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**Oxidative behavior of (+)-catechin in the presence of  
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**Running Title**

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

## 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<sub>2</sub>) 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

41 **Introduction**

42 The relationship between oxygen and wine is one of the key points of modern winemaking.  
43 Various papers have described the mechanisms of wine oxidation and the role of metal ions  
44 (iron and copper) and reactive oxygen species (ROS), in the formation of quinones (originated  
45 by reaction of such free radicals with polyphenols) and certain low molecular weight  
46 compounds, such as acetaldehyde or pyruvic acid (produced by the same radicals from  
47 ethanol or organic acids) <sup>1-4</sup>. In the presence of slow and steady aeration, such reactions may  
48 evolve with positive results on the color stability and sensory characteristics of the wine, <sup>5</sup> but  
49 if the oxygenation becomes massive and uncontrolled, the same reactions can lead to the  
50 accumulation of compounds responsible for browning and generation of off flavors <sup>6</sup>.  
51 Sulfur dioxide (SO<sub>2</sub>) is a fundamental antioxidant additive due to its ability to act at key  
52 points in such oxidation mechanisms. SO<sub>2</sub> is able to react with quinones to regenerate  
53 phenolic molecules or yield sulfonic adducts, <sup>7</sup> and it might react with hydrogen peroxide (an  
54 important intermediate compound in the reduction chain of oxygen), thus hampering the  
55 propagation of radical chains <sup>7,8</sup>. Finally, sulfur dioxide might also react with acetaldehyde  
56 and carbonyl compounds <sup>2</sup> to limit the formation of brown pigments and the genesis of off  
57 flavors. However, SO<sub>2</sub> is a toxic and allergenic substance <sup>9</sup>, and thus, despite these positive  
58 considerations, the current trend is to minimize its concentration in wine.  
59 Although different alternatives are available as a replacement for sulfites with respect to their  
60 antimicrobial activity (e.g., lysozyme, dimethyl dicarbonate), their replacement as an  
61 antioxidant is more complicated, and the solutions available are generally described as  
62 complementary tools rather than real alternatives <sup>10</sup>.  
63 Inactive dry yeasts (YDs) have been recently included among these complementary tools,  
64 probably due to their similarity to yeast lees. In recent studies, certain of these preparations,  
65 whether enriched in glutathione (GSH) or not, demonstrated their protective ability towards

aroma compounds in both wine and a model solution <sup>11,12</sup>. This effect was ascribed to their claimed antioxidant capacity and particularly to their glutathione content <sup>11</sup>, and also to the release of selected antioxidant peptides containing methionine, tryptophan, and tyrosine <sup>12</sup>. 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 <sup>13</sup>. The effect of the addition of such autolysate on color protection was second only to that of SO<sub>2</sub>, 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

prepared as reported elsewhere<sup>14</sup>. O2xyDot<sup>®</sup> oxygen sensitive sensors were sourced from OxySense Inc. (Dallas, TX, USA).

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

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

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

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

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

*Antioxidant supplementation*

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

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

#### *Oxygen consumption capacity*

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

#### *UV-Vis spectra*

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

#### *Reverse-phase HPLC*

HPLC analyses were performed on a LC-2010 AHT liquid chromatographic system (Shimadzu, Kyoto, Japan), equipped with an integrated autosampler and UV-Vis detector. Compounds were separated on a 4 µm packed 150 x 4.6 mm C<sub>18</sub> Synergi Polar column



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

#### *Cyclic voltammetry*

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

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

165 were filtered on a 0.20  $\mu\text{m}$  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 ( $\lambda_{\text{max}} = 278 \text{ nm}$  and  $442 \text{ nm}$ , 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  $53.1 \text{ mg L}^{-1}$ , corresponding to  $0.17 \text{ mM}$ ) 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  $0.17 \text{ mM}$ ) and 1:10 (antioxidant concentration  $1.70 \text{ mM}$ ). In brief, to fulfill  
186 these ratios, the potassium metabisulfite additions were  $20$  and  $200 \text{ mg L}^{-1}$  (corresponding  
187 approx. to  $11$  and  $110 \text{ mg L}^{-1}$  of sulfur dioxide), ascorbic acid was added at  $30$  and  $300 \text{ mg L}^{-1}$   
188 <sup>1</sup>, and the GSH level was set at  $52$  and  $520 \text{ mg L}^{-1}$ . For the YD preparation (for which it was  
189 not possible to operate in terms of molar concentration), two additions were performed at  $500$

190 and 5000 mg L<sup>-1</sup>, 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**

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<sup>13</sup> and in agreement with the literature<sup>16</sup>.  
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<sup>7</sup>. In their experiments,  
208 oxidation of 4-methylcatechol in a model solution containing iron and copper was accelerated  
209 by SO<sub>2</sub> 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<sup>13</sup>. 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<sup>2</sup>. Ascorbic acid also produced good color protection, and no evidence of the so-called “crossover” effect<sup>17,18</sup> 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<sup>18</sup> and the ratio of ascorbic acid to catalytic metal ions, i.e., iron and copper<sup>17</sup>. Bradshaw and colleagues<sup>18</sup> 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<sup>13</sup> 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.<sup>18</sup> in contrast with the 20 °C storage temperature set up in the current experiments.

