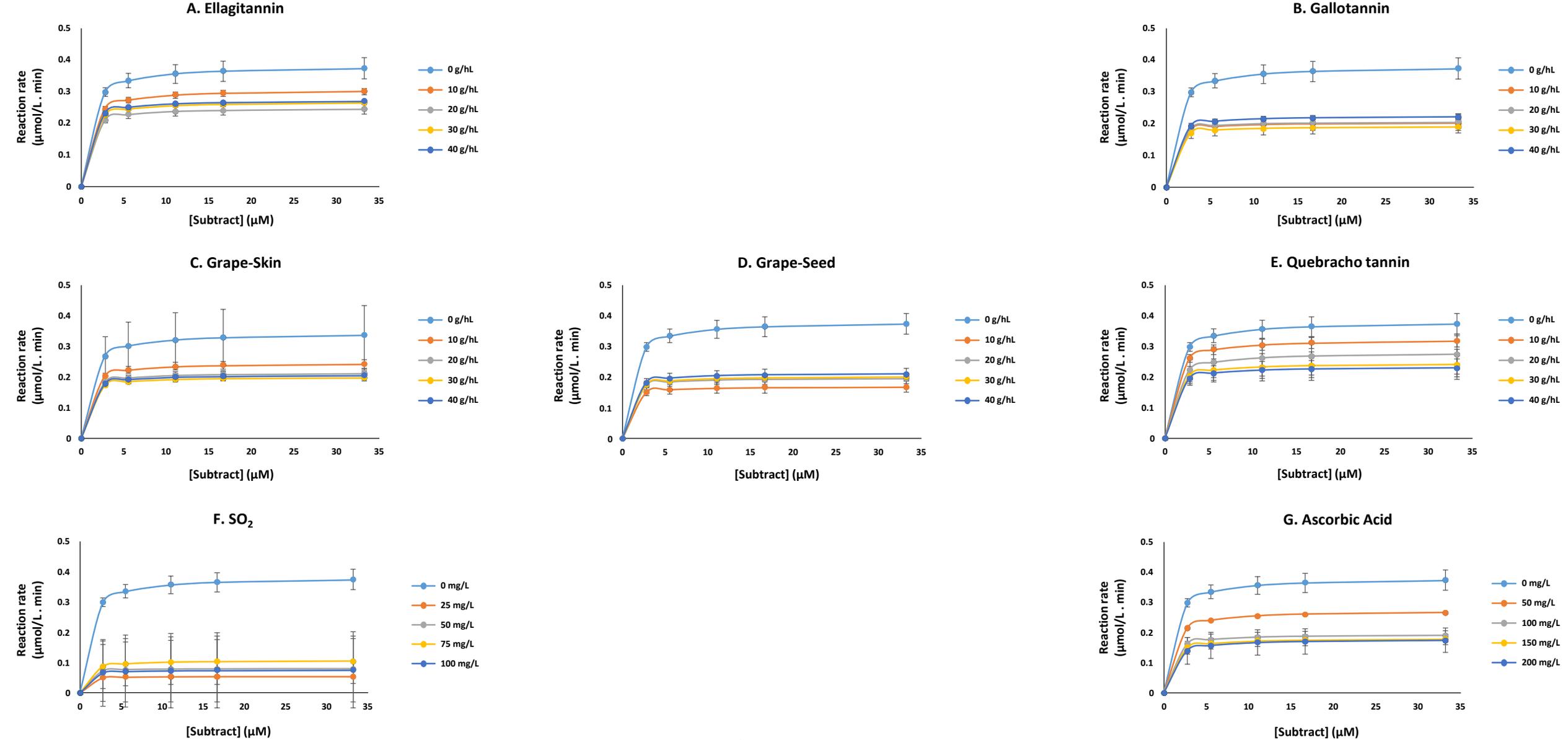
All data are the mean of triplicate. Mean ± Standard deviation

Figure 2. Lineweaver-Burk plot for determining the kinetic constants of laccase and the inhibitory effect of oenological tannins (A, B, C, D, E), SO₂ (F) or ascorbic acid (G)

