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Oxidative behavior of (+)-catechin in the presence of inactive dry yeasts: A comparison with sulfur dioxide and other wine additives and components

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Oxidative behavior of (+)-catechin in the presence of inactive dry yeasts: A comparison with sulfur dioxide and other wine additives and components

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Running Title

Antioxidant capacity of yeast derivatives compared with other wine additives and components

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Abstract

BACKGROUND: The antioxidant capacity of an inactive dry yeast preparation (YD) was investigated by conventional analytical methods (spectrophotometry, HPLC) as well as by cyclic voltammetry (CV), in a (+)-catechin model solution and compared with certain of the most common antioxidants found in wine: sulfur dioxide, ascorbic acid and glutathione.

RESULTS: Sulfur dioxide (SO_2) was the highest performing substance in protecting (+)-catechin against browning, followed by ascorbic acid and the YD preparation. Sulfites were the only antioxidant whose activity was clearly detectable in the model wines after 29 days of storage. Voltammetric studies demonstrated that the antioxidant capacity of the products tested was connected to their intrinsic characteristics and their molar concentrations (catechin/antioxidant molar ratio).

CONCLUSION: The YD preparation displayed a certain ability to protect polyphenols against browning. The antioxidant activity of YDs towards (+)-catechin appeared to be based on different mechanisms with respect to that of the other products tested: the insoluble portion of these preparations (cell wall residues) might have a non-negligible role, even if the ability of YDs to release compounds able to suppress oxidation cannot be rejected. The direct comparison of the different antioxidants led to interesting indications, concerning their mechanism of action in wine-like solution, depending on their concentration and intrinsic characteristics.

KEYWORDS: Yeast derivatives; sulfur dioxide; ascorbic acid; glutathione; (+)-catechin oxidation; wine

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Introduction

The relationship between oxygen and wine is one of the key points of modern winemaking. Various papers have described the mechanisms of wine oxidation and the role of metal ions (iron and copper) and reactive oxygen species (ROS), in the formation of quinones (originated by reaction of such free radicals with polyphenols) and certain low molecular weight compounds, such as acetaldehyde or pyruvic acid (produced by the same radicals from ethanol or organic acids) [1-4]. In the presence of slow and steady aeration, such reactions may evolve with positive results on the color stability and sensory characteristics of the wine, [5] but if the oxygenation becomes massive and uncontrolled, the same reactions can lead to the accumulation of compounds responsible for browning and generation of off flavors [6].

Sulfur dioxide (SO_2) is a fundamental antioxidant additive due to its ability to act at key points in such oxidation mechanisms. SO_2 is able to react with quinones to regenerate phenolic molecules or yield sulfonic adducts, [7] and it might react with hydrogen peroxide (an important intermediate compound in the reduction chain of oxygen), thus hampering the propagation of radical chains [7,8]. Finally, sulfur dioxide might also react with acetaldehyde and carbonyl compounds [2] to limit the formation of brown pigments and the genesis of off flavors. However, SO_2 is a toxic and allergenic substance [9], and thus, despite these positive considerations, the current trend is to minimize its concentration in wine.

Although different alternatives are available as a replacement for sulfites with respect to their antimicrobial activity (e.g., lysozyme, dimethyl dicarbonate), their replacement as an antioxidant is more complicated, and the solutions available are generally described as complementary tools rather than real alternatives [10].

Inactive dry yeasts (YDs) have been recently included among these complementary tools, probably due to their similarity to yeast lees. In recent studies, certain of these preparations, whether enriched in glutathione (GSH) or not, demonstrated their protective ability towards

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aroma compounds in both wine and a model solution [11,12]. This effect was ascribed to their claimed antioxidant capacity and particularly to their glutathione content [11], and also to the release of selected antioxidant peptides containing methionine, tryptophan, and tyrosine [12].

More recently, a thermally produced yeast autolysate was effective in reducing the color evolution of white wines during a period of fifteen days and up to eight months of storage [13]. The effect of the addition of such autolysate on color protection was second only to that of SO_2, because it was more intense than the effects of the other additives tested (ascorbic acid, glutathione and fresh lees).

Despite these interesting observations and the wide use of inactive dry yeasts in wineries, few publications have addressed their antioxidant capacity from a strictly scientific point of view. For this reason, this paper investigates the potential of an inactive dry yeast preparation obtained by thermolysis in reducing the oxidation of (+)-catechin in wine-like solution. The effect on oxygen consumption, the evolution of color and the formation of oxidation products were investigated and compared with that of sulfur dioxide and two natural wine antioxidants: glutathione and ascorbic acid. Moreover, cyclic voltammetry was used to examine how the different antioxidants tested might affect the electrocatalytic behavior of (+)-catechin, and different concentrations and catechin/antioxidant molar ratios were also considered.

Materials and Methods

Reagents and materials

Tartaric acid, glacial acetic acid, sodium hydroxide, ethanol (96% v/v), iron(II) sulfate heptahydrate, copper(II) sulfate pentahydrate and potassium metabisulfite were purchased from Carlo Erba Reagents (Milan, Italy), and (+)-catechin hydrate, ascorbic acid (ASC), glutathione (GSH) and HPLC grade acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). The inactive dry yeast (YD) was a thermally produced yeast autolysate

90 prepared as reported elsewhere [14]. O2xyDot® oxygen sensitive sensors were sourced from
91 OxySense Inc. (Dallas, TX, USA).

92 **Oxidative behavior of the (+)-catechin model solution supplemented with antioxidants at**
93 **normal wine concentration**

94 In this portion of the study, the protective capacity of the different antioxidants towards (+)-
95 catechin oxidation was investigated at the normal amounts commonly found in wine.

96 *Preparation of the (+)-catechin model solution*

97 A model wine solution was prepared by dissolving 5.00 g L^{-1} of tartaric acid in hydro-
98 alcoholic solution (ethanol 12% v/v in Milli Q grade water) and buffering the pH at 3.20 with
99 4 M sodium hydroxide. This model wine was subsequently subdivided into 100 mL
100 transparent glass bottles previously prepared by pasting an O2xyDot® sensor to the inner wall
101 of each bottle (approx. 2 cm from the bottom). After filling, the bottles were vigorously
102 shaken until the oxygen concentration (measured at 20.0 °C) was stable at 7.8 ± 0.5 mg L^{-1}
103 (saturation).

104 Catechin hydrate (531 mg L^{-1}), iron(II) sulfate heptahydrate [25 mg L^{-1}, corresponding to 5
105 mg L^{-1} of Fe(II)] and copper(II) sulfate pentahydrate [2 mg L^{-1}, corresponding to 0.5 mg L^{-1}
106 of Cu(II)] were dissolved (in the form of freshly prepared stock solutions), and antioxidant
107 products were immediately added as reported below.

108 *Antioxidant supplementation*

109 Four different products were compared, each one in three repetitions. Potassium metabisulfite
110 (90 mg L^{-1}, corresponding to approx. 50 mg L^{-1} of sulfur dioxide), ascorbic acid (50 mg L^{-1}),
111 glutathione (50 mg L^{-1}) and the YD preparation (500 mg L^{-1}) were added in the form of
112 freshly prepared stock solution, to the oxygen-saturated catechin model wine prepared as
113 described above. Nitrogen was blown into the headspace, and the bottles were sealed with
114 crown cap closures and stored at 20 °C for 29 days. During this time, the oxygen

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115 concentration in the samples was measured as reported below and compared with the (+)-
116 catechin model solution without any antioxidant addition (control sample).