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

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

To obtain additional insight on the spectral data behavior, reverse-phase HPLC analysis of (+)-catechin and its oxidation products was performed in the model solutions. The chromatographic profile of the control samples at 420 nm was modified after storage by the appearance of five new peaks (Fig. 3), in agreement with observations by Guyot and co-workers<sup>22</sup>. These oxidation products generally did not appear in the presence of SO<sub>2</sub> and were detected only in traces when catechin was supplemented with ascorbic acid. Both GSH and YD were able to reduce their formation with respect to the control, although the latter seemed slightly more effective in limiting the appearance of these colored compounds during storage. Quantitative evidence of these effects can be observed in Table 1, where the results of ANOVA analysis (performed on the absolute areas of the detected peaks) are reported. It is interesting to note that no significant differences in (+)-catechin concentration were found among the samples after 29 days of storage.

*Cyclic voltammetric analyses*

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

they are overlaid on the voltammogram recorded for the (+)-catechin model solution in the absence of antioxidants (control).

As expected, the voltammogram of the control shows two partially overlapping anodic peaks. The first one is conceivably related to oxidation of the *ortho*-hydroxyl groups of the catechol moiety of (+)-catechin (B-ring) to generate the corresponding quinone<sup>23</sup>. The second is attributable to oxidation of the –OH groups in positions 5 and 7 of the resorcinol moiety of the flavonoid (A ring)<sup>24</sup>. The former (peak 1) has a maximum at a potential (E) of 574 mV (current intensity: 12.2  $\mu$ A), and the latter (peak 2) reaches a maximum at 745 mV (intensity: 9.8  $\mu$ A). The presence in the reverse scan curve of a single low-intensity cathodic peak, located at approx. 300 mV and coupled with the anodic peak 1, offers evidence of a first quasi-reversible process related to oxidation of the catechol B-ring, and a second non-reversible one, corresponding to the oxidation of the flavonoid A-ring. The latter presumably leads to the formation of a polymeric film, which is able to inactivate the working electrode surface. For this reason, only the voltammograms recorded in the first scan were considered in this study, with a particular focus on the first anodic process (peak 1).

The addition of the different antioxidants slightly modified this voltammetric profile. Sulfur dioxide supplementation, for instance, produced a higher intensity in the two anodic peaks, particularly the first one (Fig. 4a). The current intensity detected for the control samples at the peak maximum was 12.2  $\mu$ A ( $E_{\text{max}}$ : 574 mV) on average, and this value increased to 13.0  $\mu$ A ( $E_{\text{max}}$ : 554 mV) for sulfited (+)-catechin model solutions. This difference might appear small, but the results of ANOVA analysis (performed on the intensities at the peak maximum) identified significant differences between the two sets of experiments (control vs. SO<sub>2</sub>) at  $p < 0.05$  (data not shown). In addition, these behaviors confirm the findings of Makhotkina and Kilmartin, who also observed an increase in the anodic current of the same order of magnitude when SO<sub>2</sub> was added to a catechin solution<sup>25</sup>. They explained this result by considering the ability of sulfites to react with quinones and reduce them back to catechols<sup>7</sup>.

290 This process might regenerate the oxidation substrate, which can be further oxidized at the  
 291 carbon electrode,<sup>25</sup> 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<sub>2</sub> 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  $\mu$ A, with a slight overpotential observed for peak 1 ( $E_{\text{max}}$ : 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<sup>25</sup>.

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  $\mu$ A, with a non-negligible  
 306 overpotential detected ( $E_{\text{max}}$ : 614 mV, + 40.0 mV with respect to  $E_{\text{max}}$  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<sup>25</sup>, 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.



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

Based on these behaviors, other than the sulfites, none of the other additives appeared to be present in the model solutions at the end of the storage period, and none of them were as effective as  $\text{SO}_2$  in protecting (+)-catechin from oxidation. This result might explain the reasons for the lower effectiveness of these additives in preventing the development of brown color (Fig. 2 and 3) and might also be connected with the lower molar concentration that some of them had in the model solutions. In fact, comparing the amounts of  $\text{SO}_2$ , GSH and ascorbic acid supplemented in the different trials, the former was added at 50  $\text{mg L}^{-1}$ , which corresponds to 0.78 mM, and the latter two, with an equal addition in  $\text{mg L}^{-1}$ , produced molar concentrations of 0.16 mM (GSH) and 0.28 mM (ASC), respectively. To further support these findings and investigate the effects of different molar concentrations of antioxidants on the electrocatalytic behavior of (+)-catechin, further cyclic voltammetric trials were performed.