All data are the mean of triplicate. Mean ± Standard deviation
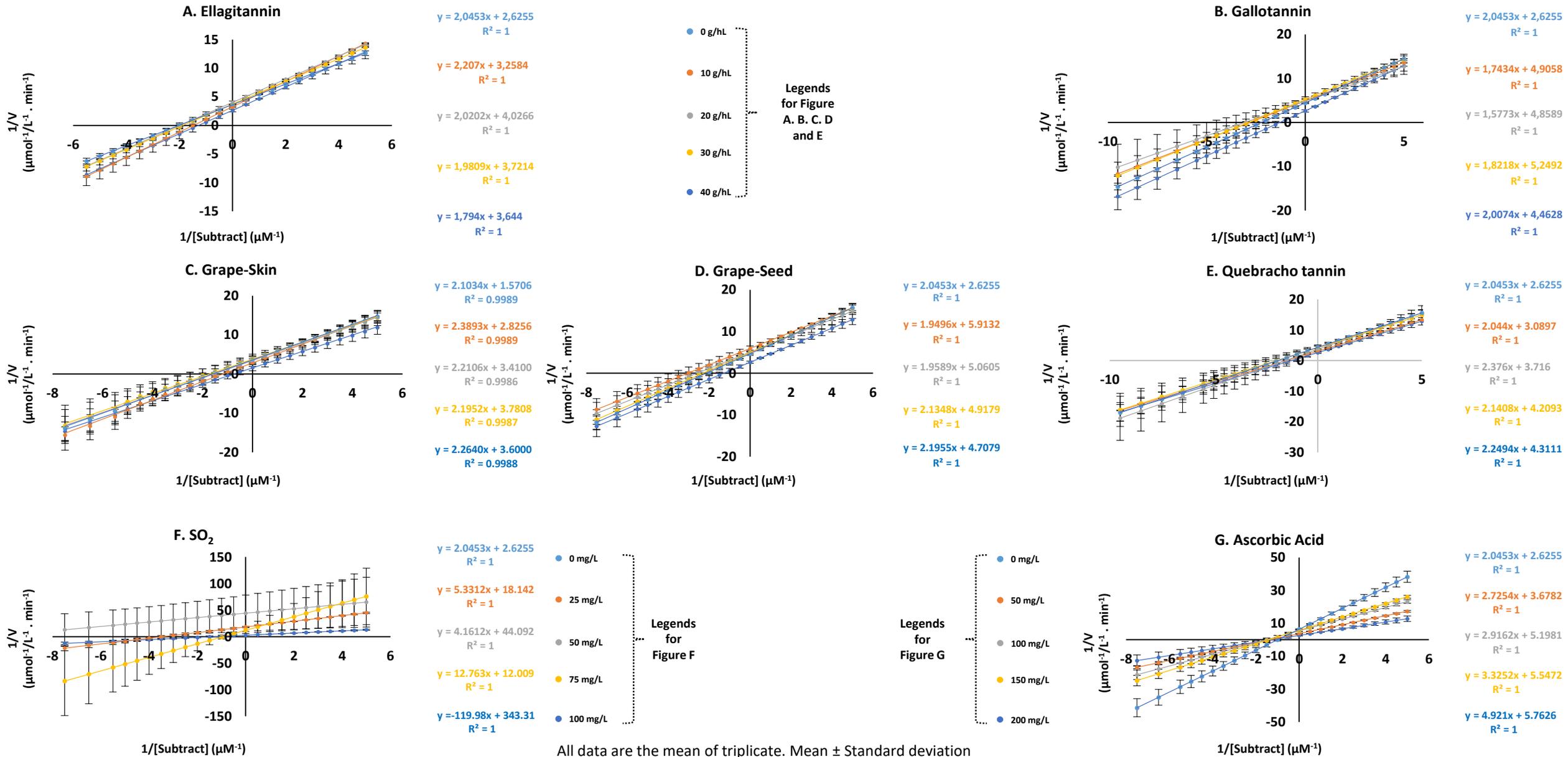
Figure 3. Influence of bentonite treatment at different