117 *Oxygen consumption capacity*

118 The system used in oxygen measurements was an OxySense® fluorimeter (OxySense Inc.,
119 Dallas, TX, USA). The O2xyDot® sensors positioned inside the bottles emit a red light via
120 fluorescence when they are illuminated by the pulsed blue light produced by the fluorimeter.
121 Oxygen molecules create a decrease in the fluorescence lifetime that is proportional to their
122 concentration (dynamic quenching). An infrared sensor located in the reader pen of the
123 fluorimeter allows simultaneous measurement of the sample temperature [15]. The instrument is
124 managed by specific software (OxySense Inc.) that facilitates immediate measurement of
125 oxygen concentration in mg L^{-1}. Oxygen measurements were performed during the entire 29-
126 day interval and were repeated daily during the first week and every two or three days later.
127 After this time, all samples were analyzed as reported below.

128 *UV-Vis spectra*

129 Spectrophotometric measurements were performed at the end of the storage period using a
130 UV-Vis spectrophotometer model V-530 (Jasco Co. Ltd., Tokyo, Japan). The UV (350-240
131 nm) and visible spectra (650-350 nm) were recorded in quartz cuvettes with a 10 mm optical
132 path length (Hellma Analytics, Mülheim, Germany) with the absorbance read against Milli Q
133 water. All samples were filtered on 0.20 μm nylon membranes (Albet-Hahnemühle,
134 Barcelona, Spain) before analysis. Additionally, a 10X dilution in Milli Q water was
135 conducted before UV measurements.

136 *Reverse-phase HPLC*

137 HPLC analyses were performed on a LC-2010 AHT liquid chromatographic system
138 (Shimadzu, Kyoto, Japan), equipped with an integrated autosampler and UV-Vis detector.
139 Compounds were separated on a 4 μm packed 150 x 4.6 mm C_{18} Synergi Polar column

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140 (Phenomenex, Torrance, CA) thermostated at 35 °C. The elution was performed in gradient
141 mode at a flow rate of 1 mL min^{-1}. The mobile phase was composed of a 1% (v/v) acetic acid
142 solution in Milli Q grade water (solvent A) and a mixture of acetonitrile/Milli Q water/acetic
143 acid, 80.0/19.5/0.5 (v/v/v) (Solvent B). The gradient was set as follows: solvent B was held at
144 5% for the first 10 min, increased to 42% in the following 30 min and further increased to
145 100% in 5 min; 100% solvent B was held for 5 min before it was decreased in 2 min to the
146 initial condition (5%). The injection volume was 5 µL. Before injection, all samples were
147 filtered on 0.20 µm nylon membranes (Albet-Hahnemühle, Barcelona, Spain). Detection was
148 performed at 280 and 420 nm. The absolute areas of the detected peaks were used in data
149 elaboration.

150 *Cyclic voltammetry*

151 Cyclic voltammetric (CV) measurements were performed at 20 ± 0.1 °C in an undivided 50
152 mL three-electrode cell using a voltammetric unit consisting of a PGSTAT 30 potentiostat
153 (Ecochemie, Utrecht, The Netherlands) driven by Ecochemie GPES 3.2 software. In all cases,
154 the counter electrode was a 1 cm^2 platinum sheet, and the reference electrode was a Ag/AgCl,
155 Cl^-_{sat} electrode connected to the cell by a salt bridge containing the electrolyte also used in the
156 test solutions. In the CV measurements, the working electrode, i.e., a disk-shaped glassy
157 carbon with a diameter of 3.0 mm, was exposed for a controlled time of 15 s to the solutions
158 analyzed (20 mL), which always contained the model wine solution as the supporting
159 electrolyte. At least 3 cyclic voltammograms were recorded for each sample.

160 Before use and prior to each CV experiment, the glassy carbon electrode was polished using
161 graded alumina powders with progressively decreasing grain sizes (from 1.0 to 0.3 µm
162 particle size), washed with Elgastat water, and inserted after drying into the voltammetric cell.
163 CV measurements were performed with a sweep rate of 20 mV s^{-1}, and the potential scan was
164 conducted from 0 to 1.3 V vs. Ag/AgCl, Cl^-_{sat}. The samples treated with the YD preparation

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165 were filtered on a 0.20 μm pore-size nylon membrane (Albet-Hahnemühle, Barcelona, Spain)
166 before CV analysis.

167 *Statistical analysis*

168 The results are averages of at least three measurements taken from three experiment
169 replications. HPLC data and the final oxygen concentration in the samples were subjected to
170 One Way ANOVA. Means and standard deviations (SD) were calculated, and significant
171 differences were evaluated using the Tukey HSD test at $p < 0.05$. The same approach (One
172 Way ANOVA and Tukey HSD test) was used in spectrophotometric measurements. The
173 absorbance recorded at the wavelength of maximum absorption for both the UV and visible
174 spectra (λ_{max} = 278 nm and 442 nm, respectively) was used in the elaboration. All analyses
175 were performed using the software Statistica for Windows, version 8.0 (StatSoft, Inc., Tulsa,
176 OK, USA).

177 **Effect of antioxidant concentration on the voltammetric behavior of (+)-catechin in the**
178 **model solution**

179 To better understand the voltammetric behavior of (+)-catechin in the presence of different
180 concentrations of each antioxidant, further CV experiments were conducted in 10X diluted
181 (+)-catechin model solution (catechin hydrate was 53.1 mg L^{-1}, corresponding to 0.17 mM) in
182 which neither iron and copper nor oxygen were supplemented.

183 Potassium metabisulfite, ascorbic acid and glutathione were added at two different levels such
184 that the catechin/antioxidant molar concentration ratio was set to 1:1 (antioxidant
185 concentration 0.17 mM) and 1:10 (antioxidant concentration 1.70 mM). In brief, to fulfill
186 these ratios, the potassium metabisulfite additions were 20 and 200 mg L^{-1} (corresponding
187 approx. to 11 and 110 mg L^{-1} of sulfur dioxide), ascorbic acid was added at 30 and 300 mg L^{-1}
188 , and the GSH level was set at 52 and 520 mg L^{-1}. For the YD preparation (for which it was
189 not possible to operate in terms of molar concentration), two additions were performed at 500

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190 and 5000 mg L^{-1}, respectively. With respect to the modalities of supplementation, all
191 antioxidants were prepared in the form of fresh stock solution and immediately used, as
192 reported above. After preparation, the samples and the control (0.17 mM catechin model
193 solution) were immediately subjected to voltammetric analysis, as reported previously. All
194 samples were prepared in three repetitions.

195 Results and Discussion

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196 Oxidative behavior of the (+)-catechin model solution supplemented with antioxidants at
197 normal wine concentration

198 As mentioned above, this part of the study was aimed to investigate the protective capacity of
199 the different antioxidants, at the normal concentrations normally found in wine.

