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Antioxidant properties of different products and additives in white wine

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Abstract: Different winemaking products (ascorbic acid, glutathione, yeast lees and a yeast autolysate) were tested in comparison with sulfur dioxide, concerning radical scavenging activity (measured by DPPH\* assay), oxygen consumption capacity and ability to reduce wine color and predisposition to browning. Trials were performed in white wines and model solution. SO<sub>2</sub> was the most active in reducing wine color development. Fresh lees and ascorbic acid were very effective in oxygen and free radical scavenging, but they both induced browning during wine storage, the former, by releasing phenolic compounds. Glutathione was also able to scavenge DPPH\* in wine, but less effective against oxygen, and it induced browning during storage. Surprisingly, the yeast derivative preparation was the treatment that behave more similarly to sulfiting; it was very active in scavenging DPPH\*, and, even without modifying oxygen consumption rate, it protected quite well wine color and phenolics over a eight months storage time.

## Cover Letter

Sulfur dioxide is considered one of the most important enological additives, but it is also toxic and allergenic. In Europe, it is mandatory to label wines with the indication “contains sulfites”, if total sulfur dioxide is higher than 10 mg/L; this has determined an increasing interest of the consumers towards the health-related aspects connected with wine consumption. For this reason, the reduction of the use of sulfur dioxide is considered one of the key objectives of modern winemaking and it is strictly connected with the priorities established by the “International Organization of Vine and Wine” (O.I.V.). Current knowledge and technologies allow to postpone sulfiting at the end of alcoholic and malolactic fermentation, with a significant reduction of the overall sulfite levels without jeopardizing wine quality. Contrary, it is more difficult to replace SO<sub>2</sub> after the fermentations, because none of the products currently available for complementing the activity of this additive during wine storage and ageing, is as effective as sulfites themselves. Some of these complementary substances, such as ascorbic acid, have been extensively studied, while some other (e.g. glutathione) were less investigated; anyway, maybe paradoxically, the most of the studies available have been published on technical journals, and the most of the few scientific publications available are focused just on one or two alternatives, sometimes without a direct comparison with sulfur dioxide; moreover, the trials have been generally performed in model solution and only few evidences have been collected on wines. This paper aims to investigate the radical scavenging activity and the oxygen consumption capacity of different enological products and additives (ascorbic acid, glutathione, yeast lees and a yeast derivative preparation), in comparison with SO<sub>2</sub>, also considering their effect on wine color and predisposition to browning. Trials were performed in model solution and in different wine typologies. We consider these results just as a preliminary investigation, but, as far as we know, this is one of the most comprehensive, direct comparisons, between sulfur dioxide and other additives, reporting effects on real wines. Moreover, surprisingly, the yeast derivative preparation used, was the additive that behave more similarly to sulfur dioxide, demonstrating its ability in protecting wine color over a 8 months storage time. As far as we know, this is the first time this kind of products has been directly compared with sulfites concerning their antioxidant characteristics.

1 **Antioxidant properties of different enological products**  
2 **and additives in comparison with sulfur dioxide**

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13

14 **Abbreviated running title:**

15 Antioxidant capacity of enological products compared with sulfites

16 **Abstract**

17 Different winemaking products (ascorbic acid, glutathione, yeast lees and a yeast autolysate)  
18 were tested in comparison with sulfur dioxide, concerning radical scavenging activity  
19 (measured by DPPH• assay), oxygen consumption capacity and ability to reduce wine color  
20 and predisposition to browning. Trials were performed in white wines and model solution.  
21 SO<sub>2</sub> was the most active in reducing wine color development. Fresh lees and ascorbic acid  
22 were very effective in oxygen and free radical scavenging, but they both induced browning  
23 during wine storage, the former, by releasing phenolic compounds. Glutathione was also able  
24 to scavenge DPPH• in wine, but less effective against oxygen, and it induced browning during  
25 storage. Surprisingly, the yeast derivative preparation was the treatment that behave more  
26 similarly to sulfiting; it was very active in scavenging DPPH•, and, even without modifying  
27 oxygen consumption rate, it protected quite well wine color and phenolics over a eight  
28 months storage time.

29

30 **KEYWORDS: sulfur dioxide; yeast derivatives; ascorbic acid; glutathione; yeast lees;**  
31 **wine**

32

## 33 **1 Introduction**

34 Despite the mechanisms involved in wine oxidation have been extensively reviewed  
35 (Singleton, 1987; du Toit, Marais, Pretorius, & du Toit, 2006; Waterhouse & Laurie, 2006;  
36 Oliveira, Ferreira, De Freitas, & Silva, 2011), the protection of wine against oxidative  
37 spoilage remains one of the main goals of modern winemaking, becoming particularly critical  
38 when low levels of sulfur dioxide are used. The chemistry of this additive in wine has been  
39 recently re-written by Danilewicz (2007; 2011) and Danilewicz, Seccombe, & Whelan  
40 (2008): they clearly demonstrated that SO<sub>2</sub> does not react directly with oxygen, as previously  
41 thought (Ribéreau-Gayon, Dubourdiou, Doneche, & Lonvaud, 2006), but, in presence of  
42 metal ions, it is able to scavenge hydrogen peroxide and the quinones formed from the  
43 oxidation of polyphenols (Danilewicz et al., 2008). Due to the toxicity and allergenic potential  
44 of sulfites, different compounds have been proposed for reducing their final concentration in  
45 wine, even if, none of them is likewise effective in protecting wine against oxidations.

46 Ascorbic acid (ASC) is the most known among these products; it is able to scavenge hydroxyl  
47 radicals (Bradshaw, Barril, Clark, Prenzler, & Scollary, 2011) and quinones (Waterhouse &  
48 Laurie, 2006; Bradshaw et al., 2011), but its metal catalyzed oxidation produces hydrogen  
49 peroxide (Zoecklein, Fugelsang, Gump, & Nury, 1995; Moreaux, Birlouez-Aragon, &  
50 Ducauze, 1996; Ribéreau-Gayon et al., 2006; Bradshaw et al., 2011) and this may trigger  
51 browning reactions if sulfites are not present (Bradshaw, Prenzler, & Scollary, 2001;  
52 Bradshaw, Cheynier, Scollary, & Prenzler, 2003). The capacity of ascorbic acid to act both as  
53 antioxidant and free-radical initiator is known as “crossover effect” (Buettner & Jurkiewicz,  
54 1996; Bradshaw et al., 2001; Bradshaw et al., 2003) and explains the reason why ASC is  
55 normally used in wine in combination with sulfites (Zoecklein et al., 1995; Ribéreau-Gayon et  
56 al., 2006; Bradshaw, et al., 2011).

57 Another traditional system to protect wine against oxidations is the use of yeast lees (Pérez-  
58 Serradilla & Luque de Castro, 2008). Fresh lees have a high oxygen consuming capacity  
59 (Fornairon-Bonnefond & Salmon, 2003), due to the presence of yeast membrane lipids and  
60 sterols (Salmon, Fornairon-Bonnefond, Mazauroic, & Moutounet, 2000; Fornairon-Bonnefond  
61 & Salmon, 2003); adsorbed polyphenols (Gallardo-Chacón, Vichi, Urpí, López-Tamames, &  
62 Buxaderas, 2010), thiol groups of cell wall proteins (Jaehrig, Rohn, Kroh, Fleischer, & Kurz,  
63 2007); Gallardo-Chacón et al., 2010) and  $\beta$ -glucans from yeast cell walls (Jaehrig et al., 2007)  
64 also contributes to their antioxidant properties. However, ageing on the lees can modify wine  
65 sensory characters, and for this reason it is not suitable for all the wine typologies; moreover,  
66 lees alone do not protect wine against microbial pollution and sulfiting is always required.

67 The possibility to use glutathione (GSH) as wine antioxidant has been considered since the  
68 role of this tripeptide in preventing must browning has been highlighted (Singleton, Salgues,  
69 Zaya, & Trousdale, 1985); nevertheless, very few studies are currently available concerning  
70 GSH addition in wine. It has been reported that glutathione supplementation could have  
71 positive effects on wine color and aroma (Dubourdieu & Lavigne-Cruege, 2003;  
72 Papadopoulou & Roussis, 2008), but high amounts of GSH in oxidative conditions can lead to  
73 color formation (Sonni, Clark, Prenzler, Riponi, & Scollary, 2011).

74 GSH can be supplemented also in form of yeast derivatives (YD): the ability of “glutathione-  
75 enriched” inactive dry yeast preparations (IDY) in reducing the loss of volatile compounds  
76 during wine storage has been reported by Andújar-Ortiz, Rodríguez-Bencomo, Moreno-  
77 Arribas, Martín-Alvarez, & Pozo-Bayon (2010): they hypothesized that this may be due to the  
78 antioxidant capacity of GSH, but also other components of the IDY preparation might be  
79 involved (Andújar-Ortiz et al., 2010).