doses on laccase activity of botrytized white must
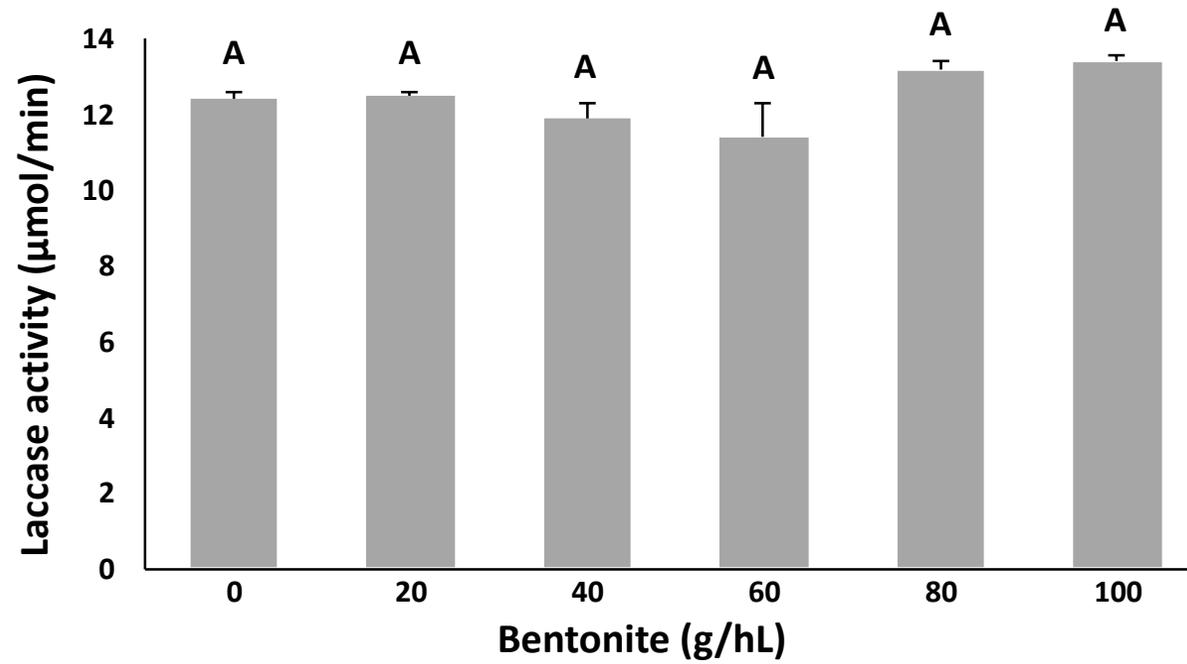
All data are the mean of triplicate. Mean ± Standard deviation