200 *Oxygen consumption capacity*

201 Figure 1 reports the behavior of oxygen consumption in the model wines, as affected by
202 supplementation with the different antioxidants. Ascorbic acid was the most active oxygen
203 scavenger, confirming previous observations in wine [13] and in agreement with the literature [16].
204 Sulfur dioxide also exhibited good ability in scavenging oxygen. Sulfiting increased the
205 oxygen consumption capacity of the model wine, and this higher consumption rate became
206 particularly evident after the fifth day of storage. The ability of sulfites to enhance oxygen
207 consumption was explained well by Danilewicz and co-workers [7]. In their experiments,
208 oxidation of 4-methylcatechol in a model solution containing iron and copper was accelerated
209 by SO_2 addition. The hypothesis they proposed to explain this behavior was that the reaction
210 of sulfur dioxide with quinones might accelerate the auto-oxidation of catechols, thus
211 increasing oxygen uptake.

212 GSH supplementation did not significantly modify the oxygen consumption capacity of the
213 (+)-catechin model buffer, even if a slightly more rapid decrease in the oxygen level was

observed during the first days of the monitoring period. This result confirms previous observations on the weak effect of GSH as an oxygen scavenger in wine [13]. Finally, YD was the less efficient additive in removing oxygen from wine-like solutions, with a behavior comparable to that of the control samples.

Browning evolution

Figure 2 presents the visible spectra of model solutions treated and untreated with different antioxidants. The ability of the tested products to protect (+)-catechin against browning was not always observed in connection with their oxygen scavenging capacities. The lowest color evolution was obtained in the presence of sulfur dioxide. This additive protected the color of the (+)-catechin solutions over the entire storage time. The control sample (catechin alone) was the most heavily affected by browning, followed by the GSH-treated samples. GSH offered a certain amount of color protection, probably as a consequence of its well-known ability to scavenge quinones [2]. Ascorbic acid also produced good color protection, and no evidence of the so-called "crossover" effect [17,18] was observed over the entire 29 days of storage. As known, the "crossover" effect is connected with the ability of ASC to act as both antioxidant and pro-oxidant, depending on the level of available ascorbic acid [18] and the ratio of ascorbic acid to catalytic metal ions, i.e., iron and copper [17]. Bradshaw and colleagues [18] found that the browning induction effect of ascorbic acid on (+)-catechin model solutions (stored in enhanced oxidative conditions at 45 °C) became evident after a lag period that ranged from 1 to 7 days, depending on the ASC concentration. In the current experiment, the lag period was longer than 7 days, in agreement with previous findings on white wine [13] in which browning induction by ASC was not observable after 15 days of storage but became evident after 8 months. This apparently longer lag period might be presumably explained by the accelerated storage conditions (temperature: 45 °C) used in the experiments by Bradshaw et al. [18] in contrast with the 20 °C storage temperature set up in the current experiments.

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239 YD also behaved quite well in terms of color protection, reducing the color evolution of
240 control sample to a greater extent with respect to pure glutathione, in agreement with our
241 previous findings [13]. Based on the amount of YD and GSH supplemented in the current
242 experiment and considering that the average content of the latter in inactive dry yeast
243 preparations ranges from few mg g^{-1} to approx. one dozen, [19,20] it may be hypothesized that
244 the ability of YDs to protect wine color could be not only ascribed to their capacity to release
245 the tripeptide. In opposition, as suggested by other papers, [12,13,21] other components of these
246 preparations might be involved with non-negligible effects.

247 *Analysis of (+)-catechin oxidation products by reverse-phase HPLC*

248 To obtain additional insight on the spectral data behavior, reverse-phase HPLC analysis of
249 (+)-catechin and its oxidation products was performed in the model solutions. The
250 chromatographic profile of the control samples at 420 nm was modified after storage by the
251 appearance of five new peaks (Fig. 3), in agreement with observations by Guyot and co-
252 workers [22]. These oxidation products generally did not appear in the presence of SO_2 and were
253 detected only in traces when catechin was supplemented with ascorbic acid. Both GSH and
254 YD were able to reduce their formation with respect to the control, although the latter seemed
255 slightly more effective in limiting the appearance of these colored compounds during storage.
256 Quantitative evidence of these effects can be observed in Table 1, where the results of
257 ANOVA analysis (performed on the absolute areas of the detected peaks) are reported. It is
258 interesting to note that no significant differences in (+)-catechin concentration were found
259 among the samples after 29 days of storage.

260 *Cyclic voltammetric analyses*

261 Voltammetric experiments were conducted on the model solutions at the end of the storage
262 period (29 days). The voltammetric profiles displayed by the supplemented sulfur dioxide,
263 ascorbic acid, glutathione and inactive dry yeast preparation are reported in Figure 4, where

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264 they are overlaid on the voltammogram recorded for the (+)-catechin model solution in the
265 absence of antioxidants (control).
266 As expected, the voltammogram of the control shows two partially overlapping anodic peaks.
267 The first one is conceivably related to oxidation of the *ortho*-hydroxyl groups of the catechol
268 moiety of (+)-catechin (B-ring) to generate the corresponding quinone [23]. The second is
269 attributable to oxidation of the –OH groups in positions 5 and 7 of the resorcinol moiety of
270 the flavonoid (A ring) [24]. The former (peak 1) has a maximum at a potential (E) of 574 mV
271 (current intensity: 12.2 μA), and the latter (peak 2) reaches a maximum at 745 mV (intensity:
272 9.8 μA). The presence in the reverse scan curve of a single low-intensity cathodic peak,
273 located at approx. 300 mV and coupled with the anodic peak 1, offers evidence of a first
274 quasi-reversible process related to oxidation of the catechol B-ring, and a second non-
275 reversible one, corresponding to the oxidation of the flavonoid A-ring. The latter presumably
276 leads to the formation of a polymeric film, which is able to inactivate the working electrode
277 surface. For this reason, only the voltammograms recorded in the first scan were considered in
278 this study, with a particular focus on the first anodic process (peak 1).
279 The addition of the different antioxidants slightly modified this voltammetric profile. Sulfur
280 dioxide supplementation, for instance, produced a higher intensity in the two anodic peaks,
281 particularly the first one (Fig. 4a). The current intensity detected for the control samples at the
282 peak maximum was 12.2 μA (E_{max}: 574 mV) on average, and this value increased to 13.0 μA
283 (E_{max}: 554 mV) for sulfited (+)-catechin model solutions. This difference might appear small,
284 but the results of ANOVA analysis (performed on the intensities at the peak maximum)
285 identified significant differences between the two sets of experiments (control vs. SO_2) at $p <$
286 0.05 (data not shown). In addition, these behaviors confirm the findings of Makhotkina and
287 Kilmartin, who also observed an increase in the anodic current of the same order of
288 magnitude when SO_2 was added to a catechin solution [25]. They explained this result by
289 considering the ability of sulfites to react with quinones and reduce them back to catechols [7].

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290 This process might regenerate the oxidation substrate, which can be further oxidized at the
291 carbon electrode, [25] thus increasing the intensity of peak 1. This effect, which was detected by
292 the authors in freshly prepared catechin solutions, is observable in the current experiment
293 after 29 days, thus highlighting that the protective effects of sulfur dioxide appear to be
294 preserved (at least partially) over the entire storage period.