80 The opportunity to reduce sulfur dioxide, by these alternative tools, is arousing more and  
81 more interest, among winemakers. Nevertheless, despite the amount of works reporting the  
82 antioxidant effects of these substances, the most of the papers regards model solutions and

83 moreover, it is currently difficult to foresee in which extent it is possible to replace sulfites  
84 with each of these alternatives, preserving wine quality, because of the lack of scientifically-  
85 based direct comparisons, among their effects and those of sulfur dioxide.

86 For this reason, the aim of this work was to carry out a preliminary investigation on the  
87 radical scavenging activity (measured by DPPH• assay) and the oxygen consumption capacity  
88 of different enological products and additives in comparison with SO<sub>2</sub>. Ascorbic acid  
89 (considered as reference standard), glutathione, yeast lees and a self-prepared yeast autolysate  
90 were tested. Trials were performed in model solution and in different wine typologies.  
91 Concerning oxygen consumption trials, wines were finally subjected to fast  
92 spectrophotometric measurements, for assessing the effect of the different antioxidants on  
93 color, total phenolics and predisposition to browning.

## 94 **2 Materials and Methods**

### 95 *2.1 Chemicals*

96 Tartaric acid, sodium hydroxide, ethanol (96 % v/v), ACS grade hydrochloric acid (37 %),  
97 hydrogen peroxide (30 % w/w), sodium acetate and potassium metabisulfite were from Carlo  
98 Erba Reagents (Milan, Italy); ascorbic acid, glutathione, 1,1-diphenyl-2-picryl-hydrazyl free  
99 radical (DPPH•) and HPLC grade methanol were purchased from Sigma-Aldrich (St. Louis,  
100 MO, USA).

### 101 *2.2 Yeast lees and yeast derivatives (YD)*

102 Fresh lees were supplied by Viticoltori Friulani “La Delizia” (Casarsa della Delizia, PN, Italy)  
103 and they were obtained after alcoholic fermentation and racking of a white table wine. The  
104 inactive dry yeast preparation (YD) used for the trials was a thermally produced yeast  
105 autolysate, prepared as reported elsewhere (Comuzzo, Tat, Liessi, Brotto, Battistutta, &  
106 Zironi, 2012).



107 *2.3 Evaluation of radical scavenging activity*

108 *2.3.1 Sample preparation*

109 Trials were performed in model solution and wine. The former was a model buffer prepared  
110 by dissolving 5 g/L (33 mM) of tartaric acid in a distilled water – ethanol mixture (12 % v/v);  
111 the pH was set at 3.20 by adding 4 M sodium hydroxide. Ascorbic acid (50 mg/L),  
112 glutathione (50 and 500 mg/L), potassium metabisulfite (100 and 1000 mg/L, corresponding  
113 respectively to 50 and 500 mg/L of sulfur dioxide), yeast lees (2.5 % v/v) and the YD  
114 preparation (2.5 % w/v) were added and the samples were immediately analyzed by DPPH•  
115 assay, as reported below.

116 The wine was a white table wine from harvest 2010, supplied by Viticoltori Friulani “La  
117 Delizia” (Casarsa della Delizia, PN, Italy); additives, lees and YD preparation were added in  
118 the same amounts reported above for wine-like solution; in addition a Control sample  
119 (untreated wine) was also included in the experimental design. Control wine and treated  
120 samples were subjected to DPPH• assay as reported below. All the experiments were carried  
121 out in three repetitions, for both wines and model solutions.

122 *2.3.2 DPPH• assay*

123 DPPH• assay was performed by a modification of the methods reported by Brand-Williams,  
124 Cuvelier, & Berset (1995) and Gallardo-Chacón et al. (2010), using a UV-vis  
125 spectrophotometer (model V-530, Jasco Co. Ltd., Tokyo, Japan). A  $6 \times 10^{-5}$  M DPPH•  
126 solution was prepared fresh daily, in a 60:40 mixture of methanol : acetate buffer (0.1 M  
127 sodium acetate, buffered at pH 4.50 with 6 M hydrochloric acid). 3 mL of this stock solution  
128 were introduced in a 10 mm optical path length glass cuvette (Hellma Analytics, Mülheim,  
129 Germany) and 100  $\mu$ L of the wine samples or fresh prepared antioxidant model solutions were  
130 added; DPPH• discoloration was followed at 515 nm during 10 min, reading the absorbance

131 against methanol: acetate buffer. Results were expressed as the percent diminution of the  
132 original absorbance [ $\Delta$ Abs 515 nm (%)].

133 For the samples treated with yeast lees and YD preparation, where insoluble particles were  
134 present, the reaction with DPPH• has been carried out as suggested by Gallardo-Chacón et al.  
135 (2010): 3 mL of DPPH• and 100  $\mu$ L of sample were introduced in a test tube; after 10 min,  
136 the reaction mixture was filtered on a 0,80  $\mu$ m nylon membrane and immediately subjected to  
137 spectrophotometric measurement. The initial value of the absorbance was read by adding 100  
138  $\mu$ L of methanol: acetate buffer, to 3 mL of DPPH• stock solution.

139 Concerning model solutions, for taking into account the effect of the solvent, a blank was also  
140 prepared, performing the DPPH• assay on the model buffer alone (tartaric acid in  
141 hydroalcoholic solution 12 % v/v, pH 3.2); the percent values measured for the  $\Delta$ Abs 515 nm  
142 were used to correct the analytical results.

## 143 *2.4 Oxygen consumption capacity*

### 144 *2.4.1 Equipment*

145 The system used for oxygen measurements was an OxySense<sup>®</sup> fluorimeter (OxySense Inc.,  
146 Dallas, TX, USA); O2xyDot<sup>®</sup> oxygen sensitive sensors (OxySense Inc., Dallas, TX, USA),  
147 were glued, by a specific silicon based oxygen permeable adhesive (OxySense Inc.), to the  
148 inner surface of each of the 750 mL colorless glass bottles used for the experiments. When  
149 O2xyDot<sup>®</sup> sensors are illuminated by a pulsed blue light, they emit a red fluorescent light,  
150 that is monitored by OxySense<sup>®</sup> fluorimeter. Dynamic quenching by oxygen molecules  
151 determines a decrease of the O2xyDot<sup>®</sup> fluorescence lifetime, that is proportional to the  
152 oxygen concentration in the bottles; the temperature is measured simultaneously, by an  
153 infrared sensor positioned in the reader pen (Li, Ashcraft, Freeman, Stewart, Jank, & Clark,  
154 2008).

155 2.4.2 *Sample preparation*

156 Two different white wines were used in two different sets of experiments. In the first one, a  
157 base wine for Prosecco D.O.C.G. Conegliano Valdobbiadene (harvest 2012), supplied by  
158 Cantina Produttori Valdobbiadene “Val d’Oca” (San Giovanni di Valdobbiadene, TV, Italy),  
159 was used; free sulfur dioxide was 13 mg/L and alcoholic strength 10.50 % v/v. The wine  
160 coming from a 20 L bulk, was shaken until oxygen saturation was reached and then  
161 transferred into the 750 mL bottles where the O2xyDot<sup>®</sup> sensors were glued (see Section  
162 2.4.1). The average oxygen concentration measured at filling was  $8.25 \pm 0.19$  mg/L and the  
163 temperature of the samples was 19.0 °C. Ascorbic acid (50 mg/L), glutathione (50 mg/L),  
164 potassium metabisulfite (100 mg/L, corresponding to 50 mg/L of sulfur dioxide), yeast lees  
165 (2.0 % v/v) and YD preparation (500 mg/L) were added to the bottles; all the treatments were  
166 replicated three times. Nitrogen was blown in the headspace of the samples and bottles were  
167 immediately sealed with crown cap closures. A control sample (untreated wine) without any  
168 antioxidant addition was also prepared in three repetitions.

169 A blend of Chardonnay, Sauvignon and Picolit from harvest 2007 (Bastianich Winery,  
170 Cividale del Friuli, UD, Italy) was used for the second set of trials; free sulfur dioxide was 5  
171 mg/L and alcoholic strength 13.50 % v/v. Wine was saturated with oxygen (as reported  
172 above) and then transferred in the 750 mL bottles with O2xyDot<sup>®</sup> sensor; the average oxygen  
173 concentration at filling was  $7.29 \pm 0.14$  mg/L and the temperature of the samples 21.0 °C.  
174 Potassium metabisulfite, ascorbic acid, glutathione, YD preparation and fresh lees were added  
175 to the bottles in the same amounts reported above; a control sample (untreated wine) without  
176 any antioxidant addition was also prepared and three repetitions were setup for each  
177 experiment. As reported for the Prosecco base wine, nitrogen was blown in the headspace of  
178 the samples and bottles were immediately crown capped. For both wine typologies, oxygen  
179 concentration into the bottles was measured daily and wines were stored at 20 °C during the  
180 whole time of the measurements.

