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. SO2 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_2 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_2, 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.

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

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Antioxidant capacity of enological products compared with sulfites

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_2 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.

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

1 Introduction

Despite the mechanisms involved in wine oxidation have been extensively reviewed (Singleton, 1987; du Toit, Marais, Pretorius, & du Toit, 2006; Waterhouse & Laurie, 2006; Oliveira, Ferreira, De Freitas, & Silva, 2011), the protection of wine against oxidative spoilage remains one of the main goals of modern winemaking, becoming particularly critical when low levels of sulfur dioxide are used. The chemistry of this additive in wine has been recently re-written by Danilewicz (2007; 2011) and Danilewicz, Seccombe, & Whelan (2008): they clearly demonstrated that SO_2 does not react directly with oxygen, as previously thought (Ribéreau-Gayon, Dubourdieu, Doneche, & Lonvaud, 2006), but, in presence of metal ions, it is able to scavenge hydrogen peroxide and the quinones formed from the oxidation of polyphenols (Danilewicz et al., 2008). Due to the toxicity and allergenic potential of sulfites, different compounds have been proposed for reducing their final concentration in wine, even if, none of them is likewise effective in protecting wine against oxidations.

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

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

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

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

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

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

For this reason, the aim of this work was to carry out a preliminary investigation on the radical scavenging activity (measured by DPPH• assay) and the oxygen consumption capacity of different enological products and additives in comparison with SO_2. Ascorbic acid (considered as reference standard), glutathione, yeast lees and a self-prepared yeast autolysate were tested. Trials were performed in model solution and in different wine typologies. Concerning oxygen consumption trials, wines were finally subjected to fast spectrophotometric measurements, for assessing the effect of the different antioxidants on color, total phenolics and predisposition to browning.

2 Materials and Methods

2.1 Chemicals

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

2.2 Yeast lees and yeast derivatives (YD)

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

2.3 Evaluation of radical scavenging activity

2.3.1 Sample preparation

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

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

2.3.2 DPPH• assay

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

against methanol: acetate buffer. Results were expressed as the percent diminution of the original absorbance [ΔAbs 515 nm (%)].

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

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

2.4 Oxygen consumption capacity

2.4.1 Equipment

The system used for oxygen measurements was an OxySense® fluorimeter (OxySense Inc., Dallas, TX, USA); O2xyDot® oxygen sensitive sensors (OxySense Inc., Dallas, TX, USA), were glued, by a specific silicon based oxygen permeable adhesive (OxySense Inc.), to the inner surface of each of the 750 mL colorless glass bottles used for the experiments. When O2xyDot® sensors are illuminated by a pulsed blue light, they emits a red fluorescent light, that is monitored by OxySense® fluorimeter. Dynamic quenching by oxygen molecules determines a decrease of the O2xyDot® fluorescence lifetime, that is proportional to the oxygen concentration in the bottles; the temperature is measured simultaneously, by an infrared sensor positioned in the reader pen (Li, Ashcraft, Freeman, Stewart, Jank, & Clark, 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® sensors were glued (see Section
162 2.4.1). The average oxygen concentration measured at filling was 8.25 ± 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® sensor; the average oxygen
173 concentration at filling was 7.29 ± 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.

2.4.3 Spectrophotometric measures and browning assay

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

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

2.5 Statistical analysis

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

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

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

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

3 Results and Discussion

3.1 Evaluation of radical scavenging activity

The results of DPPH• assay are reported in Fig. 1. Concerning model solutions (Fig. 1a), the highest radical scavenging activity was detected for yeast lees, followed by YD and the highest sulfur dioxide addition (500 mg/L). At lower amounts, such as those normally used in winemaking (50 mg/L), SO_2 was less effective in bleaching the free radical and the values reported were more similar (even if statistically lower) to those measured for the same dosage of ascorbic acid (reference standard).

Despite the reaction of sulfite (SO_3^{2-}) and bisulfite ion (HSO_3^-) with free radicals was described (Brandt & van Eldik, 1995; Neta & Huie, 1985), very few publications report data about their radical scavenging capacity in wine; moreover, the information available are generally not in agreement with the dataset presented here. In a paper published in the late 1990s, Manzocco, Mastrocola, & Nicoli (1998) found that the addition of sulfur dioxide (250 mg/L) to a model system simulating wine (water - ethanol 12 % v/v), did not affect the chain-breaking capacity of the samples towards DPPH•; the different results they obtained respect to the present study might be related to the different pH and ionic strength of the solvents used in the two experiments, for both sample preparation (hydroalcoholic solution vs. hydroalcoholic buffer, pH 3.20) and DPPH• assay (pure methanol vs. methanol : acetate buffer pH 4.50). pH might have affected the ratio between the different forms of sulfur(IV) oxides in aqueous solution and it is known that they have a different reactivity towards free radicals (Neta & Huie, 1985). Moreover, also the different ionic strength of the two model solutions might have a non-negligible role: in fact, as reported by Brandt & van Eldik