295 In effect, SO_2 is the only additive that produced an incremented anodic current with respect to
296 that registered for the control. In contrast, glutathione showed the opposite behavior, and both
297 of the anodic peaks were decreased when GSH was added to the model wines. The magnitude
298 of this decrement was 0.70 μA, with a slight overpotential observed for peak 1 (E_{max}: 594
299 mV), and peak 2 nearly disappeared (Fig. 4c). No peak attributable to GSH oxidation was
300 detected in the voltammograms. Considering that the area of the anodic peak should be
301 proportional to the amount of (+)-catechin available to be oxidized, the behavior of GSH
302 addition might confirm that the tripeptide was less effective than sulfites in protecting the
303 flavanol during storage, in agreement with the findings of other authors [25].

304 Ascorbic acid addition also produced a decrease in the intensity of the forward scan curve
305 (Fig. 4b). The magnitude of such reduction for peak 1 was 1.01 μA, with a non-negligible
306 overpotential detected (E_{max}: 614 mV, + 40.0 mV with respect to E_{max} of the control). Neither
307 the second anodic peak nor the one corresponding to the oxidation of ascorbic acid to
308 dehydroascorbic acid was detected. According to Makhotkina and Kilmartin [25], the latter peak
309 should be found at approx. 200 mV before (+)-catechin anodic peak 1, pointing out that ASC
310 is oxidized earlier than the flavonoid. Consequently, its absence in the cyclic voltammogram
311 might be a symptom of complete consumption of the additive during the storage period,
312 probably due to the intense ability of ASC to consume oxygen (Fig. 1). The lower intensity of
313 the peak 1 of (+)-catechin in the trace of the ascorbic-treated sample might suggest a lower
314 capacity for this compound with respect to sulfites in protecting catechin from oxidation.

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315 For the inactive dry yeast preparation (Fig. 4d), the shape of the voltammogram obtained for
316 the YD-treated samples was similar to that recorded for GSH, with a slightly lower intensity
317 for peak 1 (0.90 µA less intense than the control) and the same slight overpotential (E_{max}: 594
318 mV) as GSH. No peaks attributable to compounds eventually released by the preparation were
319 detected.

320 Based on these behaviors, other than the sulfites, none of the other additives appeared to be
321 present in the model solutions at the end of the storage period, and none of them were as
322 effective as SO_2 in protecting (+)-catechin from oxidation. This result might explain the
323 reasons for the lower effectiveness of these additives in preventing the development of brown
324 color (Fig. 2 and 3) and might also be connected with the lower molar concentration that some
325 of them had in the model solutions. In fact, comparing the amounts of SO_2, GSH and ascorbic
326 acid supplemented in the different trials, the former was added at 50 mg L^{-1}, which
327 corresponds to 0.78 mM, and the latter two, with an equal addition in mg L^{-1}, produced molar
328 concentrations of 0.16 mM (GSH) and 0.28 mM (ASC), respectively. To further support these
329 findings and investigate the effects of different molar concentrations of antioxidants on the
330 electrocatalytic behavior of (+)-catechin, further cyclic voltammetric trials were performed.

331 **Effect of antioxidant concentration on the voltammetric behavior of (+)-catechin in the**
332 **model solution**

333 As reported above, this second set of experiments was performed on less concentrated (+)-
334 catechin model solutions (approx. 50 mg L^{-1}) with two different levels of antioxidant
335 supplementation. Neither metal salts nor oxygen were added, and cyclic voltammograms were
336 recorded immediately after each model wine preparation (Fig. 5).

337 Sulfur dioxide was added at two dosages, 11 (0.17 mM) and 110 mg L^{-1} (1.70 mM), both
338 levels included in the range normally used at the winery scale (Fig. 5a). For the pure
339 compound, the lowest amount (0.17 mM) produced a voltammogram similar to those reported
340 in literature for slightly higher SO_2 concentrations (0.25 mM) [25]. The oxidation peak of SO_2

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341 was evident only for the 1.70 mM solution, which showed an intense anodic peak at a
342 potential close to 1100 mV, highlighting the large overpotential required to observe sulfite
343 oxidation at the carbon electrode [25]. When sulfur dioxide was added to catechin solutions, the
344 voltammograms of the control samples were modified as reported previously, with an increase
345 in the two oxidation peaks that was proportional to the antioxidant concentration. At the same
346 time, the E_{max} of the peak 1 shifted to higher values, and the shape of the curves gave
347 evidence of a process that loses its reversibility as the sulfiting level increases. According to
348 these behaviors, an increased SO_2 concentration appeared to produce a more intense
349 involvement of (+)-catechin in oxidative reactions. The sulfites themselves might act by
350 reducing back quinones in a manner that becomes more evident as their level increases. No
351 specific oxidation peak for SO_2 (at 1100 mV) was detected in the presence of catechin for
352 either of the two sulfiting levels. This result might confirm that in the presence of
353 polyphenols, the additive might be preferentially involved in the scavenging of oxidation
354 compounds (e.g., quinones or hydrogen peroxide) rather than in the direct oxidation to sulfate,
355 in agreement with the findings of Danilewicz [8].
356 The behavior of ascorbic acid alone (Fig. 5b) was similar to results reported elsewhere, [25] with
357 an anodic peak close to 300 mV and a shape of the voltammograms that highlights a non-
358 reversible process (lack of cathodic peak). Nevertheless, in the presence of (+)-catechin, the
359 two concentrations supplemented led to different situations. At the lowest level, ascorbic acid
360 did not increase the intensity of the anodic peak 1 of (+)-catechin (with respect to the intensity
361 of the control), and the oxidation peak of the additive itself was quite evident in the
362 voltammogram (close to 300 mV). This result might confirm the ability of the antioxidant to
363 be oxidized preferentially with respect to the polyphenols, and in such a manner it is less
364 effective than sulfites in reducing back quinones (anodic peak 1 was less intense after ASC
365 addition than after sulfiting). Based on such observation, at a concentration close to 30 mg L^{-1}
366 (0.17 mM, commonly found in wine), the activity of ASC appeared connected more directly

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367 with its direct oxidation at the glassy carbon electrode and less with its ability to react with
368 polyphenol oxidation products. When the concentration increased to 300 mg L^{-1} (1.70 mM),
369 ascorbic acid appeared to be preferentially involved in the scavenging of quinones. The
370 anodic peak at 300 mV was less evident in the voltammogram, whereas the intensity of the
371 anodic peak 1 of (+)-catechin significantly increased, indicating a possible minor involvement
372 of ASC in direct oxidation. These observations might be connected with the so-called
373 "crossover" effect [26]. Nevertheless, the reasons for such different behaviors in the
374 voltammetric traces should be further confirmed and investigated in the future.
375 For pure glutathione (Fig. 5c), at a concentration close to 50 mg L^{-1} (0.17 mM, commonly
376 found in wine), the tripeptide gave rise to voltammetric curves similar to those described in
377 literature, with no peaks detected below 1000 mV and an increasing anodic current observed
378 as the potential exceeded approximately 600 mV [25]. In contrast, at 520 mg L^{-1} (1.70 mM), in
379 addition to the described broad increase of current above 600 mV, the traces showed an
380 additional anodic peak at 292 mV. Such a peak was detected in all repetitions performed for
381 1.70 mM pure GSH as well as when the same concentration of the tripeptide was added to
382 (+)-catechin solutions.
383 The presence of this oxidation peak was not reported in other voltammetric studies related to
384 glutathione, and according to the literature, it is unlikely to correspond to the oxidation of
385 GSH itself to disulfide (GSSG). In fact, Huang, Yan, and Tong [27] report that amperometric
386 detection of GSH at common electrodes (including glassy carbon electrodes) is difficult due
387 to the slow electron transfer rate of the tripeptide, which results in a high anodic potential.
388 Based on such considerations, the peak might be connected with adsorption phenomena that
389 potentially involve GSH or impurity traces present in the model solution. However, due to the
390 interest in the supposed antioxidant activity of glutathione in wines, the presence of this peak
391 should be further investigated in the future.