181 2.4.3 *Spectrophotometric measures and browning assay*

182 All the wines were analyzed 15 days after bottling; in addition, for the blended wine, analyses  
183 were repeated after 8 months. Wine color and total phenolics were assessed by measuring the  
184 absorbance of the samples at 420 and 280 nm respectively, using 10 mm optical path length  
185 quartz cuvettes (Hellma Analytics, Mülheim, Germany); readings were performed against  
186 distilled water. Concerning the UV measures, wine samples were previously diluted ten times  
187 and total phenolic index (TPI) was calculated multiplying by 10 the absorbance measured at  
188 280 nm.

189 The predisposition of wines towards browning was determined by slightly modifying the so  
190 called POM-test, a browning test reported by Müller-Späth (1992); briefly, 5 mL of wine  
191 were heated at 60 °C for one hour, after addition of 25 µL of a 3 % hydrogen peroxide  
192 solution; the browning produced was estimated on the basis of the percent increase of the  
193 absorbance at 420 nm. All the analyses were carried out by using a UV-vis  
194 spectrophotometer, model V-530 (Jasco Co. Ltd., Tokyo, Japan).

195 2.5 *Statistical analysis*

196 As concerns DPPH• trials, One Way ANOVA was carried out on the percent diminutions of  
197 absorbance, measured during the assay [ $\Delta$ Abs 515 nm (%)]; means and standard deviations  
198 (SD) were calculated and significant differences were evaluated by Tukey HSD test, at  $p <$   
199 0.05. Variances were homogeneous according to Brown-Forsythe test.

200 To assess differences in oxygen consumption rates, Factorial ANOVA was used on the  
201 oxygen concentrations measured for the different treatments; means and standard errors (SE)  
202 were calculated, and significant differences were assessed by Tukey HSD test ( $p <$  0.05).  
203 Variances were homogeneous according to Cochran C, Hartley F-max and Bartlett test.

204 Finally, as regards spectrophotometric measurements (Abs 280 nm, 420 nm and POM-test),  
205 One Way ANOVA and Tukey HSD test were carried out as reported for DPPH• assay;

206 results were considered significant at  $p < 0.05$ . All the analyses were carried out by using the  
207 software Statistica for Windows, version 8.0 (StatSoft, Inc., Tulsa, OK, USA).

### 208 **3 Results and Discussion**

#### 209 *3.1 Evaluation of radical scavenging activity*

210 The results of DPPH• assay are reported in Fig. 1. Concerning model solutions (Fig. 1a), the  
211 highest radical scavenging activity was detected for yeast lees, followed by YD and the  
212 highest sulfur dioxide addition (500 mg/L). At lower amounts, such as those normally used in  
213 winemaking (50 mg/L), SO<sub>2</sub> was less effective in bleaching the free radical and the values  
214 reported were more similar (even if statistically lower) to those measured for the same dosage  
215 of ascorbic acid (reference standard).

216 Despite the reaction of sulfite (SO<sub>3</sub><sup>2-</sup>) and bisulfite ion (HSO<sub>3</sub><sup>-</sup>) with free radicals was  
217 described (Brandt & van Eldik, 1995; Neta & Huie, 1985), very few publications report data  
218 about their radical scavenging capacity in wine; moreover, the information available are  
219 generally not in agreement with the dataset presented here. In a paper published in the late  
220 1990s, Manzocco, Mastrocola, & Nicoli (1998) found that the addition of sulfur dioxide (250  
221 mg/L) to a model system simulating wine (water - ethanol 12 % v/v), did not affect the chain-  
222 breaking capacity of the samples towards DPPH•; the different results they obtained respect to  
223 the present study might be related to the different pH and ionic strength of the solvents used  
224 in the two experiments, for both sample preparation (hydroalcoholic solution vs.  
225 hydroalcoholic buffer, pH 3.20) and DPPH• assay (pure methanol vs. methanol : acetate  
226 buffer pH 4.50). pH might have affected the ratio between the different forms of sulfur(IV)  
227 oxides in aqueous solution and it is known that they have a different reactivity towards free  
228 radicals (Neta & Huie, 1985). Moreover, also the different ionic strength of the two model  
229 solutions might have a non-negligible role: in fact, as reported by Brandt & van Eldik

230 (1995), the reaction rate between sulfur(IV) oxides and radicals increases with the increase of  
231 the ionic strength. Anyway, it is important to underline that the hydroalcoholic tartaric buffer  
232 we used for dissolving or suspending the different products tested, gave a very poor effect in  
233 bleaching DPPH• solution ( $\Delta\text{Abs } 515 \text{ nm} = -3.4 \%$ ).

234 Also the results of Vivas and colleagues (Vivas, Saint-Cricq de Gaulejac, & Glories, 1997)  
235 are in disagreement with the radical scavenging capacity of sulfur dioxide, reported in Fig. 1a.  
236 Studying the influence of SO<sub>2</sub> and ascorbic acid on the scavenging effect of tannins, they  
237 wrote that, at usual enological concentrations, sulfites do not have a scavenging effect on  
238 superoxide anion. Nevertheless, according to literature, superoxide radical reacts very slowly  
239 with sulfite (SO<sub>3</sub><sup>2-</sup>) and the rate constant of the reaction is very low ( $82 \text{ M}^{-1} \text{ s}^{-1}$ ) (Neta & Huie,  
240 1985).

241 As observed for SO<sub>2</sub>, also glutathione activity in model wine depends on the concentration.  
242 GSH levels in wine range from non-detectable values to 70 mg/L (Kritzinger, Bauer, & du  
243 Toit, 2013a), with a relatively high variability; at a concentration close to these amounts (50  
244 mg/L), glutathione demonstrated the lowest ability in bleaching DPPH• and a dosage ten  
245 times higher (500 mg/L) was needed to significantly overtake the effect of the reference  
246 sample (ASC 50 mg/L); nevertheless, regardless of the amounts added, GSH activity was  
247 always significantly lower than that measured for the same addition of sulfur dioxide.

248 This last considerations is not in full agreement with the data published by Vivas, Vivas de  
249 Gaulejac, & Nonier (2001); they measured the scavenging activity towards superoxide anion  
250 of different antioxidants in model solution, in a range of concentration from 0.1 to 1 g/L; they  
251 also found that ascorbic acid was more effective than an equal amount of sulfur dioxide and  
252 glutathione, but in their experiment, the scavenging activities observed for the latter additives  
253 were very similar.

254 Speaking from the chemical point of view, the effects reported for such molecules in Fig. 1a  
255 seem connected quite well with their molar concentration. Glutathione molar concentration

256 in the sample at 50 mg/L, the one with the lowest radical scavenging activity, corresponds to  
257 approx. 0.16 mM. Ascorbic acid and sulfur dioxide at 50 mg/L, were 0.28 and 0.78 mM  
258 respectively and determined a greater percentage of discoloration; the higher efficiency of the  
259 former, despite its lower molar concentration, confirms the higher capacity of ASC to  
260 scavenge free radicals (Vivas et al., 2001). Finally, GSH at 500 mg/L (1.6 mM) and the same  
261 quantity of SO<sub>2</sub> (7.8 mM) showed an increasing activity in DPPH• bleaching.

262 On the basis of this trend, a non-negligible part of the effects observable when comparing  
263 different antioxidants in winemaking conditions (therefore, in the amounts normally used and  
264 speaking in terms of mg/L) should be attributed not only to the chemical characteristics of  
265 each antioxidant molecule in itself, but also to its molar concentration. This is certainly a key  
266 point in evaluating antioxidant additives in comparison with sulfur dioxide; in fact, due to its  
267 average concentration in wine (0-40 mg/L as free SO<sub>2</sub>) and to the lower molecular weight  
268 respect to the most suggested alternatives (ACS or GSH), the molar concentration of free SO<sub>2</sub>  
269 (considering both the molecular fraction and bisulfite ion) is normally higher, respect to that  
270 of these latter molecules. For instance, 240 mg/L of GSH and 137 mg/L of ascorbic acid  
271 would be required for obtaining a 0.78 mM solution, the same molar concentration of sulfur  
272 dioxide in the sample at 50 mg/L; these amounts are more than a half of the European legal  
273 limit established for ASC (250 mg/L, according to the Regulation (EC) No 606/2009) and far  
274 from the normal quantity of GSH naturally detectable in wine (Dubourdieu & Lavigne-  
275 Cruege, 2003; Kritzinger et al., 2013a; Fracassetti, Lawrence, Tredoux, Tirelli, Nieuwoudt, &  
276 du Toit, 2011; Sonni et al., 2011).