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

Also the results of Vivas and colleagues (Vivas, Saint-Cricq de Gaulejac, & Glories, 1997) are in disagreement with the radical scavenging capacity of sulfur dioxide, reported in Fig. 1a. Studying the influence of SO_2 and ascorbic acid on the scavenging effect of tannins, they wrote that, at usual enological concentrations, sulfites do not have a scavenging effect on superoxide anion. Nevertheless, according to literature, superoxide radical reacts very slowly with sulfite (SO_3^{2-}) and the rate constant of the reaction is very low (82 M^{-1} s^{-1}) (Neta & Huie, 1985).

As observed for SO_2, also glutathione activity in model wine depends on the concentration. GSH levels in wine range from non-detectable values to 70 mg/L (Kritzinger, Bauer, & du Toit, 2013a), with a relatively high variability; at a concentration close to these amounts (50 mg/L), glutathione demonstrated the lowest ability in bleaching DPPH• and a dosage ten times higher (500 mg/L) was needed to significantly overtake the effect of the reference sample (ASC 50 mg/L); nevertheless, regardless of the amounts added, GSH activity was always significantly lower than that measured for the same addition of sulfur dioxide.

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

Speaking from the chemical point of view, the effects reported for such molecules in Fig. 1a 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_2 (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_2) and to the lower molecular weight
268 respect to the most suggested alternatives (ACS or GSH), the molar concentration of free SO_2
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,

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

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

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

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

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

These behaviors might be explained considering a sort of "matrix-related effect", connected to the ability of the different antioxidants, to interact with other components present in wine. In effects, the wine itself, demonstrated a non-negligible capacity in scavenging DPPH•; the presence of the antioxidant products might have affected this capacity, not only by directly reacting with the free radical, but also by interacting with certain wine compounds. For instance, it is reported that ascorbic acid, glutathione and SO_2 can hamper oxidation chains by regenerating phenolic species from *o*-quinones (Waterhouse & Laurie, 2006; Danilewicz, 2007; Bradshaw et al., 2011); phenolics are well known radical scavengers and this regeneration could be connected just with the increased chain-breaking activity measured for the treated samples.

It is also interesting to observe, that comparing the activity of GSH with those of ascorbic acid and SO_2 (samples at 50 mg/L), the former is no more the less effective in promoting DPPH• discoloration (as it was in model buffer): in Fig. 1b, at a low dosage, glutathione

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

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

3.2 Oxygen consumption capacity

The ability of the different antioxidant products to affect oxygen consumption was investigated in two different wines. The curves describing oxygen consumption vs. time, in a young wine (harvest 2012), are reported in Fig. 2. As one can observe, the amount of oxygen dissolved at saturation was completely depleted in the untreated wine (Control) in 192 hours (8 days). The most active oxygen scavenging substances among the tested products were fresh lees (2 % v/v) and ascorbic acid (50 mg/L). At the concentration used, pure glutathione (50 mg/L) and the yeast derivative preparation (500 mg/L) gave results similar to those observed in the Control samples. On the contrary, sulfur dioxide (50 mg/L) allowed to significantly reduce oxygen consumption, with a final average level close to 3 mg/L. In Fig. 3, the statistical confirmation of these behaviors is reported, on the basis of the results of Factorial ANOVA. Concerning SO_2, 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_2 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_2 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_2 (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_2, 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,

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

Another interesting observation can be done comparing the oxygen consumption curves detected for the aged wine (Fig. 4) and the youngest one (Fig. 2); as one can observe, oxygen disappearance was initially faster in the former, but the slope of the curves progressively decreased, and after 216 hours the average level of residual oxygen (in the most of the treatments) was still close to 1-1.5 mg/L; otherwise, the curves reported for the young wine were less steep in their first part, but the complete oxygen consumption was obtained in less than 200 hours (with the only exception of the sulfited samples). This different behavior is probably related with the different phenolic content and "oxidative history" of the two wines. The aged one had an average TPI of 11.6, while for the youngest one TPI was lower (only 3.5); the former was produced by prolonged barrel ageing, while the latter was stored in stainless steel vats, so in a more protective environment with respect to oxygen contact.