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

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

Sulfur dioxide was added at two dosages, 11 (0.17 mM) and 110  $\text{mg L}^{-1}$  (1.70 mM), both levels included in the range normally used at the winery scale (Fig. 5a). For the pure compound, the lowest amount (0.17 mM) produced a voltammogram similar to those reported in literature for slightly higher  $\text{SO}_2$  concentrations (0.25 mM)<sup>25</sup>. The oxidation peak of  $\text{SO}_2$



was evident only for the 1.70 mM solution, which showed an intense anodic peak at a potential close to 1100 mV, highlighting the large overpotential required to observe sulfite oxidation at the carbon electrode<sup>25</sup>. When sulfur dioxide was added to catechin solutions, the voltammograms of the control samples were modified as reported previously, with an increase in the two oxidation peaks that was proportional to the antioxidant concentration. At the same time, the  $E_{\max}$  of the peak 1 shifted to higher values, and the shape of the curves gave evidence of a process that loses its reversibility as the sulfiting level increases. According to these behaviors, an increased  $\text{SO}_2$  concentration appeared to produce a more intense involvement of (+)-catechin in oxidative reactions. The sulfites themselves might act by reducing back quinones in a manner that becomes more evident as their level increases. No specific oxidation peak for  $\text{SO}_2$  (at 1100 mV) was detected in the presence of catechin for either of the two sulfiting levels. This result might confirm that in the presence of polyphenols, the additive might be preferentially involved in the scavenging of oxidation compounds (e.g., quinones or hydrogen peroxide) rather than in the direct oxidation to sulfate, in agreement with the findings of Danilewicz<sup>8</sup>.

The behavior of ascorbic acid alone (Fig. 5b) was similar to results reported elsewhere,<sup>25</sup> with an anodic peak close to 300 mV and a shape of the voltammograms that highlights a non-reversible process (lack of cathodic peak). Nevertheless, in the presence of (+)-catechin, the two concentrations supplemented led to different situations. At the lowest level, ascorbic acid did not increase the intensity of the anodic peak 1 of (+)-catechin (with respect to the intensity of the control), and the oxidation peak of the additive itself was quite evident in the voltammogram (close to 300 mV). This result might confirm the ability of the antioxidant to be oxidized preferentially with respect to the polyphenols, and in such a manner it is less effective than sulfites in reducing back quinones (anodic peak 1 was less intense after ASC addition than after sulfiting). Based on such observation, at a concentration close to  $30 \text{ mg L}^{-1}$  (0.17 mM, commonly found in wine), the activity of ASC appeared connected more directly

with its direct oxidation at the glassy carbon electrode and less with its ability to react with polyphenol oxidation products. When the concentration increased to  $300 \text{ mg L}^{-1}$  ( $1.70 \text{ mM}$ ), ascorbic acid appeared to be preferentially involved in the scavenging of quinones. The anodic peak at  $300 \text{ mV}$  was less evident in the voltammogram, whereas the intensity of the anodic peak 1 of (+)-catechin significantly increased, indicating a possible minor involvement of ASC in direct oxidation. These observations might be connected with the so-called “crossover” effect<sup>26</sup>. Nevertheless, the reasons for such different behaviors in the voltammetric traces should be further confirmed and investigated in the future.

For pure glutathione (Fig. 5c), at a concentration close to  $50 \text{ mg L}^{-1}$  ( $0.17 \text{ mM}$ , commonly found in wine), the tripeptide gave rise to voltammetric curves similar to those described in literature, with no peaks detected below  $1000 \text{ mV}$  and an increasing anodic current observed as the potential exceeded approximately  $600 \text{ mV}$ <sup>25</sup>. In contrast, at  $520 \text{ mg L}^{-1}$  ( $1.70 \text{ mM}$ ), in addition to the described broad increase of current above  $600 \text{ mV}$ , the traces showed an additional anodic peak at  $292 \text{ mV}$ . Such a peak was detected in all repetitions performed for  $1.70 \text{ mM}$  pure GSH as well as when the same concentration of the tripeptide was added to (+)-catechin solutions.

The presence of this oxidation peak was not reported in other voltammetric studies related to glutathione, and according to the literature, it is unlikely to correspond to the oxidation of GSH itself to disulfide (GSSG). In fact, Huang, Yan, and Tong<sup>27</sup> report that amperometric detection of GSH at common electrodes (including glassy carbon electrodes) is difficult due to the slow electron transfer rate of the tripeptide, which results in a high anodic potential. Based on such considerations, the peak might be connected with adsorption phenomena that potentially involve GSH or impurity traces present in the model solution. However, due to the interest in the supposed antioxidant activity of glutathione in wines, the presence of this peak should be further investigated in the future.

When GSH was added in the presence of (+)-catechin at the lowest supplementation amount, it caused an increase in anodic peak 1 of the polyphenol, in agreement with the literature<sup>25</sup>. This increase might be linked to the ability of GSH itself in reducing back quinones. If we compare the height of anodic peak 1 registered for GSH/ catechin (Fig. 5c) with those detected for sulfur dioxide/catechin (Fig. 5a) and ascorbic acid/catechin (Fig. 5b), it might be argued that (at equimolar concentration) the ability of the tripeptide in scavenging quinones is the intermediate between the other two antioxidants. When the concentration of GSH increased (1.70 mM), the oxidation peak 1 of (+)-catechin also increased, presumably because a greater amount of the tripeptide is involved in the scavenging of quinones.