277 Another interesting observation can be made, comparing the effects reported in Fig. 1a for the  
278 yeast autolysate (YD) and the two samples supplemented with glutathione. As reported by  
279 Kritzinger and colleagues (Kritzinger et al., 2013a), commercial YDs claim to preserve wine  
280 aroma, delaying the development of browning and oxidized notes; reduced GSH is generally  
281 considered the YD's most active component from this point of view (Pozo-Bayón,

282 Andújar-Ortiz, & Moreno-Arribas, 2009; Andújar-Ortiz et al., 2010; Kritzinger et al., 2013a).  
283 According to Tirelli, Fracassetti, & De Noni (2010), glutathione content in yeast autolysates  
284 ranges from 1 to 14 mg/g (0.33-4.60 mmol/100 g), a value close to the 0.1-1 % (1-10 mg/g) of  
285 the *Saccharomyces cerevisiae* dry cell weight (Bachhawat et al., 2009). These quantities also  
286 agree with the information available about the ability of such products to release the tripeptide  
287 in model solution: Andújar-Ortiz and co-workers (Andújar-Ortiz, Pozo-Bayón, Moreno-  
288 Arribas, Martín-Alvarez, & Rodríguez-Bencomo, 2012) detected 1-2 mg/L of reduced GSH,  
289 after addition of 0.3 g/L of a glutathione-enriched inactive dry yeast (GSH-IDY); these data  
290 were confirmed by Kritzinger and colleagues (Kritzinger, Stander, & Du Toit, 2013b), who  
291 detected a glutathione release between 1.45 and 2.53 mg/L, after the same supplementation  
292 (0.3 g/L) with five different GSH-IDY preparations. On the basis of these quantities, we could  
293 roughly quantify in 3-8 mg the average amount of free glutathione released by 1 g of YD  
294 preparation.

295 In the present study, we made a yeast autolysate addition of 25 g/L (2.5 % w/v), 50-100 times  
296 higher respect to the normal amounts used in winemaking (250-500 mg/L). According to the  
297 calculations above, this would be able to release approx. 75-200 mg/L of free GSH, so a lower  
298 quantity respect to the highest amount of glutathione we have added to the model wine (500  
299 mg/L); nevertheless, the DPPH• discoloration promoted by YD in Fig. 1a is significantly  
300 higher respect to that determined by 500 mg/L of GSH.

301 On the basis of these results, we can hypothesize that, probably, glutathione was not the only  
302 factor in determining the radical scavenging capacity of the yeast derivative, but something  
303 else among YD components might be involved, with a non-negligible contribution, in the  
304 effects observed. This hypothesis was also considered in the paper published by Andújar-  
305 Ortiz et al. (2010): reporting the effects of glutathione-enriched inactive dry yeast  
306 preparations on the aroma of wines, besides the antioxidant capacity of GSH, they also



307 mentioned “the activation of different types of chemical reactions promoted by other  
308 components from the IDY preparations”.

309 The most accredited among these “other components” might be proteins: Jaehrig et al. (2007)  
310 consider proteins from yeast cell walls as one of the most active components from the anti-  
311 oxidative point of view, due to their aromatic side chains and thiol groups of cysteine  
312 residues. In support of this postulate, Tirelli and colleagues (Tirelli et al., 2010) quantified the  
313 amounts of reducing proteins with cysteine residues (RPC), in several YD products, in  
314 comparison with the content of free glutathione (GSH) and free cysteine (Cys); RPC were  
315 estimated at non-negligible concentrations in the four yeast autolysates evaluated (0.73-1.40  
316 mmol in 100 g of product), with values close, or even higher, respect to that of reduced GSH.  
317 Obviously, in the light of these few bibliographic evidences, our current dataset must be  
318 considered only as a preliminary result, being still quite poor to allow a certain conclusion;  
319 further investigations will be needed to confirm these hypotheses, as well as to better  
320 elucidate the role of the different YD components and fractions in scavenging free radicals.

321 A final consideration about Fig. 1a is related to the comparison between the radical  
322 scavenging activity of the YD preparation and that of yeast lees. The high amount of yeast  
323 autolysate used in this study was selected to make it comparable with lees addition (2.5 %  
324 v/v). Obviously, we can expect that fresh lees were less concentrated in yeast cell residues,  
325 because of their higher humidity content; nevertheless, the anti-radical activity of the model  
326 wines treated with lees was significantly higher respect to that observed for the samples  
327 treated with the yeast derivative. This could be due to the presence of residual phenolic  
328 substances in the lees, that could have contributed to DPPH• discoloration, but also to the  
329 production process of yeast derivatives, which could have reduced the radical scavenging  
330 capacity of yeast cell components. This last observation could confirm the conclusions of  
331 Tirelli et al. (2010); in the study mentioned above, they also reported the levels of overall Cys  
332 (Cys + 2 × Cystine), that is to say a parameter which also takes into account the thiol

333 groups in form of disulfide; this fraction was generally the most representative among those  
334 estimated (free GSH, free Cys, RPC and overall Cys), with a big variation among the  
335 analyzed YD formulations; for this reason, the authors hypothesized that the technologies  
336 applied for the production of yeast derivatives are not suitable for preserving the thiol groups  
337 of the proteins with cysteine residues, as well as the antioxidant capacity of these products.

338 When the radical scavenging activity was measured in a white wine, the results were different  
339 respect to what observed in model solution (Fig. 1b). First of all, all the tested substances  
340 significantly increased the DPPH• bleaching capacity of the wine itself (Control sample).  
341 Lees was the most active product in promoting discoloration, but the differences among the  
342 treatments seemed less intense than those observed in the model buffer. The concentration  
343 affected in a lower extent the scavenging ability of the products: for example, the two levels  
344 tested for sulfur dioxide gave, statistically, similar performances in terms of  $\Delta\text{Abs} \%$  and also  
345 the two dosages of glutathione were not as different as those reported in Fig. 1a.

346 These behaviors might be explained considering a sort of “matrix-related effect”, connected to  
347 the ability of the different antioxidants, to interact with other components present in wine. In  
348 effects, the wine itself, demonstrated a non-negligible capacity in scavenging DPPH•; the  
349 presence of the antioxidant products might have affected this capacity, not only by directly  
350 reacting with the free radical, but also by interacting with certain wine compounds. For  
351 instance, it is reported that ascorbic acid, glutathione and  $\text{SO}_2$  can hamper oxidation chains by  
352 regenerating phenolic species from *o*-quinones (Waterhouse & Laurie, 2006; Danilewicz,  
353 2007; Bradshaw et al., 2011); phenolics are well known radical scavengers and this  
354 regeneration could be connected just with the increased chain-breaking activity measured for  
355 the treated samples.

356 It is also interesting to observe, that comparing the activity of GSH with those of ascorbic  
357 acid and  $\text{SO}_2$  (samples at 50 mg/L), the former is no more the less effective in promoting  
358 DPPH• discoloration (as it was in model buffer): in Fig. 1b, at a low dosage, glutathione

359 behaves similarly to sulfur dioxide, being not statistically different respect to the  
360 performances of ascorbic acid. In addition, at 500 mg/L, despite the five-folds lower molar  
361 concentration and in opposition to what seen in wine-like solution, GSH demonstrated a  
362 higher efficacy, respect to sulfites.

363 Finally, a last consideration concerns the YD preparation; if compared just with glutathione  
364 (500 mg/L), one can observe that, in wine, the differences between the two treatments are  
365 basically nil. This might be explained considering the previously mentioned “matrix-related  
366 effect”, so that the supplementation with pure glutathione, in a real wine, seemed to be as  
367 effective as the YD product, probably due to the capacity of the tripeptide to improve the  
368 overall radical scavenging activity of the wine itself. This might be connected with the ability  
369 of GSH to react with quinones, regenerating phenolic molecules (Waterhouse & Laurie,  
370 2006), but further investigations shall be done to better clarify the role of glutathione and YDs  
371 in hampering radical chains in both model solution and different wine typologies.

### 372 *3.2 Oxygen consumption capacity*

373 The ability of the different antioxidant products to affect oxygen consumption was  
374 investigated in two different wines. The curves describing oxygen consumption vs. time, in a  
375 young wine (harvest 2012), are reported in Fig. 2. As one can observe, the amount of oxygen  
376 dissolved at saturation was completely depleted in the untreated wine (Control) in 192 hours  
377 (8 days). The most active oxygen scavenging substances among the tested products were fresh  
378 lees (2 % v/v) and ascorbic acid (50 mg/L). At the concentration used, pure glutathione (50  
379 mg/L) and the yeast derivative preparation (500 mg/L) gave results similar to those observed  
380 in the Control samples. On the contrary, sulfur dioxide (50 mg/L) allowed to significantly  
381 reduce oxygen consumption, with a final average level close to 3 mg/L. In Fig. 3, the  
382 statistical confirmation of these behaviors is reported, on the basis of the results of Factorial  
383 ANOVA. Concerning SO<sub>2</sub>, it is interesting to underline that the reduction of oxygen

384 depletion rate occurred in the last 48 hours of the monitoring period, while during the first six  
385 days, sulfites did not basically affect the kinetic of oxygen consumption. This behavior seems  
386 in contrast with that reported by Danilewicz and colleagues (Danilewicz et al., 2008):  
387 studying the interaction of oxygen, sulfur dioxide and 4-methylcatechol in a model wine  
388 containing iron and copper, they found that the rate of reaction of oxygen was accelerated by  
389 SO<sub>2</sub> addition; the explanation they gave, is that sulfites can react with quinones accelerating  
390 catechol autoxidation. They also confirmed this effect on a red wine where sulfites were  
391 eliminated by adding hydrogen peroxide; this elimination significantly reduced the ability of  
392 the wine to consume oxygen (Danilewicz et al., 2008).