So, the faster oxygen consumption, detected at the beginning of the monitoring period in the samples from harvest 2007, was probably due to their higher phenolic content, that might have initially increased oxygen consumption capacity (Danilewicz et al., 2008); nevertheless, the more intense contact that this wine had with oxygen during the whole production chain, might have been the most important factor, responsible of the decrease of oxygen consumption rate in the last part of the monitoring period. Contrary, in the youngest wine, which had a lower TPI, but was probably subjected to a less intense aeration during processing, oxygen depletion started slower, but it was completed in a shorter time, denoting 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 ± 0. 2 in the Control wines, to 15.0 ± 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 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

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_2 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_2 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

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

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

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Figure Captions

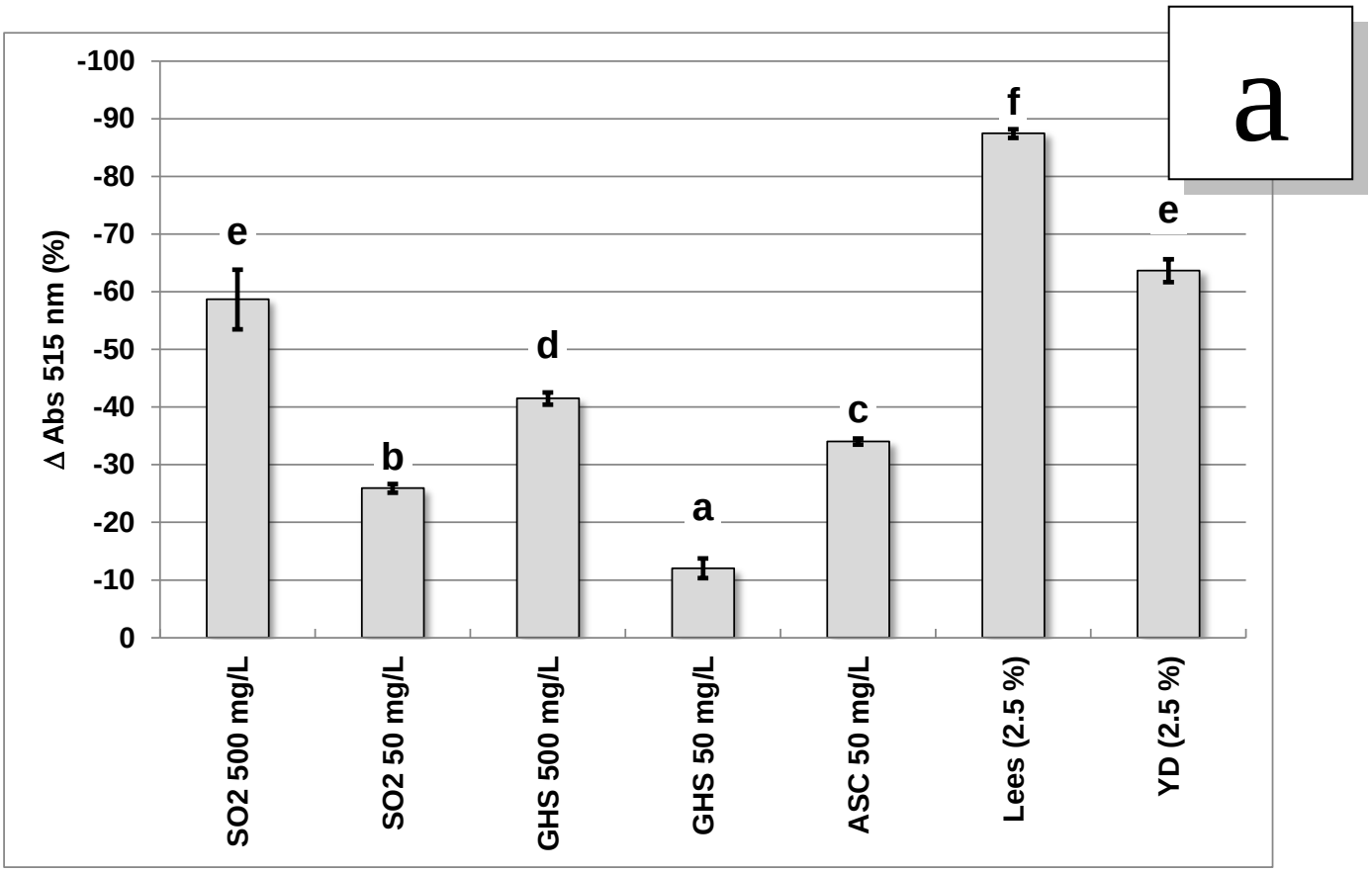
Fig. 1. Radical scavenging activity of different enological additives and products in model solution **(a)** and in white table wine **(b)**. Results of ANOVA analysis and Tukey HSD test; means and standard deviations of three repetitions are reported. Different letters mark significant differences at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