Finally, Figure 5d reports the cyclic voltammograms recorded for the samples supplemented with the inactive dry yeast preparation. As shown, the profiles collected in the absence of (+)-catechin are similar to the traces reported for pure GSH, with no anodic peaks detected in the range of potential scanned. Moreover, no differences were found between the curves registered for the two levels of supplementation of 500 and 5000 mg L<sup>-1</sup>.

When YD was added to the (+)-catechin model wine, the behavior of the anodic trace of the polyphenol was quite different with respect to that observed for the other antioxidants tested. Independent of the amount added, YD caused a decreased intensity of the oxidation peak 1 of the polyphenol and a slight shift of the peak maximum towards more positive potentials.

This effect might be ascribed to adsorption phenomena related to the ability of inactive dry yeasts to release macromolecules, particularly proteins and glucidic colloids<sup>14</sup>. Such macromolecules might have been adsorbed on the glassy carbon surface, thus hampering the (+)-catechin voltammetric response. Moreover, certain of these substances (e.g., proteins) are well known to have a binding capacity towards phenolics and quinones,<sup>2,28</sup> and this might have facilitated an increased adsorption of the flavonoid oxidation products onto the working electrode. The observation that proteins demonstrated a certain ability to mask the antioxidant capacity of catechin might support this hypothesis<sup>28</sup>.

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

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

## Conclusions

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

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.

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**Figure Captions**

Figure 1. Oxygen consumption capacity of (+)-catechin model solution (Control) as affected by antioxidant supplementation: sulfur dioxide 50 mg L<sup>-1</sup> (SO<sub>2</sub>); ascorbic acid 50 mg L<sup>-1</sup> (ASC); glutathione 50 mg L<sup>-1</sup> (GSH); inactive dry yeast 500 mg L<sup>-1</sup> (YD). Mean values of three repetitions are reported; vertical bars represent standard deviation. (+)-catechin concentration in the samples: 500 mg L<sup>-1</sup>.

Figure 2. Visible spectra of (+)-catechin model solution (Control) as affected by antioxidant supplementation: sulfur dioxide 50 mg L<sup>-1</sup> (SO<sub>2</sub>); ascorbic acid 50 mg L<sup>-1</sup> (ASC); glutathione 50 mg L<sup>-1</sup> (GSH); inactive dry yeast 500 mg L<sup>-1</sup> (YD). Each curve represents the average spectrum of three repeated samples. (+)-catechin concentration in the samples: 500 mg L<sup>-1</sup>.

Figure 3. Chromatograms (recorded at 420 nm) of (+)-catechin model solution (Control) supplemented and not with the different antioxidants: sulfur dioxide 50 mg L<sup>-1</sup> (SO<sub>2</sub>); ascorbic acid 50 mg L<sup>-1</sup> (ASC); glutathione 50 mg L<sup>-1</sup> (GSH); inactive dry yeast 500 mg L<sup>-1</sup> (YD). Antioxidant addition determined differences among the samples, in the retention time range between 28 and 45 min. (+)-catechin concentration in the samples: 500 mg L<sup>-1</sup>.

Figure 4. Cyclic voltammograms (0.0 – 1.3 V) recorded for (+)-catechin model solution (Control) supplemented and not with the different antioxidants: sulfur dioxide 50 mg L<sup>-1</sup> (SO<sub>2</sub>); ascorbic acid 50 mg L<sup>-1</sup> (ASC); glutathione 50 mg L<sup>-1</sup> (GSH); inactive dry yeast 500 mg L<sup>-1</sup> (YD). Each curve represents the average voltammogram of three repeated samples. (+)-catechin concentration in the samples: 500 mg L<sup>-1</sup>.