393 On the other hand, a further confirmation of these results comes also from Fig. 4, where  
394 oxygen consumption vs. time is reported for the aged wine (harvest 2007). As one can  
395 observe, fresh lees and ascorbic acid were again the most active substances in scavenging  
396 oxygen, but sulfur dioxide also increased the O<sub>2</sub> consumption rate; it was only a slight  
397 acceleration, but according to the results of Factorial ANOVA, oxygen depletion was  
398 significantly faster in the sulfited samples than in the Control wines (Fig. 5).

399 On the basis of these evidences, it is difficult to explain the reason why sulfites reduced the  
400 oxygen consumption rate in the young wine (Fig. 2). This product was quite poor in phenolic  
401 compounds (the average total phenolic index of the Control samples was 3.5) and had a  
402 relatively low content of free SO<sub>2</sub> (see Section 2.4.2). If we consider that sulfites can  
403 accelerate catechol autoxidation, it can be hypothesized that the addition of potassium  
404 metabisulfite has actually accelerated the conversion of polyphenols into quinonic species. It  
405 has been reported that, when “4-methylcatechol is oxidized in presence of SO<sub>2</sub>, the 38 %  
406 approximately of the quinone formed reacts with bisulfite to produce the sulfonic adduct and  
407 the most of the remainder is reduced back to catechol” (Danilewicz et al., 2008); so, we  
408 should expect a progressive reduction of the concentration of available polyphenols during the  
409 storage time. Due to the low TPI of the wine, and due to the fact that, as already mentioned,

410 this reduction should have been faster in the sulfited samples, we can hypothesize that oxygen  
411 consumption, was slowed down, in the last part of the curves of the latters (see Fig. 2), just  
412 because of this faster consumption of reactive substances.

413 Another interesting observation can be done comparing the oxygen consumption curves  
414 detected for the aged wine (Fig. 4) and the youngest one (Fig. 2); as one can observe, oxygen  
415 disappearance was initially faster in the former, but the slope of the curves progressively  
416 decreased, and after 216 hours the average level of residual oxygen (in the most of the  
417 treatments) was still close to 1-1.5 mg/L; otherwise, the curves reported for the young wine  
418 were less steep in their first part, but the complete oxygen consumption was obtained in less  
419 than 200 hours (with the only exception of the sulfited samples). This different behavior is  
420 probably related with the different phenolic content and “oxidative history” of the two wines.

421 The aged one had an average TPI of 11.6, while for the youngest one TPI was lower (only  
422 3.5); the former was produced by prolonged barrel ageing, while the latter was stored in  
423 stainless steel vats, so in a more protective environment with respect to oxygen contact.

424 So, the faster oxygen consumption, detected at the beginning of the monitoring period in the  
425 samples from harvest 2007, was probably due to their higher phenolic content, that might  
426 have initially increased oxygen consumption capacity (Danilewicz et al., 2008); nevertheless,  
427 the more intense contact that this wine had with oxygen during the whole production chain,  
428 might have been the most important factor, responsible of the decrease of oxygen  
429 consumption rate in the last part of the monitoring period. Contrary, in the youngest wine,  
430 which had a lower TPI, but was probably subjected to a less intense aeration during  
431 processing, oxygen depletion started slower, but it was completed in a shorter time, denoting  
432 a higher capacity of such product to scavenge oxygen.

### 433 *3.3 Effect on wine color and predisposition to browning*

434 The effects of the different treatments on color and predisposition to browning of the young  
435 wine (harvest 2012) are reported in Table 1 (Section a). As one can observe, sulfiting allowed  
436 the lowest color development, followed by the treatment with YD preparation. GSH and  
437 ascorbic acid gave intermediate results, while the highest color formation was detected for  
438 Control samples and the wines treated with fresh lees.

439 The POM-test values confirm these behaviors: the highest levels mark out samples in which  
440 phenolic fraction is more preserved, while oxidative phenomena tend to reduce the POM-test  
441 index. It is interesting to underline that the lowest POM-tests were detected for the wines  
442 treated with GSH and ASC, while oxidizable phenolics were better protected by sulfur  
443 dioxide and YD preparation.

444 Concerning the aged wine (harvest 2007), the results were less evident (Table 1, Section b);  
445 15 days after the treatments, only fresh lees gave a significant increase of the color, probably  
446 due to their ability to release phenolic compounds (TPI significantly increased, according to  
447 ANOVA, from a value of  $11.6 \pm 0.2$  in the Control wines, to  $15.0 \pm 0.3$  in the lees added  
448 samples). Anyway, according to the POM-Test levels, after 15 days, potassium metabisulfite  
449 and YD were the additives that allowed the best preservation of oxidizable phenolics;  
450 concerning sulfites, this confirms the behaviors observed in Table 1a for the young wine,  
451 while, in this case (Table 1b), the effects given by the yeast autolysate were less evident, and  
452 very close to those of glutathione and ascorbic acid.

453 The situation of the aged wine changed 8 months after the treatments (Table 1, Section b).  
454 Sulfites demonstrated their effectiveness in protecting wine against browning, allowing only a  
455 slight increase of the color during storage time. YD was the additive that behave more  
456 similarly to  $\text{SO}_2$ : the color index (Abs 420 nm) of the samples treated with the autolysate  
457 preparation was significantly higher than those of the sulfited wines, but significantly lower  
458 than in all the other experiments, highlighting a certain ability of such kind of preparations<sub>19</sub>

459 in protecting wine against color development, over a medium-long storage time. Finally, fresh  
460 lees and particularly ascorbic acid were the treatments which gave the most intense browning.  
461 POM-test results confirm these considerations: SO<sub>2</sub> gave the highest index, followed by YD  
462 and GSH, while the lowest values were detected just for ASC and fresh lees.

463 In conclusion, the products tested in this study confirmed to have different effects concerning  
464 their antioxidant properties, and this reflects in a very different behavior towards the  
465 modifications they can induce in wine.

466 Fresh lees were very effective in oxygen and free radical scavenging, but they increased wine  
467 color by the release of phenolic compounds. Ascorbic acid confirmed its effectiveness in the  
468 removal of oxygen and DPPH• free radical, but as reported elsewhere (Bradshaw et al., 2001),  
469 it induced an intense browning during wine storage. Glutathione also demonstrated its ability  
470 in scavenging DPPH• in wine; nevertheless, it showed a poor capacity in scavenging oxygen,  
471 and its activity in protecting wine against color formation was lower than that given by  
472 sulfiting (particularly after 8 months of storage). This confirms the data published by Sonni et  
473 al. (2011), who reported that GSH can initially provide protection against oxidative  
474 coloration, but eventually induced color formation.

475 Strictly speaking in terms of the possibility to replace sulfur dioxide, the analyses carried out  
476 on wine color and predisposition to browning, highlighted that SO<sub>2</sub> remains the most  
477 performing additive. Nevertheless, maybe surprisingly, the addition of the yeast derivative  
478 preparation (YD), was the treatment that behave more similarly to sulfiting: YD was very  
479 active in scavenging DPPH• free radical in both model solution and wine, and, even without  
480 significantly affecting the ability of the wine to consume oxygen, it protected quite well color  
481 and phenolics over a medium-length storage time (8 months). Due to the effects we have  
482 observed for GSH, and the average levels reported for glutathione in inactive dry yeasts, we  
483 are oriented to think that this ability of YDs might be not only connected with their capacity  
484 to release the tripeptide in wine; the glutathione released might contribute, but we can

485 hypothesize that other components of these complex preparations might be involved in the  
486 effects observed, with a non-negligible role.

487 Obviously, the bottleneck in using yeast derivatives for partially replacing sulfites in bottled  
488 wines is connected with the presence of insoluble particles in the commercial preparations,  
489 but this does not jeopardize the possibility to use these products during the storage in stainless  
490 steel containers. From this point of view, these specific results seem interesting, because such  
491 kind of preparations might allow a significant reduction of sulfite levels during wine storage,  
492 with the possibility of postponing sulfiting in the production steps immediately preceding  
493 bottling. Further investigations will make it possible to confirm these behaviors, clarifying the  
494 mechanisms through which YDs carry out their protective action, and allowing both the  
495 optimization of their use in winemaking as antioxidant formulations and the development of  
496 suitable technologies for producing specific preparations for this specific winemaking use.

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## 657 **Figure Captions**

658 **Fig. 1.** Radical scavenging activity of different enological additives and products in model solution  
659 (a) and in white table wine (b). Results of ANOVA analysis and Tukey HSD test; means and  
660 standard deviations of three repetitions are reported. Different letters mark significant differences at  
661  $p < 0.05$ . See the text for abbreviations.