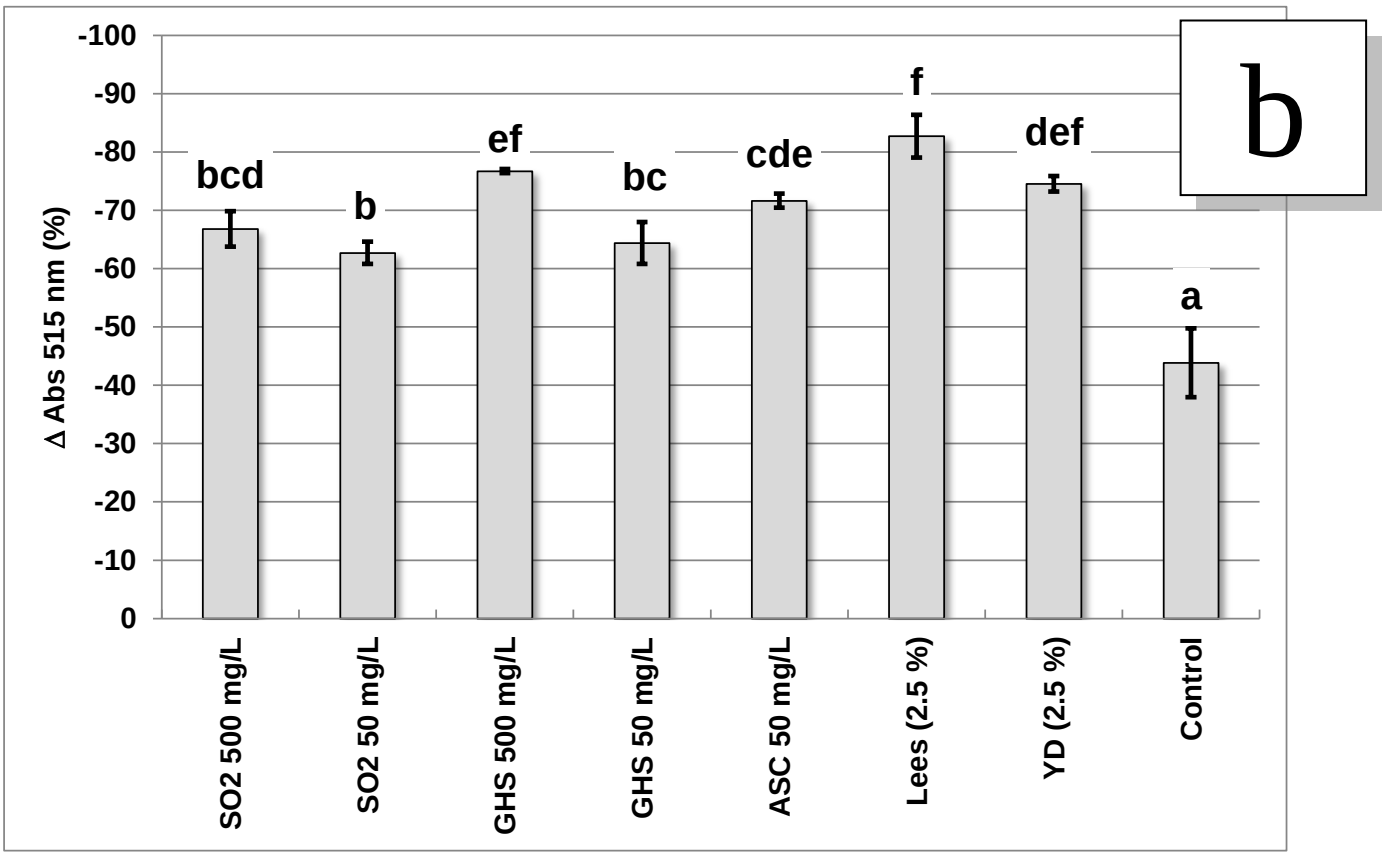
Fig. 2. Oxygen consumption versus time, measured for the samples prepared in Prosecco base wine (harvest 2012). Mean values of three repetitions are reported. Vertical bars represent standard deviations. See the text for abbreviations.