1  
2 557 Figure 5. Cyclic voltammograms (0.0 – 1.3 V) recorded in model wine (pH 3.2, ethanol 12 %  
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4 558 v/v) for (+)-catechin ( $50 \text{ mg L}^{-1}$  – 0.17 mM), the different antioxidants at different molar  
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6 559 concentration (0.17 and 1.70 mM) and the catechin / antioxidant mixtures at different molar  
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8 560 ratios (1:1 and 1: 10). YD preparation was added at 500 and 5000  $\text{mg L}^{-1}$ . Each curve  
9  
10 561 represents the average voltammogram of three repeated samples. See the text for  
11  
12 562 abbreviations.  
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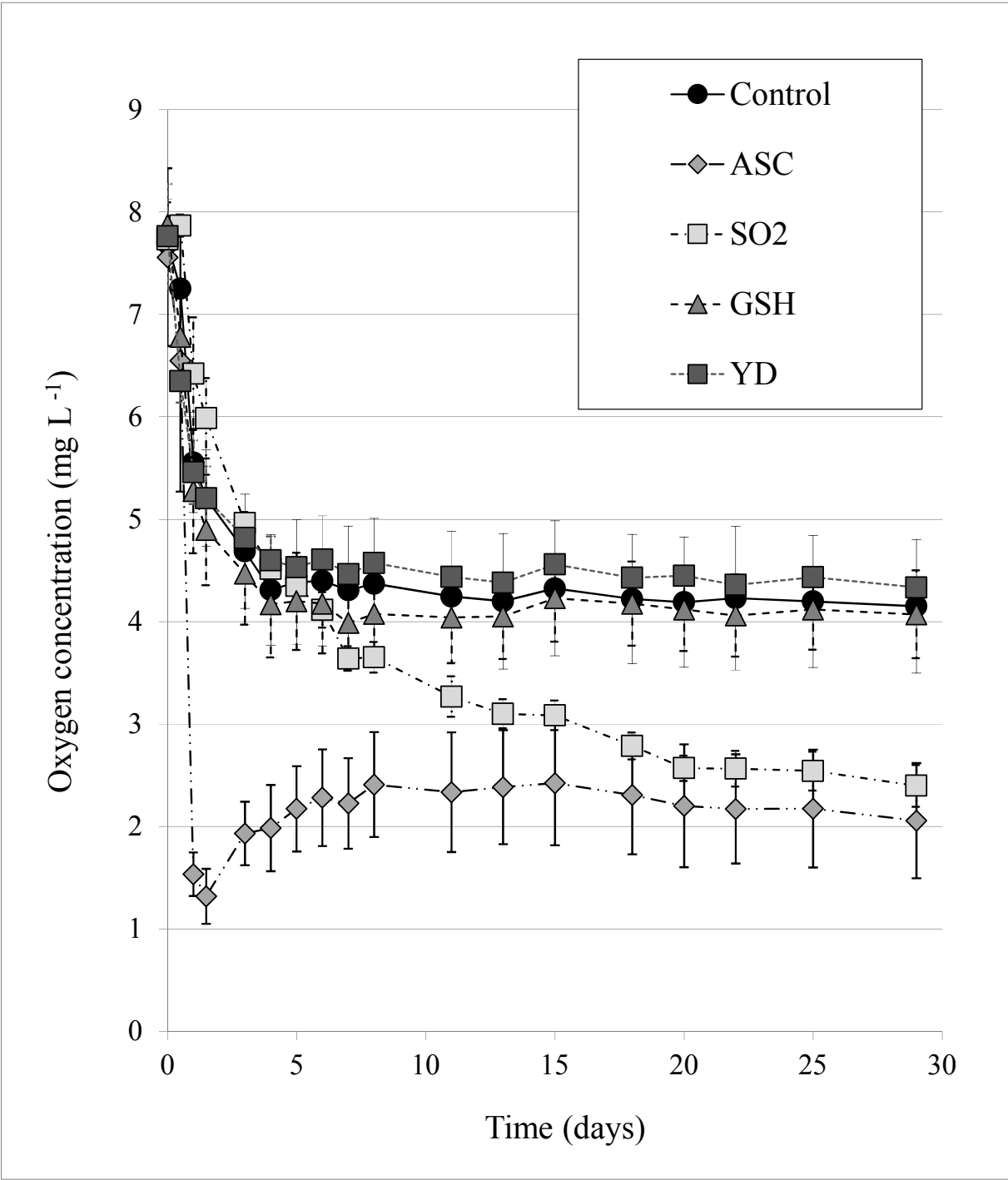