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392 When GSH was added in the presence of (+)-catechin at the lowest supplementation amount,
393 it caused an increase in anodic peak 1 of the polyphenol, in agreement with the literature [25].
394 This increase might be linked to the ability of GSH itself in reducing back quinones. If we
395 compare the height of anodic peak 1 registered for GSH/ catechin (Fig. 5c) with those
396 detected for sulfur dioxide/catechin (Fig. 5a) and ascorbic acid/catechin (Fig. 5b), it might be
397 argued that (at equimolar concentration) the ability of the tripeptide in scavenging quinones is
398 the intermediate between the other two antioxidants. When the concentration of GSH
399 increased (1.70 mM), the oxidation peak 1 of (+)-catechin also increased, presumably because
400 a greater amount of the tripeptide is involved in the scavenging of quinones.
401 Finally, Figure 5d reports the cyclic voltammograms recorded for the samples supplemented
402 with the inactive dry yeast preparation. As shown, the profiles collected in the absence of (+)-
403 catechin are similar to the traces reported for pure GSH, with no anodic peaks detected in the
404 range of potential scanned. Moreover, no differences were found between the curves
405 registered for the two levels of supplementation of 500 and 5000 mg L^{-1}.
406 When YD was added to the (+)-catechin model wine, the behavior of the anodic trace of the
407 polyphenol was quite different with respect to that observed for the other antioxidants tested.
408 Independent of the amount added, YD caused a decreased intensity of the oxidation peak 1 of
409 the polyphenol and a slight shift of the peak maximum towards more positive potentials.
410 This effect might be ascribed to adsorption phenomena related to the ability of inactive dry
411 yeasts to release macromolecules, particularly proteins and glucidic colloids [14]. Such
412 macromolecules might have been adsorbed on the glassy carbon surface, thus hampering the
413 (+)-catechin voltammetric response. Moreover, certain of these substances (e.g., proteins) are
414 well known to have a binding capacity towards phenolics and quinones, [2,28] and this might
415 have facilitated an increased adsorption of the flavonoid oxidation products onto the working
416 electrode. The observation that proteins demonstrated a certain ability to mask the antioxidant
417 capacity of catechin might support this hypothesis [28].

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418 Based on such observations, it appears clear that the mechanisms that make YDs able to 419 reduce color development in wines [13] are probably different from those shown by the other 420 antioxidants tested, and CV analyses did not allow a clear elucidation of these mechanisms. In 421 particular, considering the voltammetric results from the current experiment, the negligible 422 differences found between the voltammetric traces collected for the two levels of YD 423 supplementation and considering that the YD-treated samples were all filtered before CV 424 analysis, it might appear that the ability of inactive dry yeasts to reduce color development 425 (Fig. 2) is more likely to be connected with the presence of the solid particles of cell wall 426 residues rather than the release of soluble compounds.

427 In fact, if the antioxidant capacity was connected with the release of soluble antioxidant 428 compounds, the increase in the YD concentration should have produced a different shape of 429 the anodic traces in CV analysis due to the presence of greater amounts of such antioxidant 430 molecules. In contrast, if the protective effect of YDs was related to the cell wall residues, the 431 elimination of the solid particles might justify the reason for why YD actually protected (+)- 432 catechin from color development (Fig. 2), but CV was unable to detect any evident 433 antioxidant effect.

434 Conclusions

435 In conclusion, YD demonstrated a certain effect in protecting (+)-catechin against browning 436 in a wine-like medium, thus confirming previous findings [13]. Although this effect was not 437 comparable to that of sulfur dioxide, the yeast-derived product tested was more efficient than 438 pure glutathione. Cyclic voltammetry demonstrated that the protection conferred by the 439 different additives examined was connected with their molar concentration and also with the 440 intrinsic antioxidant capacity of the single product, and differences among the behaviors of 441 the substances tested were found at the equimolar concentration. Indeed, sulfites were the 442 only additive demonstrated to be present in the samples after 29 days of storage. With respect

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443 to the properties of YDs, voltammetric analyses showed that their mechanism of action is
444 probably different than those of the other compounds. In the current discussion, a non-
445 negligible role for the insoluble portion of these preparations (cell wall residues) has been
446 hypothesized, even if the ability of YDs to release antioxidant molecules or compounds able
447 to suppress oxidation cannot be rejected. In any case, further investigations are required to
448 better explain the mode of action of such interesting additives in protecting wine phenolics.
449 This might lead to a more detailed knowledge of specific production processes, tailored to
450 maximize their antioxidant capacity.

451 Acknowledgements

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533 # Figure Captions

534 Figure 1. Oxygen consumption capacity of (+)-catechin model solution (Control) as affected
535 by antioxidant supplementation: sulfur dioxide 50 mg L^{-1} (SO_2); ascorbic acid 50 mg L^{-1}
536 (ASC); glutathione 50 mg L^{-1} (GSH); inactive dry yeast 500 mg L^{-1} (YD). Mean values of
537 three repetitions are reported; vertical bars represent standard deviation. (+)-catechin
538 concentration in the samples: 500 mg L^{-1}.

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540 Figure 2. Visible spectra of (+)-catechin model solution (Control) as affected by antioxidant
541 supplementation: sulfur dioxide 50 mg L^{-1} (SO_2); ascorbic acid 50 mg L^{-1} (ASC); glutathione
542 50 mg L^{-1} (GSH); inactive dry yeast 500 mg L^{-1} (YD). Each curve represents the average
543 spectrum of three repeated samples. (+)-catechin concentration in the samples: 500 mg L^{-1}.

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545 Figure 3. Chromatograms (recorded at 420 nm) of (+)-catechin model solution (Control)
546 supplemented and not with the different antioxidants: sulfur dioxide 50 mg L^{-1} (SO_2); ascorbic
547 acid 50 mg L^{-1} (ASC); glutathione 50 mg L^{-1} (GSH); inactive dry yeast 500 mg L^{-1} (YD).
548 Antioxidant addition determined differences among the samples, in the retention time range
549 between 28 and 45 min. (+)-catechin concentration in the samples: 500 mg L^{-1}.

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551 Figure 4. Cyclic voltammograms (0.0 – 1.3 V) recorded for (+)-catechin model solution
552 (Control) supplemented and not with the different antioxidants: sulfur dioxide 50 mg L^{-1}
553 (SO_2); ascorbic acid 50 mg L^{-1} (ASC); glutathione 50 mg L^{-1} (GSH); inactive dry yeast 500
554 mg L^{-1} (YD). Each curve represents the average voltammogram of three repeated samples.
555 (+)-catechin concentration in the samples: 500 mg L^{-1}.