662

663 **Fig. 2.** Oxygen consumption versus time, measured for the samples prepared in Prosecco base wine  
664 (harvest 2012). Mean values of three repetitions are reported. Vertical bars represent standard  
665 deviations. See the text for abbreviations.

666

667 **Fig. 3.** Factorial ANOVA and Tukey HSD test, carried out on the oxygen concentrations measured  
668 for the curves in Fig. 2. Means and standard errors (SE) of three repetitions are reported; different  
669 letters mark significant differences at  $p < 0.05$ . See the text for abbreviations.

670

671 **Fig. 4.** Oxygen consumption versus time, measured for the samples prepared in the blended wine  
672 from harvest 2007 (Chardonnay, Sauvignon, Picolit). Mean values of three repetitions are reported.  
673 Vertical bars represent standard deviations. See the text for abbreviations.

674

675 **Fig. 5.** Factorial ANOVA and Tukey HSD test, carried out on the oxygen concentrations measured  
676 for the curves in Fig. 4. Means and standard errors (SE) of three repetitions are reported; different  
677 letters mark significant differences at  $p < 0.05$ . See the text for abbreviations.

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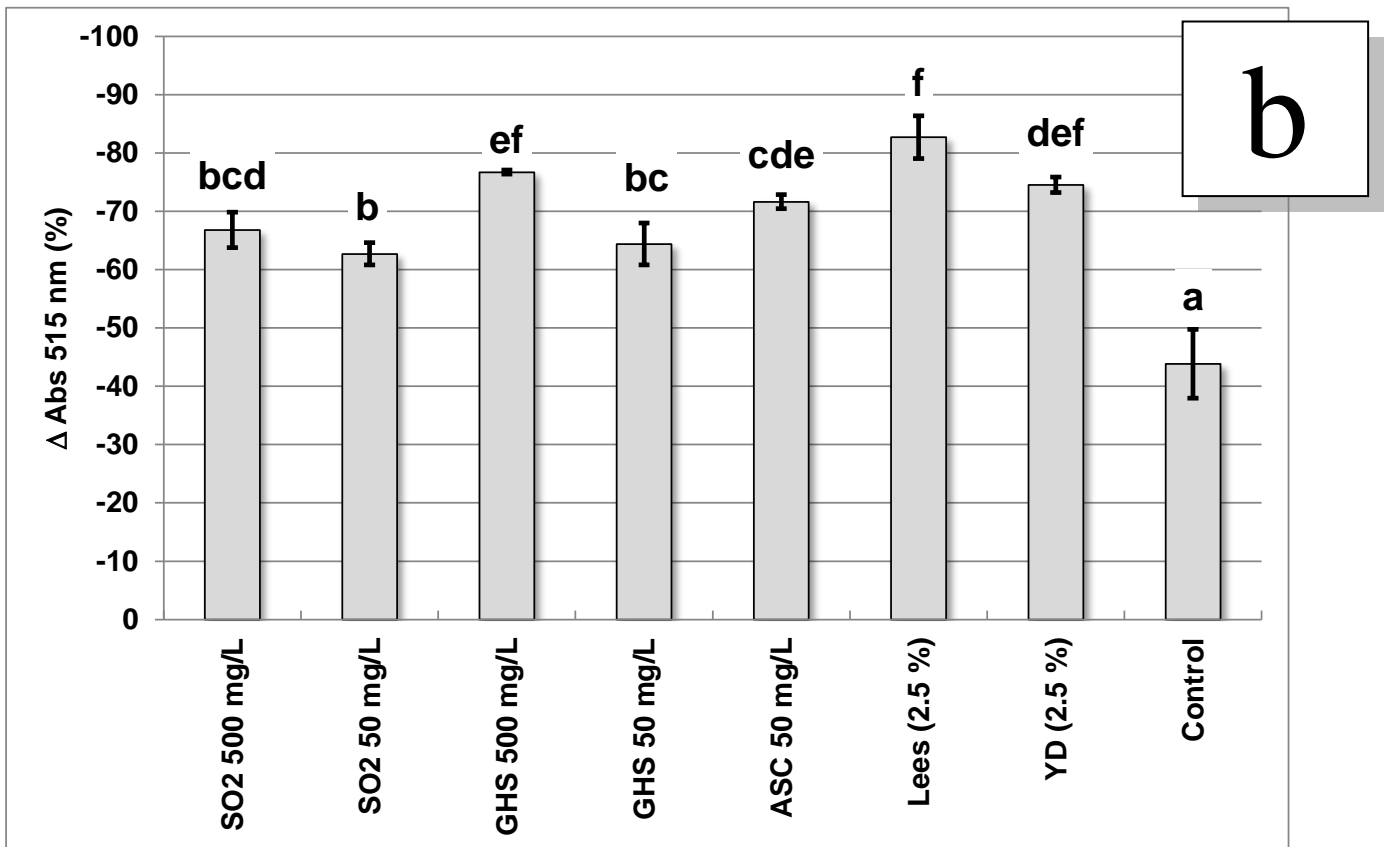
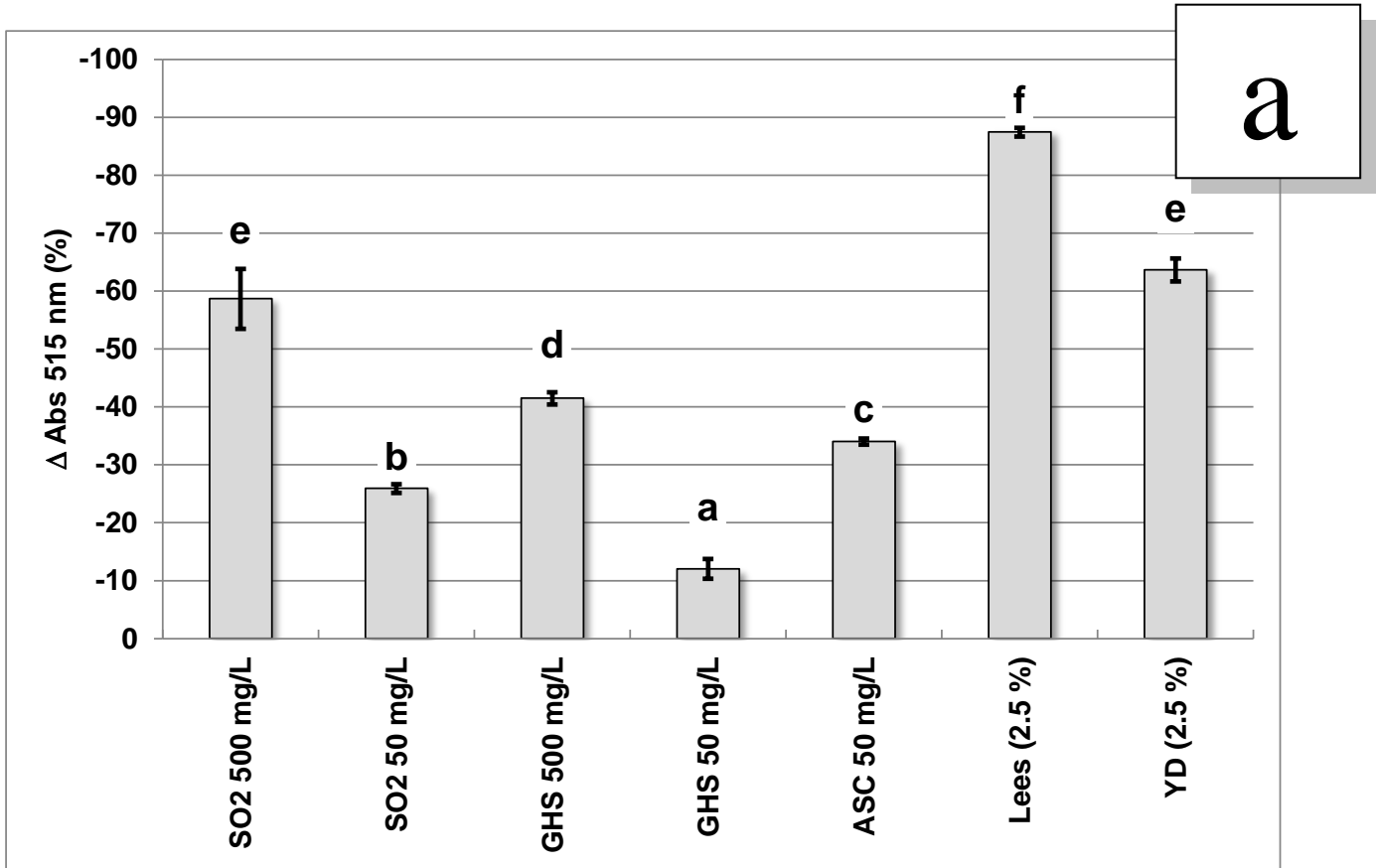
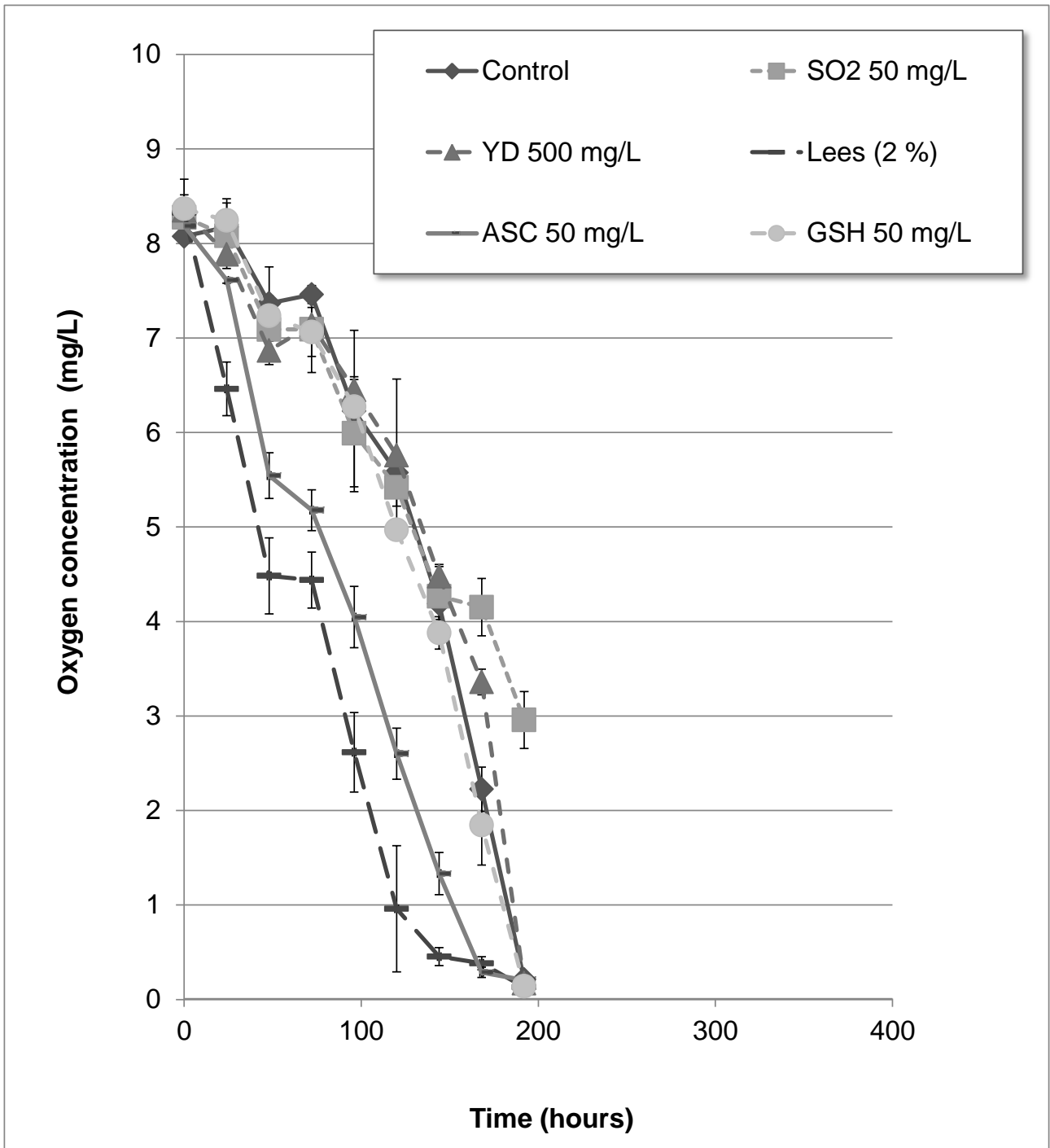