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

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

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


a
Δ Abs 515 nm (%)
-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
0
e
b
d
a
c
f
e
SO2 500 mg/L
SO2 50 mg/L
GHS 500 mg/L
GHS 50 mg/L
ASC 50 mg/L
Lees (2.5 %)
YD (2.5 %)


b
Δ Abs 515 nm (%)
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-90
-80
-70
-60
-50
-40
-30
-20
-10
0
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b
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bc
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f
def
a
SO2 500 mg/L
SO2 50 mg/L
GHS 500 mg/L
GHS 50 mg/L
ASC 50 mg/L
Lees (2.5 %)
YD (2.5 %)
Control

Fig. 1

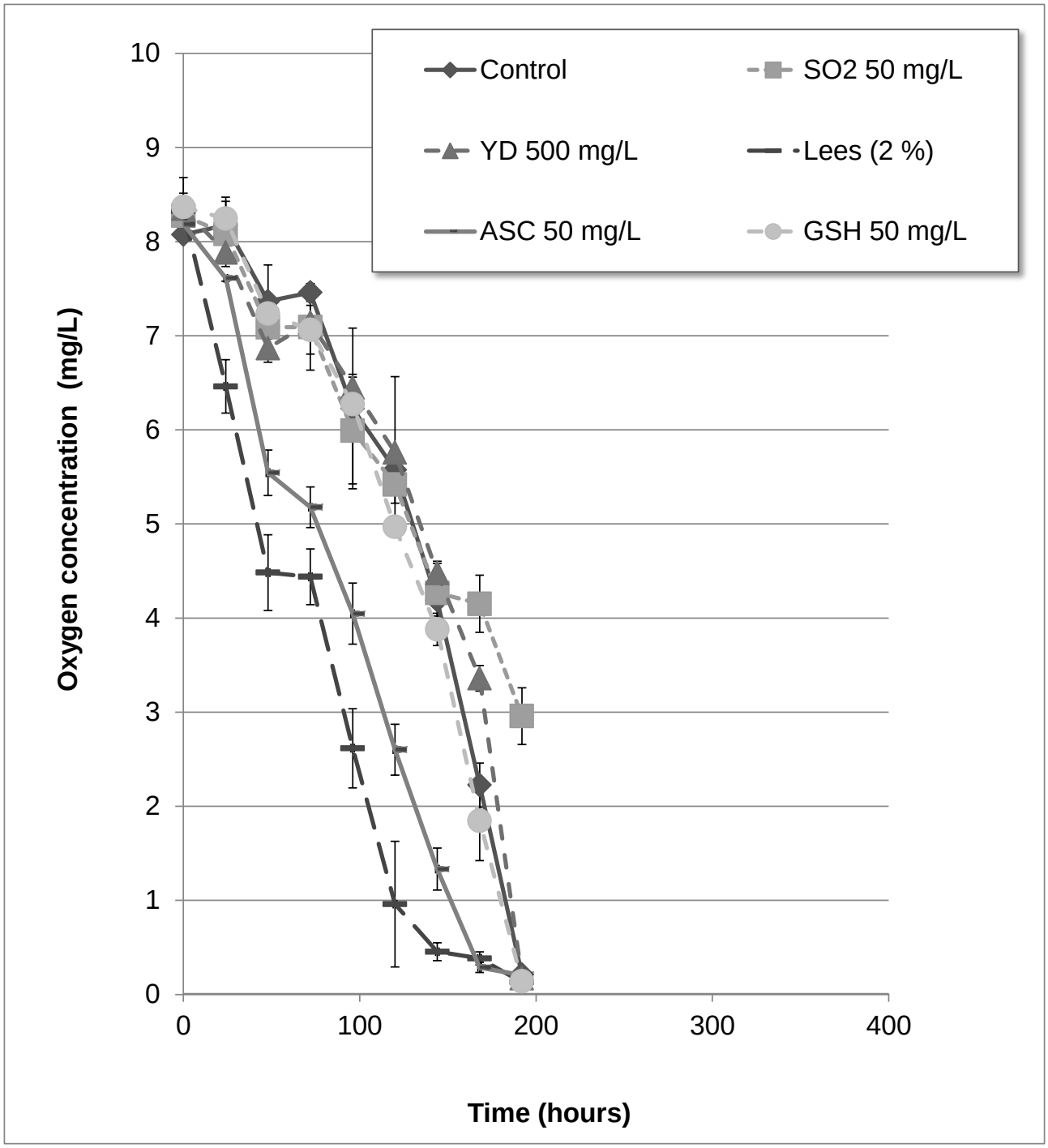
Control
SO2 50 mg/L
YD 500 mg/L
Lees (2 %)
ASC 50 mg/L
GSH 50 mg/L
Oxygen concentration (mg/L)
Time (hours)
0
1
2
3
4
5
6
7
8
9
10
0
100
200
300
400