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557 Figure 5. Cyclic voltammograms (0.0 – 1.3 V) recorded in model wine (pH 3.2, ethanol 12 %
558 v/v) for (+)-catechin (50 mg L^{-1} – 0.17 mM), the different antioxidants at different molar
559 concentration (0.17 and 1.70 mM) and the catechin / antioxidant mixtures at different molar
560 ratios (1:1 and 1: 10). YD preparation was added at 500 and 5000 mg L^{-1}. Each curve
561 represents the average voltammogram of three repeated samples. See the text for
562 abbreviations.

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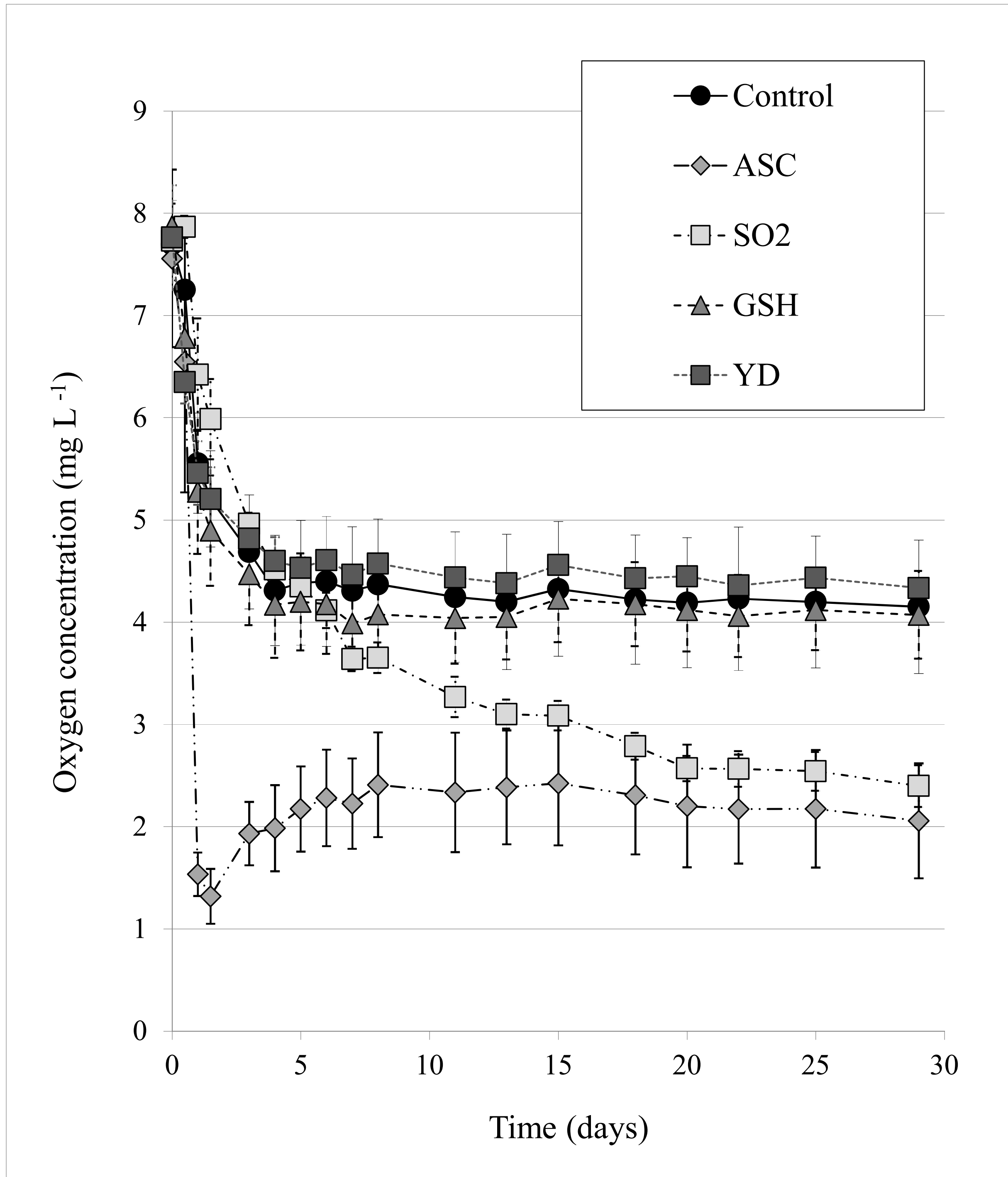

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566 **Figure 1**

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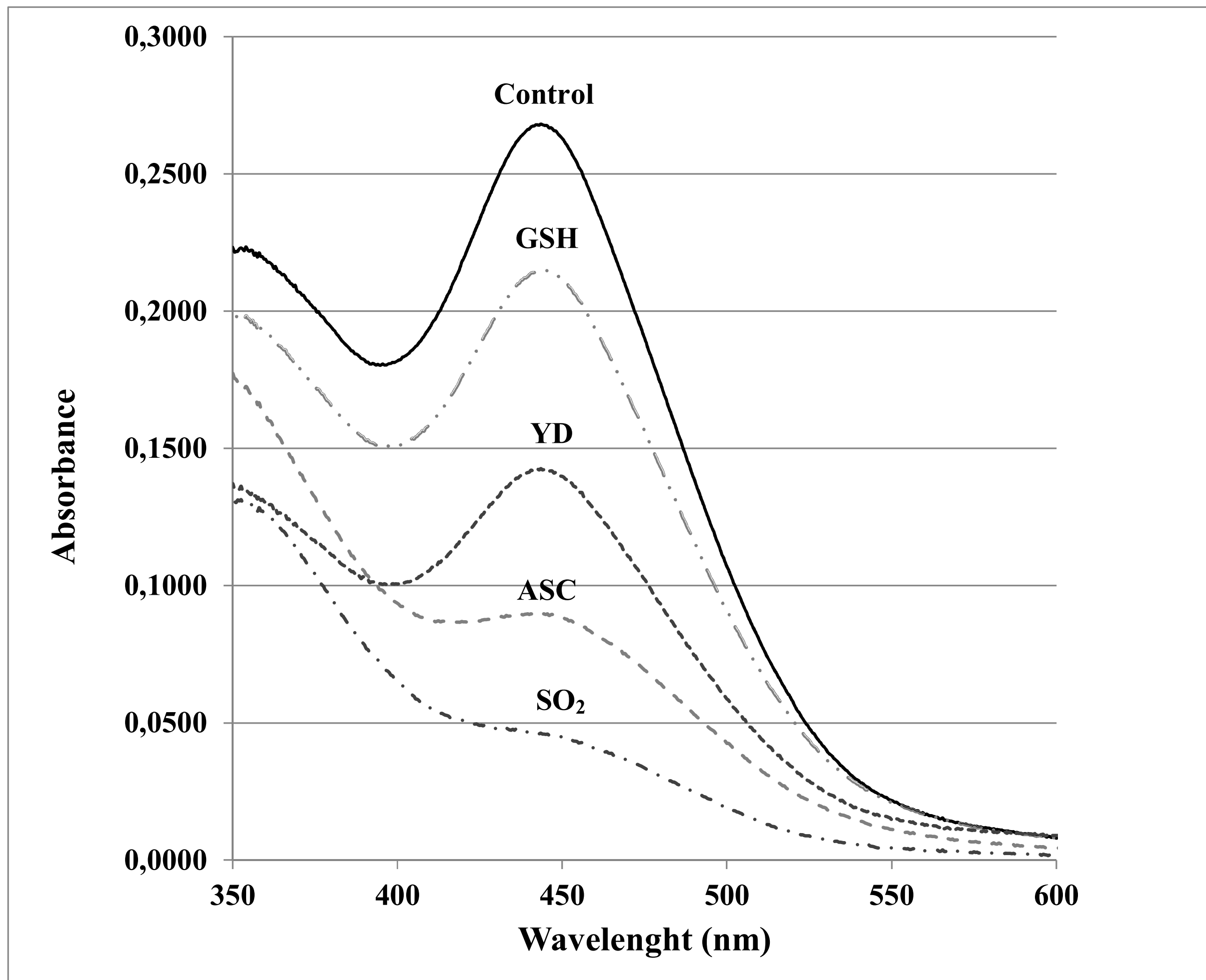