Fig. 1



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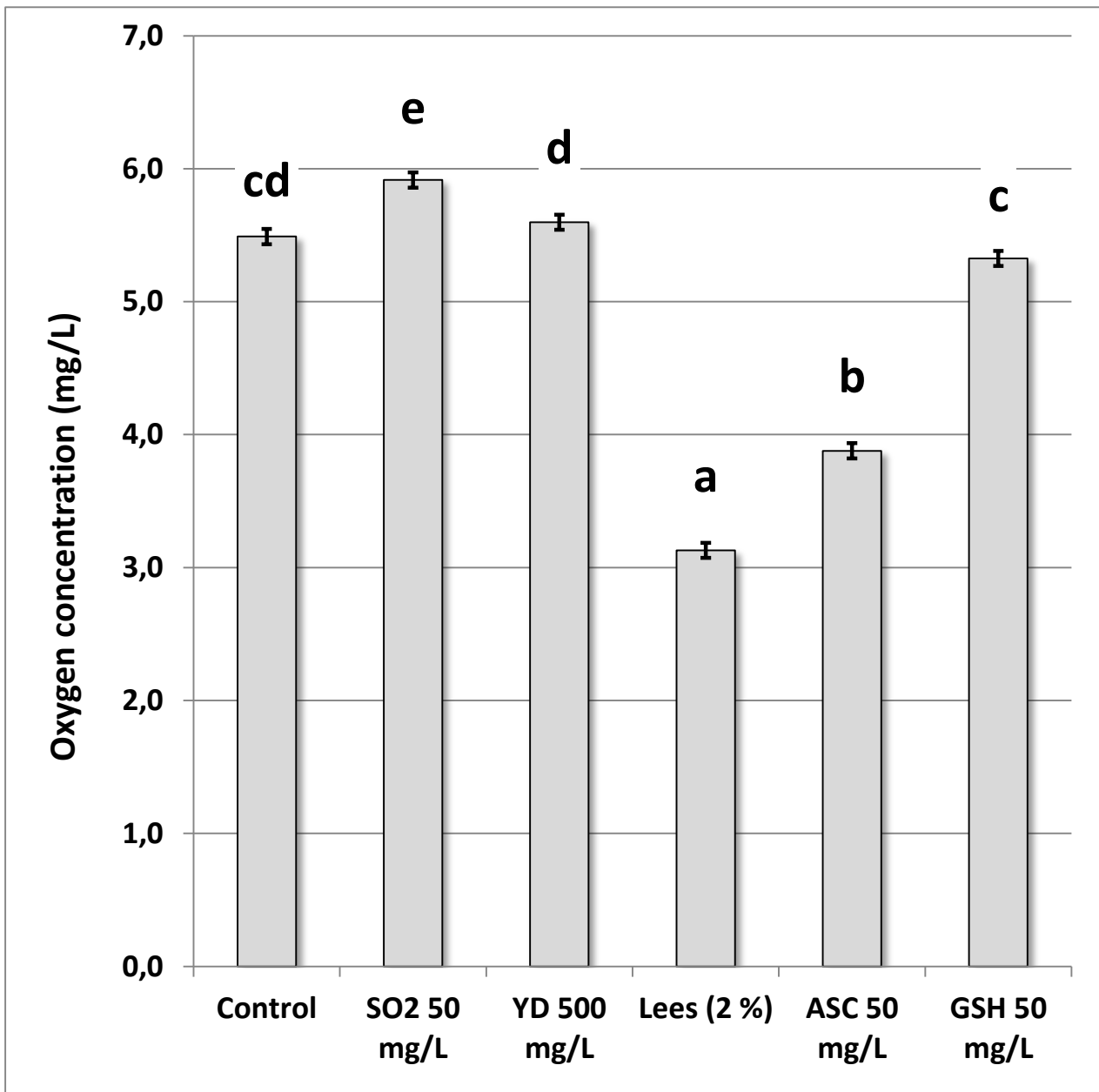
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Fig. 2