Figure 4. Electrophoresis gels of laccases produced by *Botrytis cinerea* and supplemented by oenological tannins (40 g/hL) with a bentonite treatment (200 g/hL)

M: Molecular Weight marker
1: Laccase (L)
2: Laccase + Bentonite (B)
3: White wine (Ww)
4: Ww+ B
5: Ww+ L
6: Ww+ L + B
7: Ww+ L + B + Gallotannin 40 g/hL
8: Ww+ L + B
9: Ww+ L + B + Ellagitannin 40 g/hL
10: Ww+ L + B + Grape-seed tannin 40 g/hL
11: Ww+ L + B + Grape-skin tannin 40 g/hL
12: Ww+ L + B + Quebracho tannin 40 g/hL
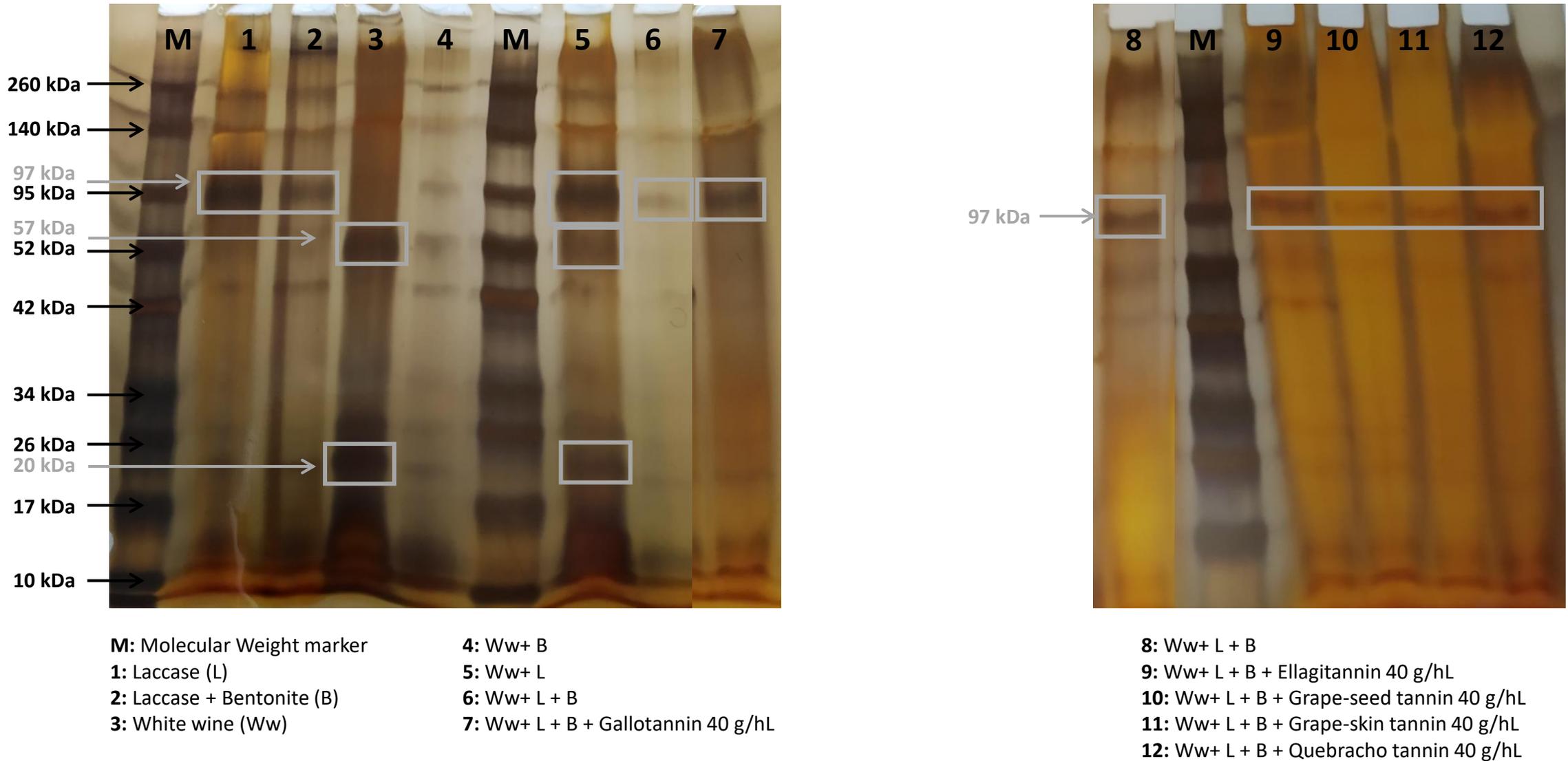
The arrows indicate the different molecular weight of the corresponding bands. The brackets correspond to the different bands of interest.

Table 1. Kinetics parameters (Vmax and Km) and residual laccase activity of botrytized white must added by oenological tannins or SO$_2$ or ascorbic acid at different concentration