Fig. 2

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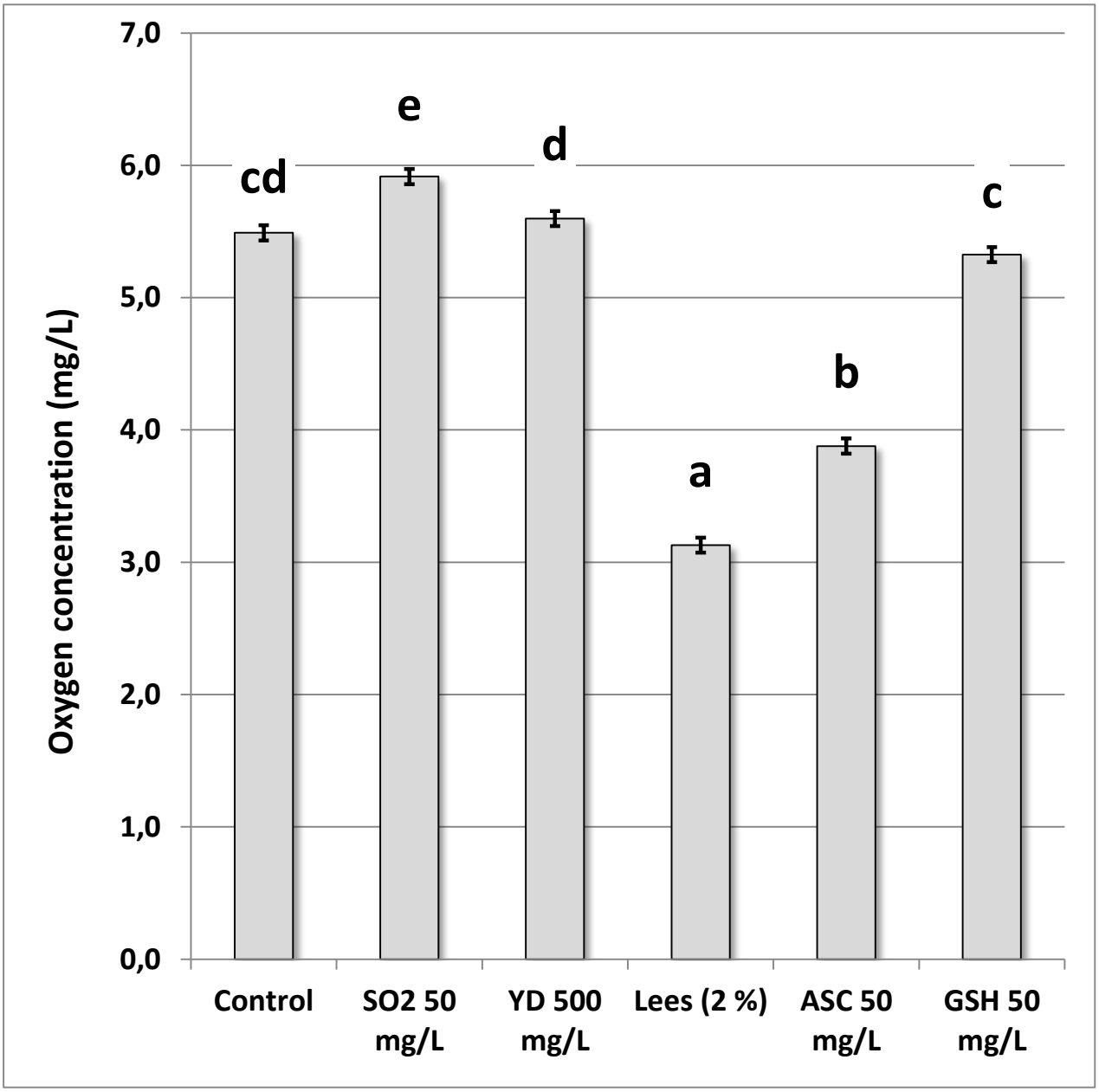
713