Figure 1

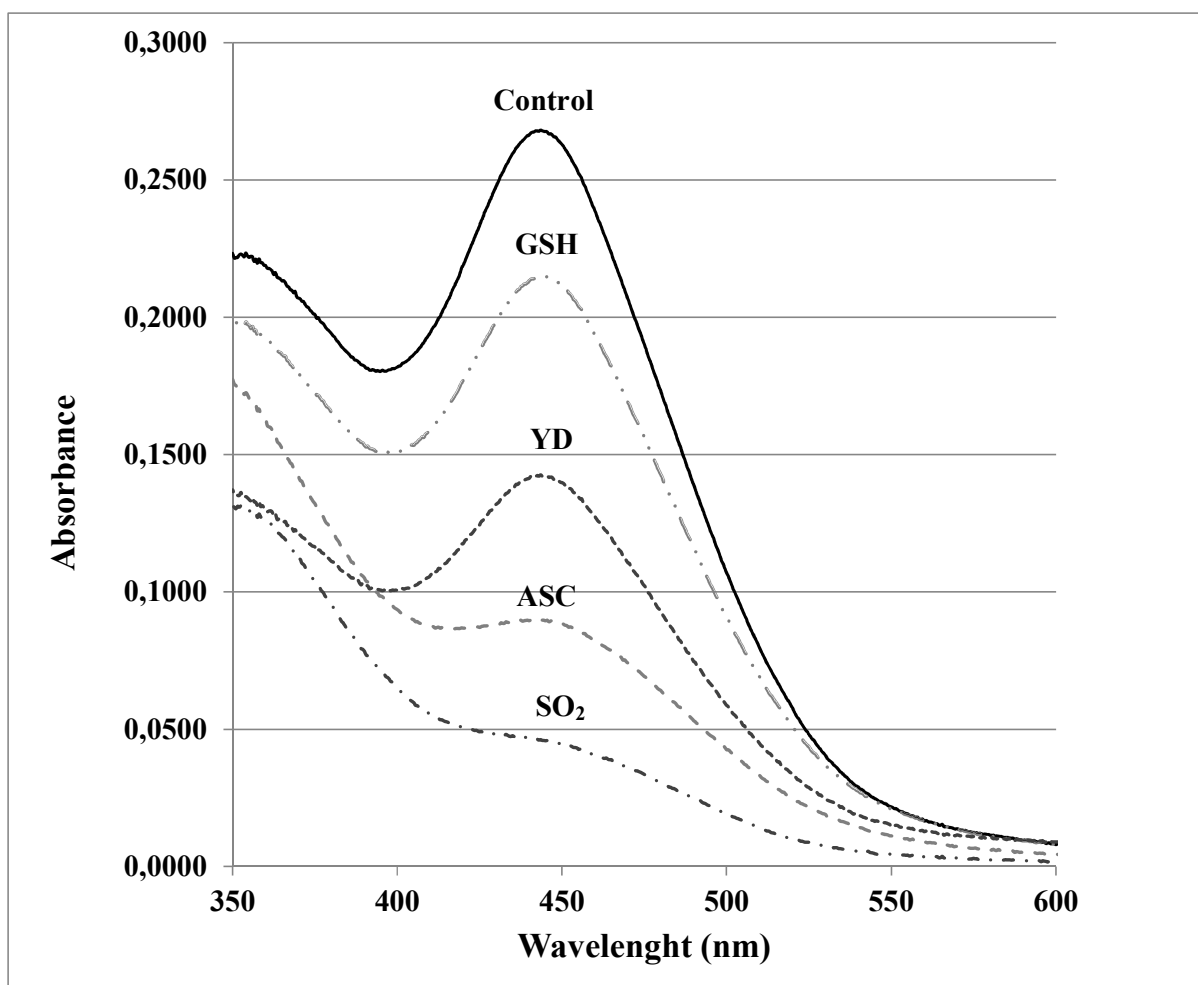


Figure 2

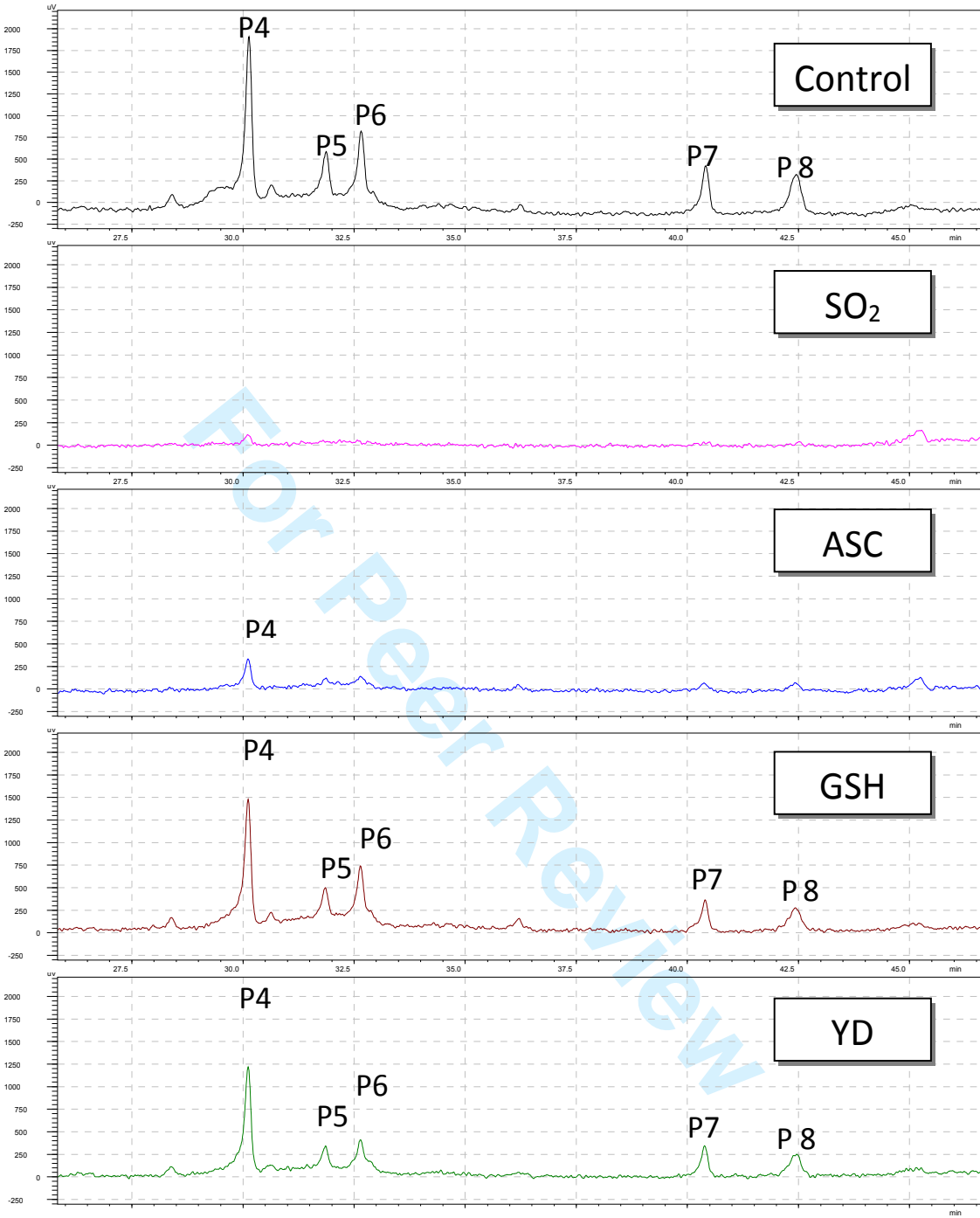


Figure 3

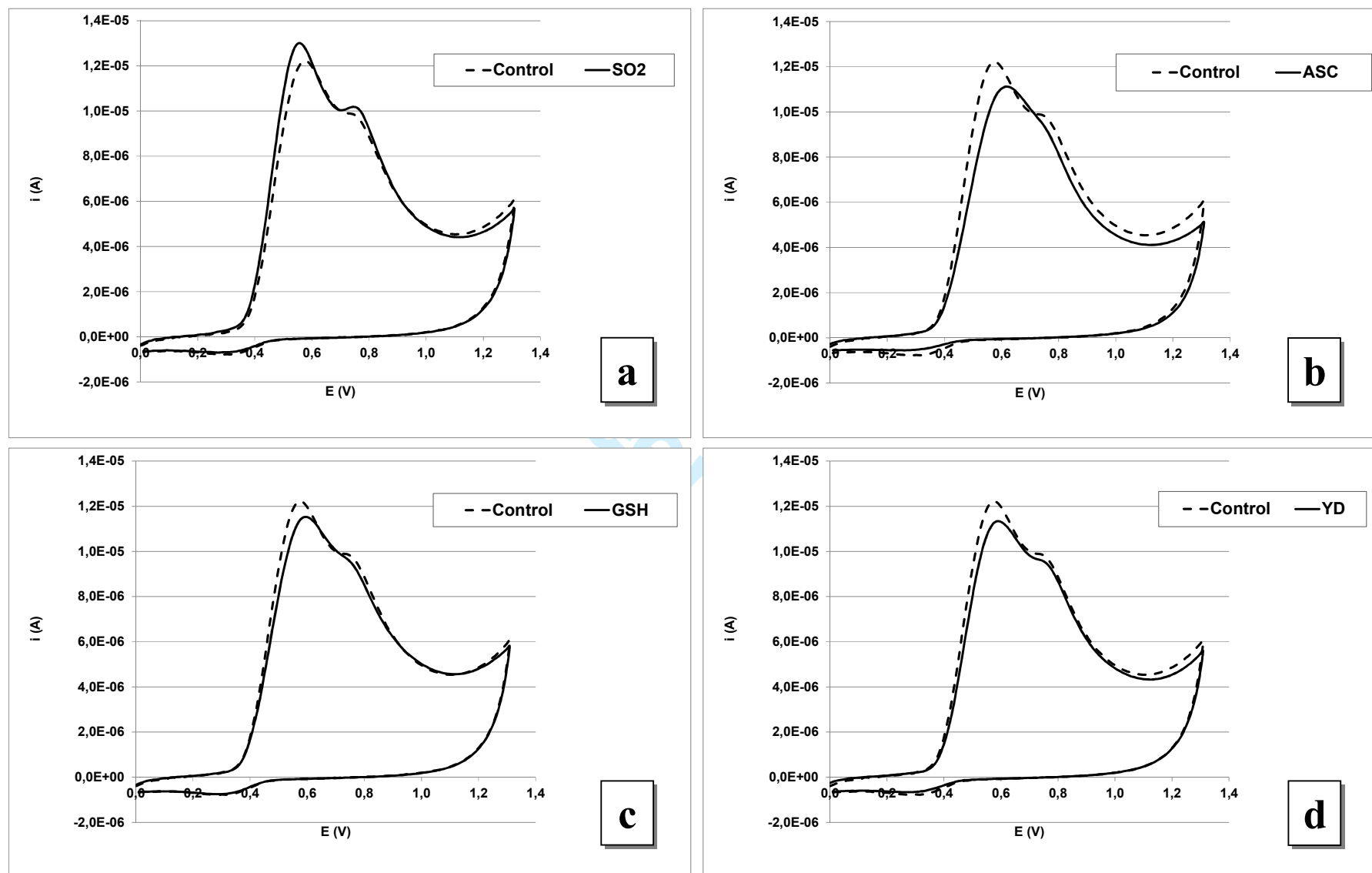


Figure 4

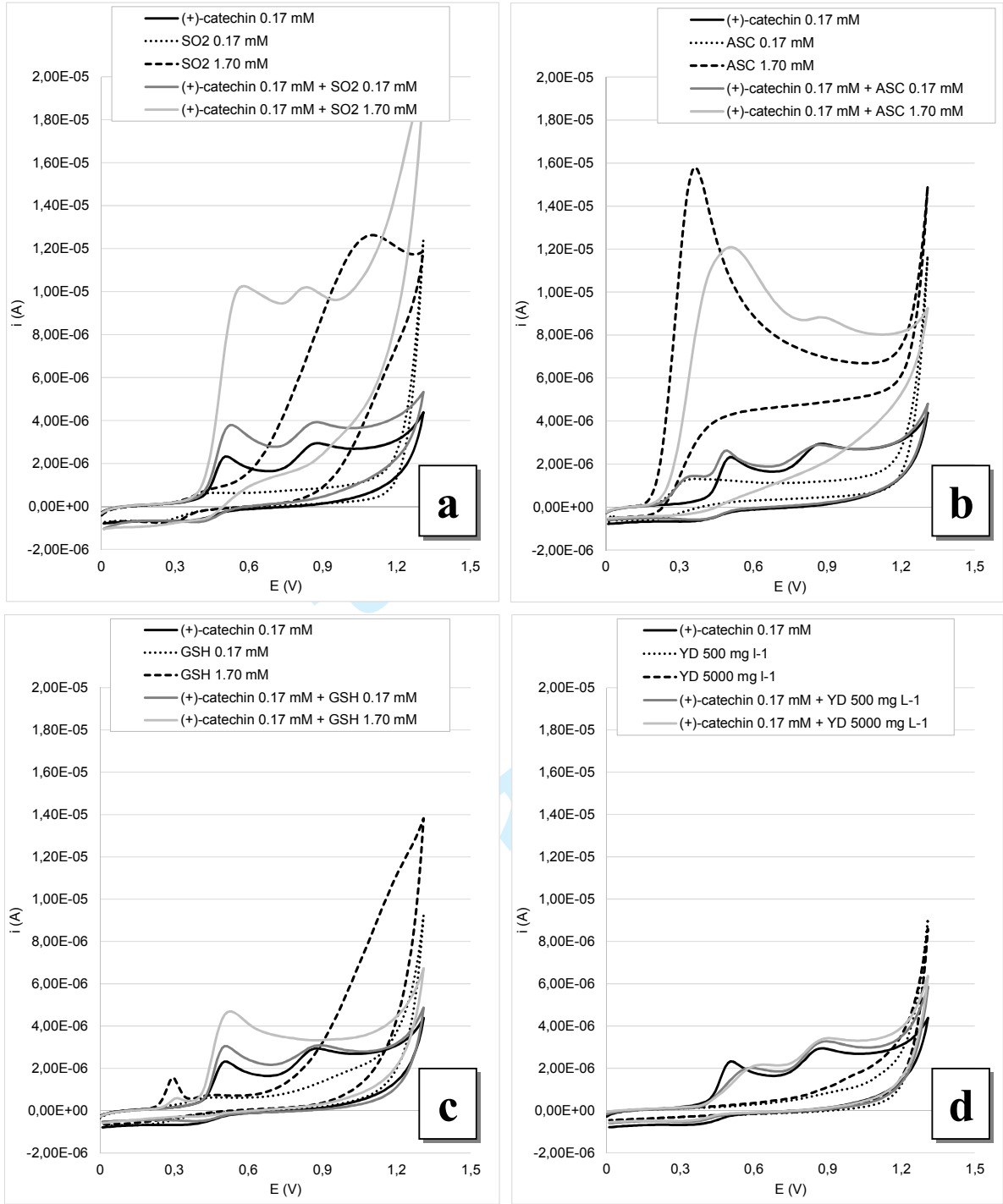


Figure 5

Table 1. HPLC data (absolute areas / 1000) obtained from (+)-catechin model solution (Control) supplemented and not with the different antioxidants: sulfur dioxide 50 mg L<sup>-1</sup> (SO<sub>2</sub>); ascorbic acid 50 mg L<sup>-1</sup> (ASC); glutathione 50 mg L<sup>-1</sup> (GSH); inactive dry yeast 500 mg L<sup>-1</sup> (YD). Results of ANOVA and Tukey HSD Test are reported: different letters mark significant differences at p< 0.05. (+)-catechin concentration in the samples: 500 mg L<sup>-1</sup>. For Peak number, refer to Figure 3.

sample	(+) catechin_UV (absolute area / 1000)			peak 4_Vis (absolute area / 1000)			peak 5_Vis (absolute area / 1000)		
	mean ± SD			mean ± SD			mean ± SD		
control	1451	± 48	<b>a</b>	33	± 8	<b>c</b>	12	± 5	<b>c</b>
SO <sub>2</sub>	1487	± 100	<b>a</b>	2	± 1	<b>a</b>	0	± 0	<b>ab</b>
ASC	1429	± 64	<b>a</b>	5	± 1	<b>a</b>	1	± 1	<b>a</b>
GSH	1448	± 97	<b>a</b>	23	± 2	<b>bc</b>	9	± 1	<b>c</b>
YD	1490	± 56	<b>a</b>	21	± 3	<b>b</b>	7	± 3	<b>bc</b>

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