Figure 2

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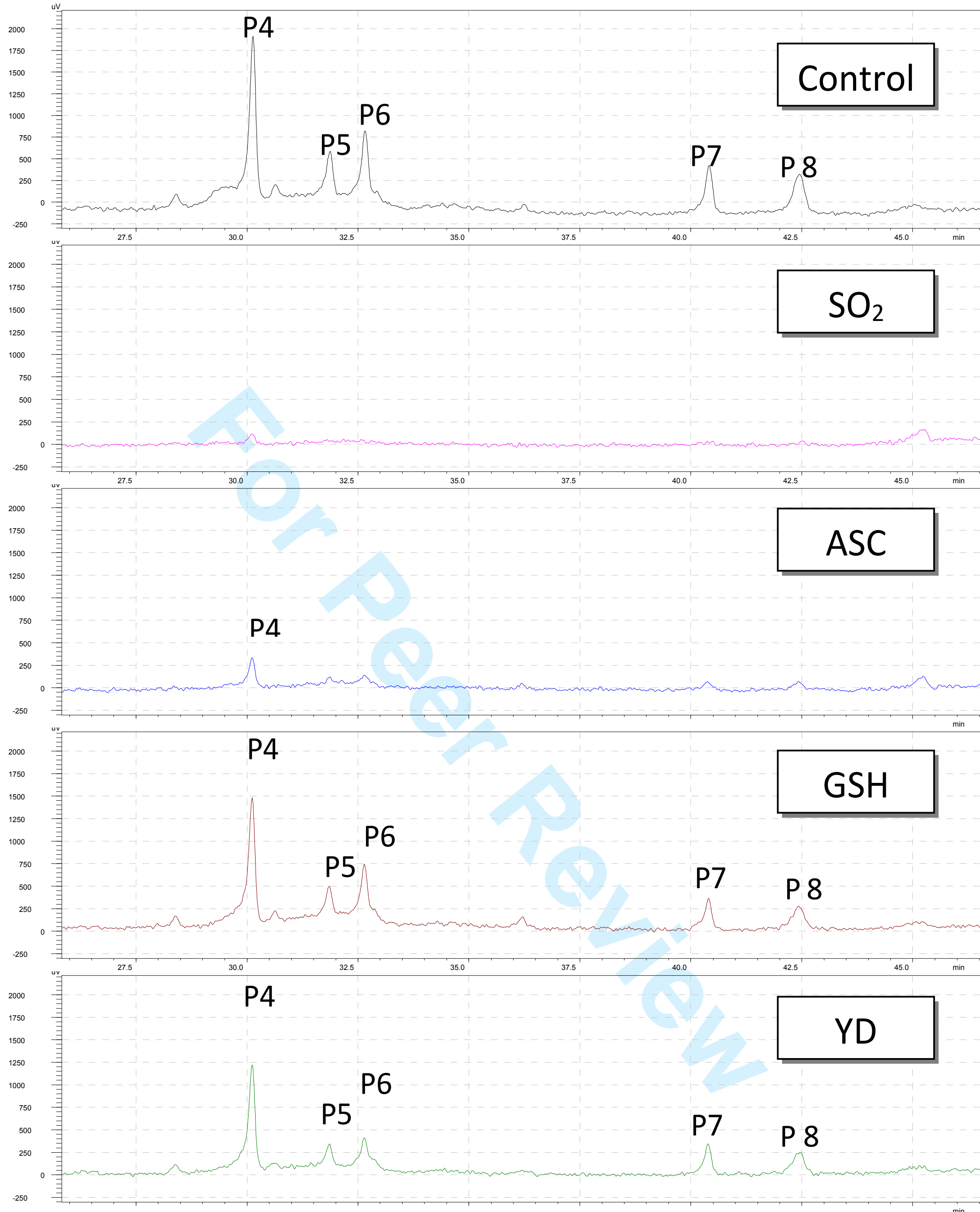

608 **Figure 3**

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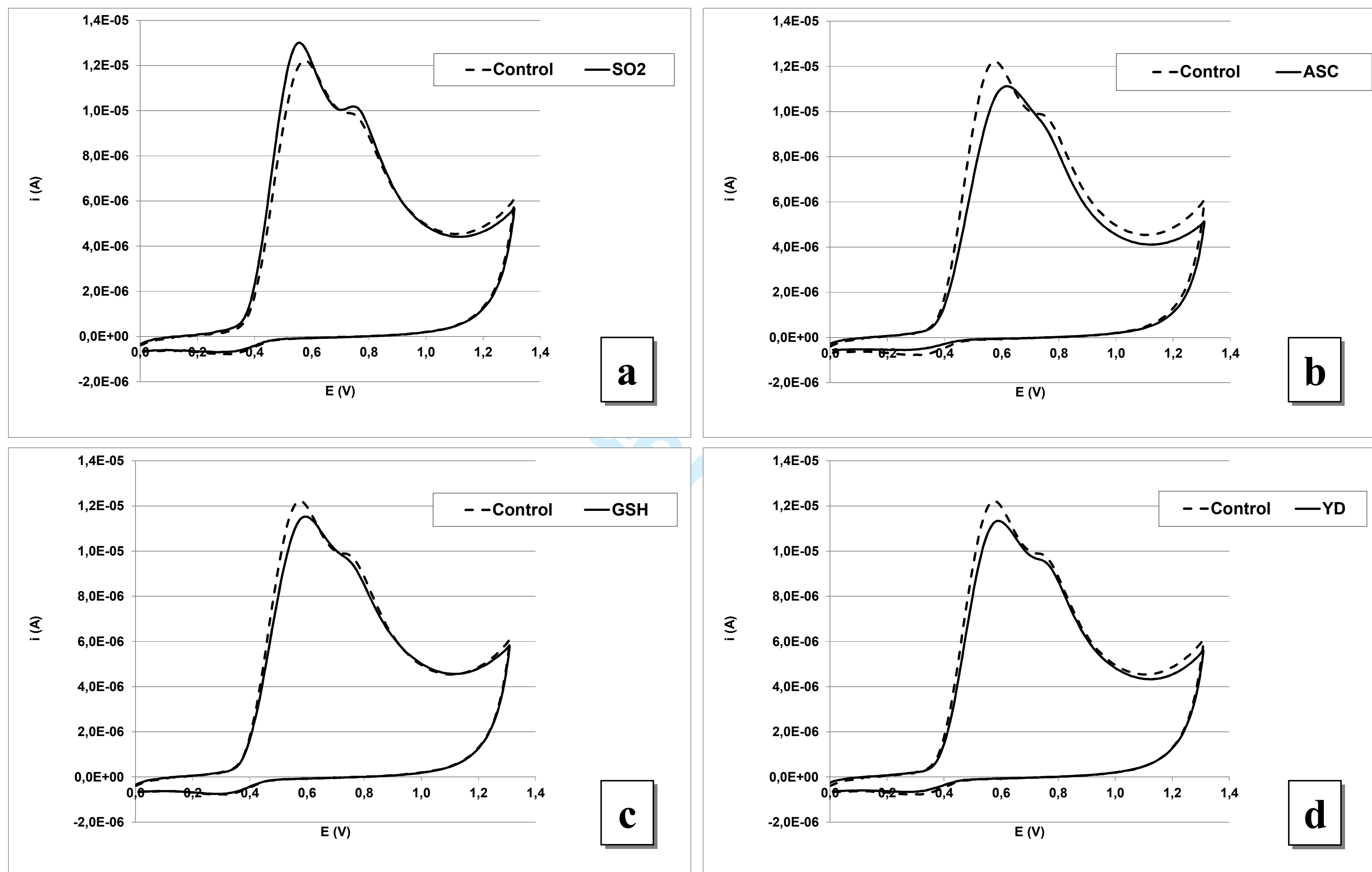