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715 **Fig. 3**

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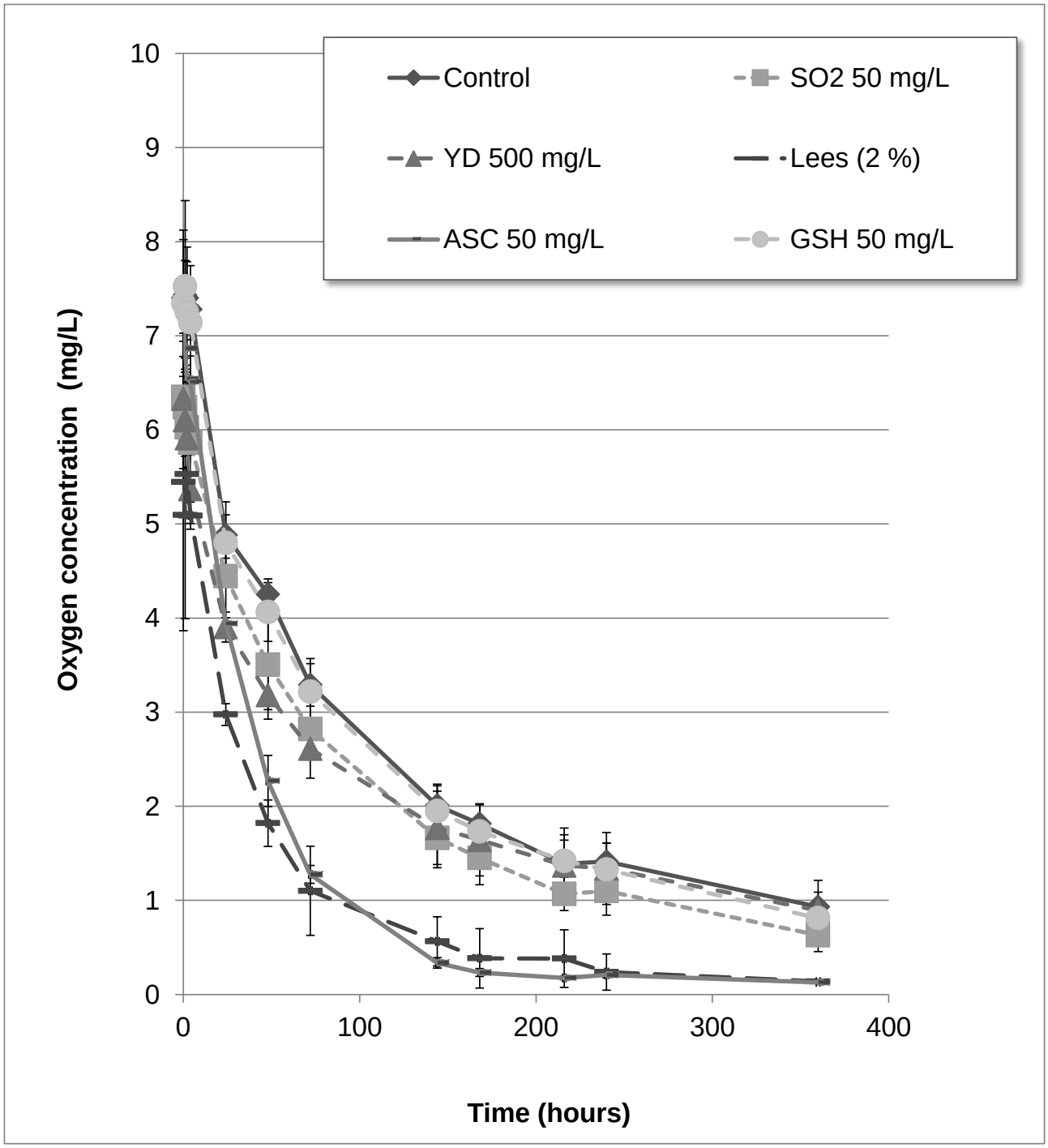
Control
SO2 50 mg/L
YD 500 mg/L
Lees (2 %)
ASC 50 mg/L
GSH 50 mg/L
Oxygen concentration (mg/L)
Time (hours)
0
1
2
3
4
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6
7
8
9
10
0
100
200
300
400