Samples	Doses	V_{max} (µmol/L . min)			K_M (µmol/L)			Residual activity (%)		
Ellagitannin (g/hL)	0	0.364 ± 0.026	A	α	0.697 ± 0.133	A	α	100.0 ± 6.40	A	α
	10	0.307 ± 0.010	B	α	0.678 ± 0.024	A	α	78.4 ± 6.4	B	α
	20	0.257 ± 0.011	C	β	0.534 ± 0.042	AB	αβ	80.4 ± 3.2	B	α
	30	0.269 ± 0.006	C	α	0.525 ± 0.011	AB	β	76.0 ± 3.9	B	α
	40	0.274 ± 0.002	C	α	0.506 ± 0.019	B	β	81.8 ± 3.2	B	α
Gallotannin (g/hL)	0	0.364 ± 0.026	A	α	0.697 ± 0.133	A	α	100.0 ± 6.4	A	α
	10	0.212 ± 0.010	B	γ	0.396 ± 0.053	B	β	41.5 ± 3.9	B	γ
	20	0.221 ± 0.019	B	βγ	0.407 ± 0.100	B	αβ	39.9 ± 6.4	B	γ
	30	0.202 ± 0.015	B	β	0.364 ± 0.020	B	β	45.6 ± 3.9	B	γ
	40	0.220 ± 0.006	B	β	0.417 ± 0.048	B	β	44.6 ± 7.8	B	γ
Quebracho (g/hL)	0	0.364 ± 0.026	A	α	0.697 ± 0.133	A	α	100.0 ± 6.4	A	α
	10	0.334 ± 0.013	A	α	0.663 ± 0.037	A	α	73.6 ± 3.2	B	αβ
	20	0.318 ± 0.043	AB	α	0.571 ± 0.032	B	αβ	80.4 ± 8.5	B	α
	30	0.245 ± 0.051	BC	αβ	0.411 ± 0.088	B	β	73.6 ± 17.8	B	αβ
	40	0.217 ± 0.010	C	β	0.462 ± 0.015	B	β	67.9 ± 11.7	B	αβ
Grape-skin (g/hL)	0	0.364 ± 0.026	A	α	0.697 ± 0.133	A	α	100.0 ± 6.4	A	α
	10	0.248 ± 0.015	B	β	0.620 ± 0.061	AB	α	65.6 ± 3.2	B	β
	20	0.215 ± 0.015	BC	βγ	0.386 ± 0.070	C	αβ	61.5 ± 3.2	BC	β
	30	0.200 ± 0.010	C	β	0.386 ± 0.052	C	β	53.7 ± 3.9	BC	βγ
	40	0.201 ± 0.007	C	β	0.426 ± 0.036	BC	β	50.7 ± 5.5	C	βγ
Grape-seed (g/hL)	0	0.364 ± 0.026	A	α	0.697 ± 0.133	A	α	100.0 ± 6.4	A	α
	10	0.180 ± 0.013	B	δ	0.295 ± 0.057	B	β	13.5 ± 3.2	B	δε
	20	0.209 ± 0.001	B	βγ	0.343 ± 0.009	B	αβ	16.2 ± 0.0	B	δ
	30	0.210 ± 0.005	B	β	0.481 ± 0.025	B	β	15.6 ± 3.2	B	δ
	40	0.214 ± 0.019	B	β	0.470 ± 0.072	B	β	12.8 ± 6.4	B	δ
SO$_2$ (mg/L)	0	0.364 ± 0.026	A	α	0.697 ± 0.133	A	α	100.0 ± 6.4	A	α
	25	0.055 ± 0.003	B	ε	0.274 ± 0.031	B	β	4.1 ± 0.0	B	δε
	50	0.016 ± 0.001	C	δ	0.237 ± 0.141	B	β	2.0 ± 0.0	B	ε
	75	0.065 ± 0.000	B	γ	0.349 ± 0.000	B	β	1.4 ± 1.0	B	δ
	100	0.003 ± 0.002	C	δ	0.451 ± 0.000	B	β	0.9 ± 0.4	B	δ
Ascorbic acid (mg/L)	0	0.364 ± 0.026	A	α	0.697 ± 0.133	A	α	100.0 ± 6.4	A	α
	50	0.272 ± 0.003	B	β	0.741 ± 0.030	A	α	21.3 ± 3.9	B	δ
	100	0.203 ± 0.007	C	γ	0.667 ± 0.121	A	α	12.8 ± 0.0	B	δε
	150	0.193 ± 0.001	C	β	0.724 ± 0.153	A	α	19.6 ± 3.2	B	δ
	200	0.156 ± 0.004	D	γ	0.996 ± 0.137	A	α	11.5 ± 3.2	B	δ

All data are the mean of triplicate. Mean ± Standard deviation. Capital letters indicate significant differences between the different doses for the same sample (p<0.05). Greek letters indicate significant differences between samples for the same doses (p<0.05)

Table 2. Molecular weight and intensity of the bands of laccases produced by Botrytis cinerea; Influence of treatment with oenological tannins (40 g/hL) and with bentonite (200 g/hL)

		1	2	3	4	6	7	9	10	11	12
Band 1 (laccase)	MW (kDa)	97	97			97	97	97	97	97	97
	Quantity (%)	100.0	31.6			100.0	48.3	94.4	22.8	25.5	105.3
Band 2 (wine)	MW (kDa)			57	57	57					
	Quantity (%)			100.0	n.d	n.d					
Band 3 (wine)	MW (kDa)			20	20	20					
	Quantity (%)			100.0	n.d	n.d					

1: Laccase (L)
2: Laccase + Bentonite (B)
3: White wine (Ww)
4: Ww+ B
6: Ww+ L + B

7: Ww+ L + B + Gallotannin 40 g/hL
9: Ww+ L + B + Ellagitannin 40 g/hL
10: Ww+ L + B + Grape-seed tannin 40 g/hL
11: Ww+ L + B + Grape-skin tannin 40 g/hL
12: Ww+ L + B + Quebracho tannin 40 g/hL