Figure 4

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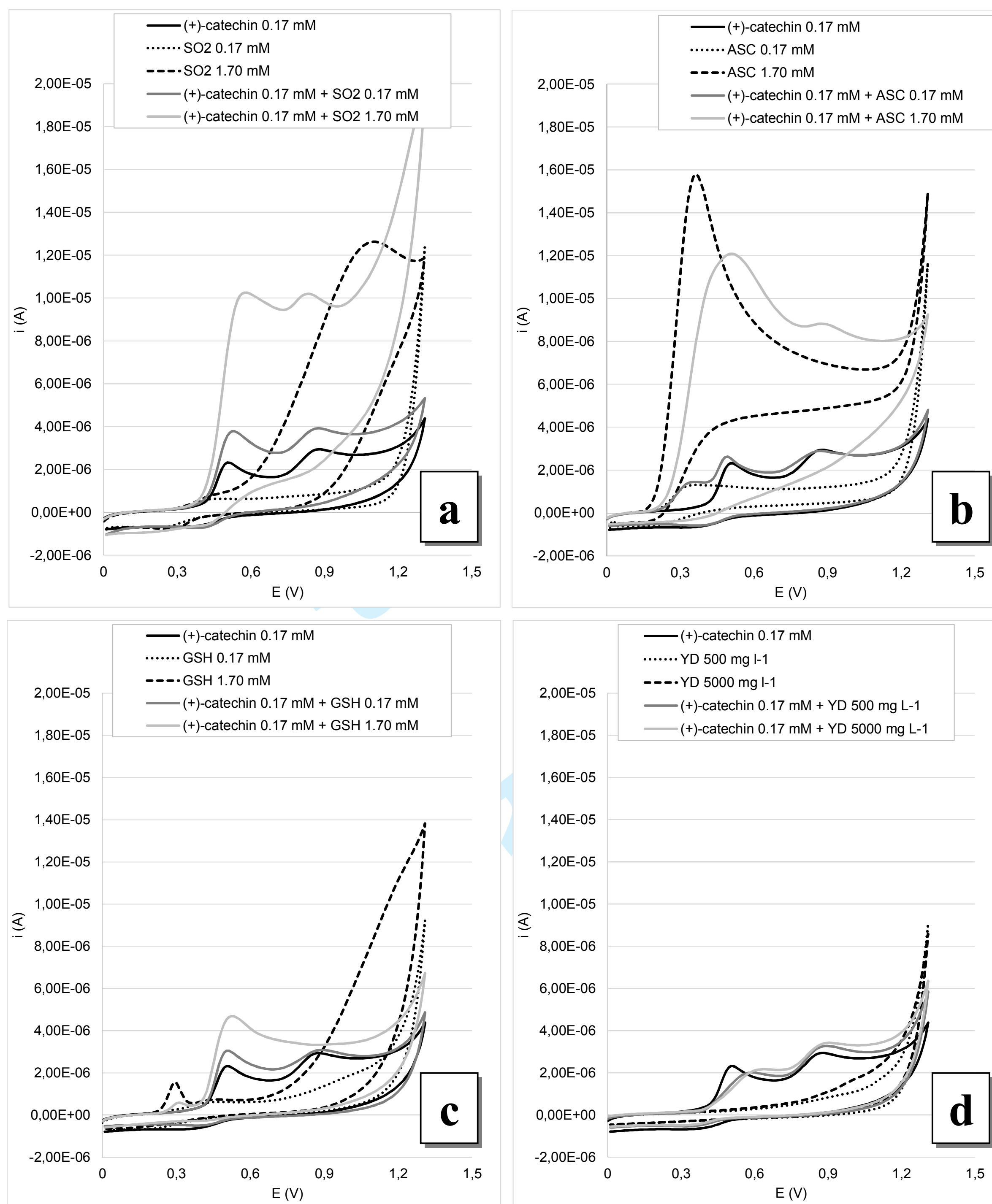

Figure 5

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6 649 Table 1. HPLC data (absolute areas / 1000) obtained from (+)-catechin model solution (Control) supplemented and not with the different antioxidants:
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8 650 sulfur dioxide 50 mg L^{-1} (SO_2); ascorbic acid 50 mg L^{-1} (ASC); glutathione 50 mg L^{-1} (GSH); inactive dry yeast 500 mg L^{-1} (YD). Results of ANOVA
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10 651 and Tukey HSD Test are reported: different letters mark significant differences at $p< 0.05$. (+)-catechin concentration in the samples: 500 mg L^{-1}. For
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12 652 Peak number, refer to Figure 3.
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sample	(+)-catechin_UV (absolute area / 1000) mean ± SD		peak 4_Vis (absolute area / 1000) mean ± SD		peak 5_Vis (absolute area / 1000) mean ± SD	
control	1451 ± 48	**a**	33 ± 8	**c**	12 ± 5	**c**
SO_2	1487 ± 100	**a**	2 ± 1	**a**	0 ± 0	**ab**
ASC	1429 ± 64	**a**	5 ± 1	**a**	1 ± 1	**a**
GSH	1448 ± 97	**a**	23 ± 2	**bc**	9 ± 1	**c**
YD	1490 ± 56	**a**	21 ± 3	**b**	7 ± 3	**bc**

sample	peak 6_Vis (absolute area / 1000) mean ± SD		peak 7_Vis (absolute area / 1000) mean ± SD		peak 8_Vis (absolute area / 1000) mean ± SD	
control	21 ± 6	**c**	10 ± 3	**c**	12 ± 4	**c**
SO_2	0 ± 0	**a**	0 ± 0	**a**	0 ± 0	**a**
ASC	3 ± 0	**a**	2 ± 1	**ab**	2 ± 1	**ab**
GSH	16 ± 4	**bc**	7 ± 1	**bc**	8 ± 2	**bc**
YD	10 ± 3	**ab**	8 ± 2	**c**	7 ± 4	**abc**

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