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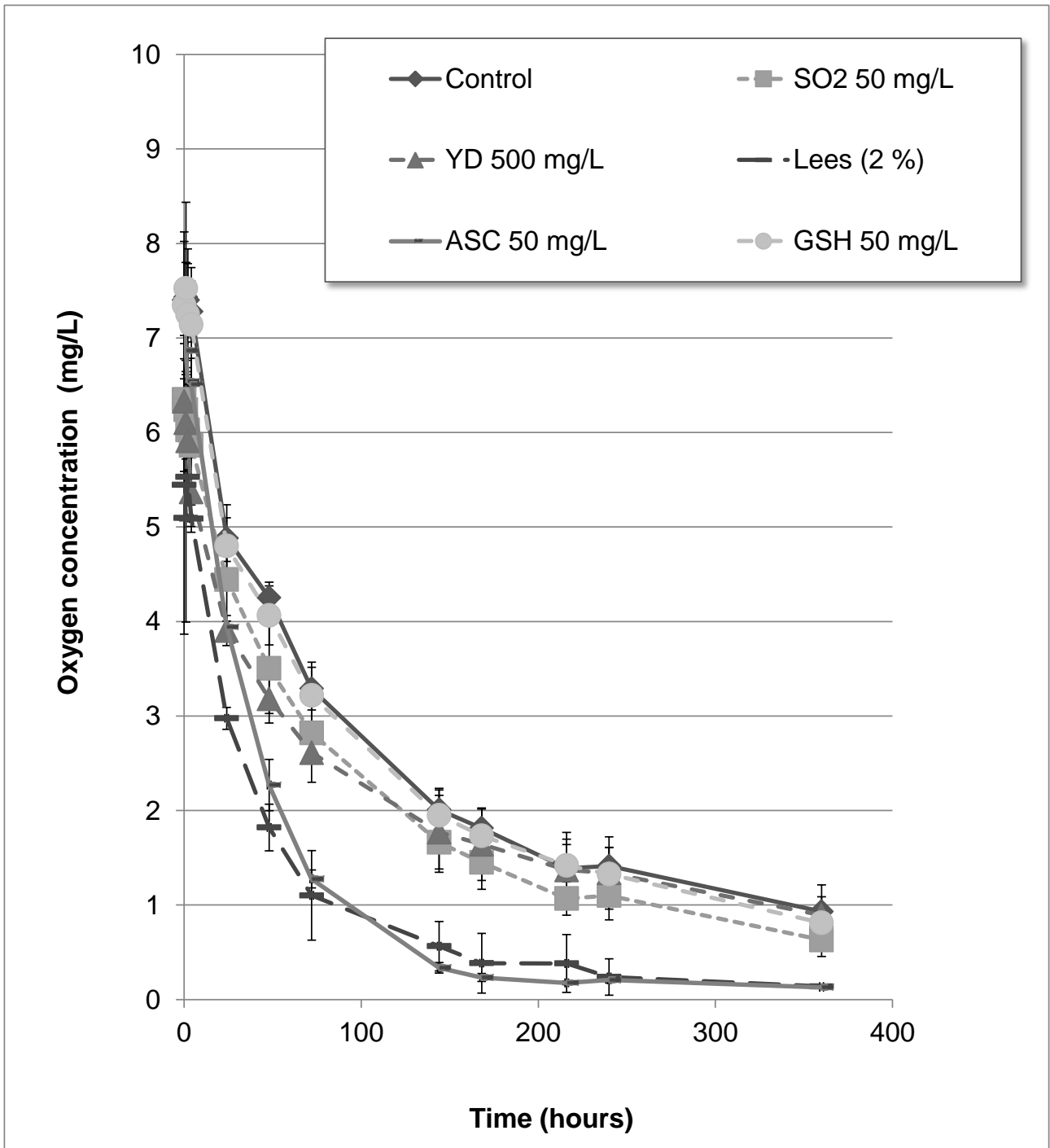
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Fig. 3

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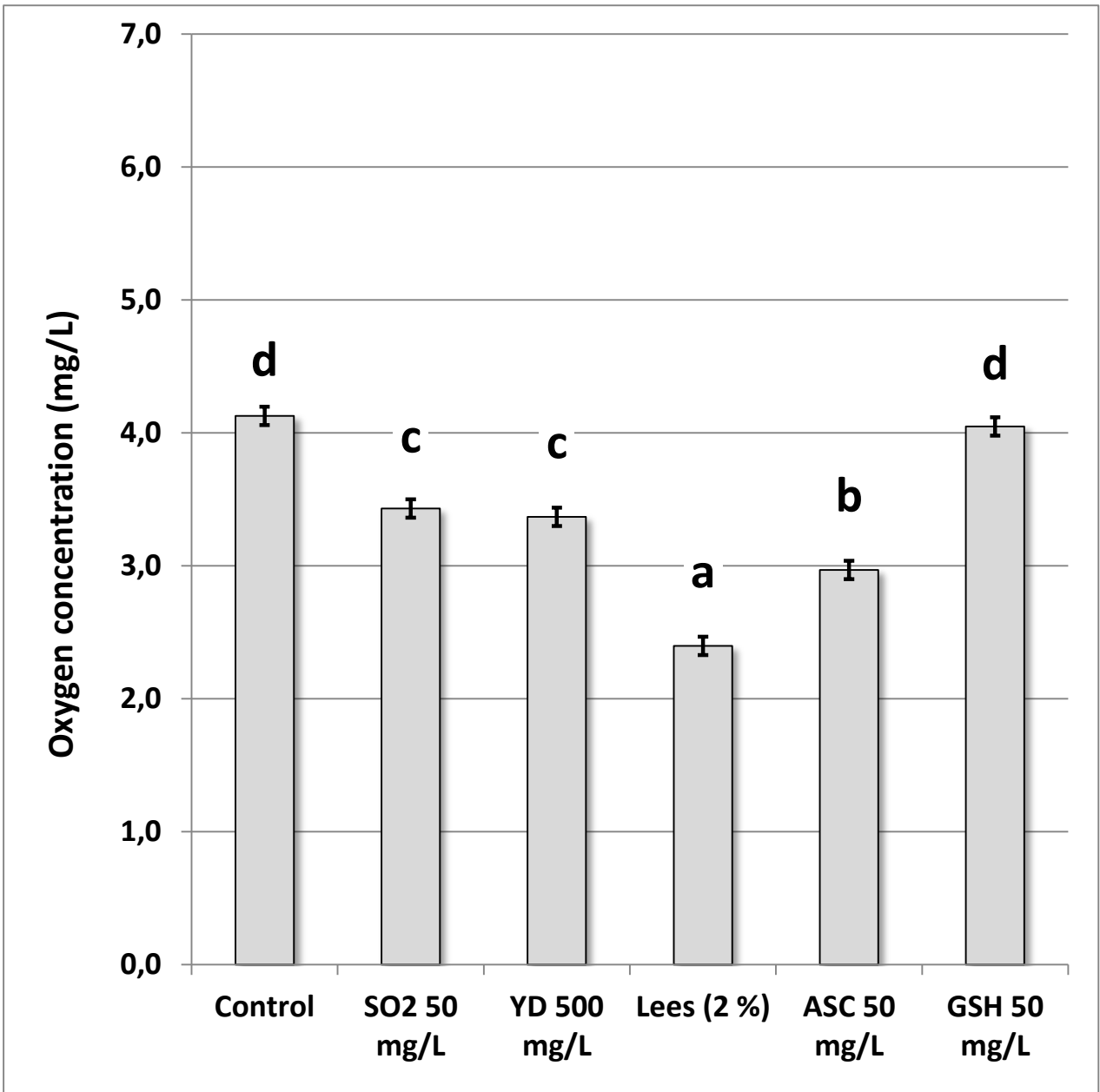
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Fig. 4

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725

Fig. 5

726 **Table 1**

727 Absorbance at 420 nm and POM-test values detected for the two wines used in the experiments; samples were analyzed 15 days after bottling, and  
 728 limitedly to the aged wine (harvest 2007), after 8 months. Means, standard deviations (SD) and the results of One Way ANOVA and Tukey HSD test are  
 729 reported; different letters represent significant differences at  $p < 0.05$ .

(a) Young wine (harvest 2012)			(b) Aged wine (harvest 2007)						
Sample	Abs 420 nm		Sample	Abs 420 nm					
	15 days			15 days		8 months			
	Mean	± SD	Mean	± SD	Mean	± SD			
Control	0.038	± 0.001	e	0.204	± 0.002	a	0.251	± 0.005	c
SO2 50 mg/L	0.019	± 0.000	a	0.182	± 0.002	a	0.200	± 0.000	a
YD 500 mg/L	0.024	± 0.000	b	0.206	± 0.024	a	0.214	± 0.009	b
Lees (2 %)	0.039	± 0.000	e	0.273	± 0.010	b	0.280	± 0.005	d
ASC 50 mg/L	0.033	± 0.001	d	0.198	± 0.001	a	0.288	± 0.001	d
GSH 50 mg/L	0.028	± 0.000	c	0.205	± 0.001	a	0.258	± 0.005	c

POM-test			POM-test						
Sample	15 days		Sample	15 days		8 months			
	Mean	± SD		Mean	± SD	Mean	± SD		
Control	90	± 10	b	35	± 15	a	24	± 2	bc
SO2 50 mg/L	167	± 36	cd	69	± 5	b	45	± 0	e
YD 500 mg/L	181	± 6	d	43	± 14	ab	29	± 3	d
Lees (2 %)	111	± 17	bc	30	± 9	a	21	± 1	ab
ASC 50 mg/L	20	± 35	a	42	± 1	a	19	± 0	a
GSH 50 mg/L	25	± 2	a	42	± 5	a	27	± 1	cd

730

## **Highlights**

Sulfur dioxide was the most active additive in reducing wine color development

Ascorbic acid was a good O<sub>2</sub> and radical scavenger but induced browning during storage

Lees were good O<sub>2</sub> and radical scavengers but increased color by releasing phenolics

Glutathione had radical scavenging ability in wine but increased color during storage

Yeast derivative behave more similarly to SO<sub>2</sub> protecting wine color over 8 months