Fig. 4

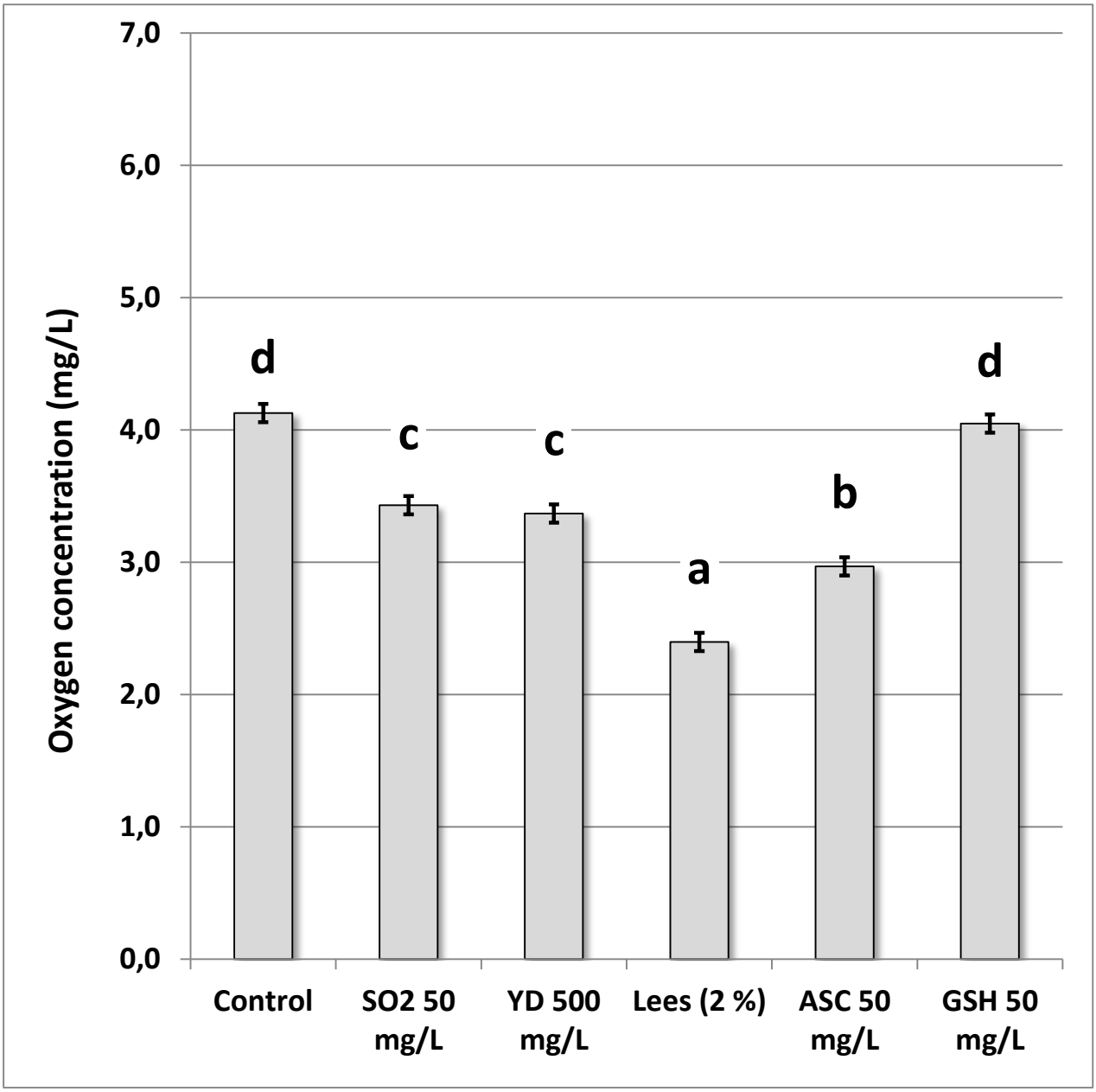
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
Oxygen concentration (mg/L)
d
c
c
a
b
d
Control
SO2 50 mg/L
YD 500 mg/L
Lees (2 %)
ASC 50 mg/L
GSH 50 mg/L

Fig. 5

Table 1

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 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 reported; different letters represent significant differences at $p < 0.05$.

(a) Young wine (harvest 2012)

Sample	Abs 420 nm 15 days Mean ± SD	
Control	0.038 ± 0.001	e
SO2 50 mg/L	0.019 ± 0.000	a
YD 500 mg/L	0.024 ± 0.000	b
Lees (2 %)	0.039 ± 0.000	e
ASC 50 mg/L	0.033 ± 0.001	d
GSH 50 mg/L	0.028 ± 0.000	c

(b) Aged wine (harvest 2007)

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

Sample	POM-test 15 days Mean ± SD	
Control	90 ± 10	b
SO2 50 mg/L	167 ± 36	cd
YD 500 mg/L	181 ± 6	d
Lees (2 %)	111 ± 17	bc
ASC 50 mg/L	20 ± 35	a
GSH 50 mg/L	25 ± 2	a

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

Highlights

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

Ascorbic acid was a good O_2 and radical scavenger but induced browning during storage

Lees were good O_2 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_2 protecting wine color over 8 months