

1 **Inhibitory effect of fungoid chitosan in the generation of aldehydes**  
2 **relevant to photooxidative decay in a sulphite-free white wine**

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## 23 Abstract

24 The reaction pathways were investigated by which a fungoid chitosan (CsG) may protect  
25 against photooxidative decay of model solutions and a sulphite-free white wine. Samples  
26 containing CsG were dark incubated for 2 days before exposure to fluorescent lighting for  
27 up to 21 days in the presence of wine like (+)-catechin and/or iron doses. In both systems  
28 CsG at winemaking doses significantly reduced the photoproduction of acetaldehyde and,  
29 to a better extent, glyoxylic acid, two key reactive aldehydes implicated in wine oxidative  
30 spoilage. After 21 days, CsG was two-fold more effective than sulphur dioxide in  
31 preventing glyoxylic acid formation and minimizing the browning of white wine. Among the  
32 antioxidant mechanisms involved in CsG protective effect, iron chelation, and hydrogen  
33 peroxide quenching were demonstrated. Besides, the previously unreported tartrate  
34 displacement from the [iron(III)-tartrate] complex was revealed as an additional inhibitory  
35 mechanism of CsG under photo-Fenton oxidation conditions.

36

## 37 Highlights

- 38 1. Fungoid chitosan reduces the generation of aldehydes during wine photooxidation
- 39 2. Chitosan reduces iron amounts in solution by adsorbing [carboxylates-Fe(III)]  
40 complexes
- 41 3. In extended oxidative conditions SO<sub>2</sub> is a poorer wine anti-browning agent than  
42 chitosan
- 43 4. Sulphites better control free acetaldehyde but not glyoxylic acid amounts
- 44 5. In wines chitosan can mitigate the browning while preserving catechin amounts

45

46 **Keywords:** Chitosan, sulphite-free white wine, photo-Fenton oxidation, aldehydes, iron  
47 chelation, antioxidant, browning, iron-tartrate complex

48 **Abbreviations:** ANOVA, analysis of variance; CsG, chitosan.

49

## 50 **1. Introduction**

51 The optimal management of wine oxidation represents a huge challenge for  
52 winemakers. From a sensory point of view, controlled oxidation could be beneficial for red  
53 wines due to the reduction of astringency and the enhancement of colour stabilization.  
54 However, white wines are usually damaged by air exposure which can lead to unwanted  
55 non-enzymatic browning, and the decay of both the sensory characteristics (aromatic  
56 defects, increase of astringency), and nutritional properties (Waterhouse & Laurie, 2006).

57 In oenology, oxidation involves enzymatic or non-enzymatic reactions. However, after  
58 the inactivation of most polyphenol oxidases during the alcoholic fermentation, non-  
59 enzymatic cascade becomes the main oxidative pathway throughout the winemaking  
60 process commonly carried out in wineries, and it remains still active upon bottling and at  
61 storage. During wine oxidation, a sequence of univalent reduction steps settles from  
62 oxygen up to water, via a Fe(II)/Fe(III) redox cycle where hydrogen peroxide ( $H_2O_2$ ), and  
63 the highly reactive hydroxyl radical ( $HO\cdot$ ) are formed (Waterhouse & Laurie, 2006;  
64 Oliveira, Ferreira, De Freitas, & Silva, 2011; Danilewicz, 2012). The most conclusive proof  
65 of  $HO\cdot$  formation in wine oxidation came from electron paramagnetic resonance (EPR)  
66 studies (Elias, Andersen, Skibsted, & Waterhouse, 2009; Nikolantonaki et al., 2019; Castro  
67 Marín et al., 2019; Marchante et al., 2020) showing that 1-hydroxyethyl radical (1-HER) is  
68 transiently generated during the conversion of ethanol into acetaldehyde, a reaction  
69 forming  $H_2O_2$  (Fig. 1).

70 The reduction of  $H_2O_2$  to  $HO\cdot$  is mediated by metal ions (e.g., by the Fe(II)/Fe(III)  
71 redox couple) according to the so-called 'thermal' (as opposed to light-induced) Fenton  
72 mechanism (black pathway in Fig. 1). In this process wine polyphenols play a major role

73 by redox cycling iron (Oliveira et al., 2011; Danilewicz, 2012). Fenton-derived HO• will also  
74 react with wine carboxylic acids such as tartaric acid (a major component in grape juice),  
75 forming a carbon-centered radical derivative which undergoes oxidation and  
76 decarboxylation steps to yield glyoxylic acid. Hence, acetaldehyde and glyoxylic acid are  
77 main intermediates in the oxidative evolution of wine (Drinkine, Glories, & Saucier, 2005).  
78 These compounds have been shown to cross-link wine flavan-3-ols to yield methyne-  
79 bridged dimers, which in turn decompose into (i) 8-vinylflavan-3-ols adducts (Fulcrand,  
80 Dueñas, Salas, & Cheynier, 2006) in the case of acetaldehyde, and (ii) yellow-brown  
81 xanthylium cation pigments that contribute to the chemical browning of white wines (Es-  
82 Safi et al., 1999; Bührle, Gohl, & Weber, 2017), in the case of glyoxylic acid. Figure 1  
83 stresses on 8-vinyl-(+)-catechin, the unstable acetaldehyde adduct formed when (+)-  
84 catechin is the flavan-3-ol substrate, as this class of compounds brings about browning  
85 upon condensing with anthocyanins (Mateus et al., 2002; Cruz et al., 2009) and/or reacting  
86 with quinones through electron transfer reactions (Cruz et al., 2009).

87 Accordingly, reducing the development of aldehydic oxidation intermediates would be  
88 a relevant strategy to limit browning spoilage of wines. Possessing antimicrobial  
89 properties, sulphur dioxide (SO<sub>2</sub>) is widely used in oenology for its global antioxidant  
90 power, acting upstream (by scavenging H<sub>2</sub>O<sub>2</sub> and reducing quinones back to their phenolic  
91 precursors) or downstream (by binding acetaldehyde) to HO• formation (Danilewicz, 2007;  
92 Oliveira et al., 2011). However, an increasing number of consumers are turning toward  
93 non-sulphited wines because sulphites have been associated with many adverse health  
94 effects, (Vally, Misso, & Madan, 2009).

95 Another strategy not involving SO<sub>2</sub> to preserve shelf life of finished wines and to  
96 prevent chemical browning would be inhibiting specifically free radical processes shown in  
97 Figure 1. This has promoted the use of antioxidant additives such as glutathione or  
98 ascorbic acid (Sonni, Clark, Prenzler, Riponi et al., 2011a; Barril, Clark, & Scollary, 2012;

99 Marchante et al., 2020). Alternatively, use of metal chelators to prevent the Fenton  
100 reaction is gaining popularity. Among the few biocompatible, biodegradable and non-toxic  
101 potential candidates chitosan, the deacetylated derivative of chitin found in the carapace of  
102 shellfish, insects, green algae or fungi, is of great interest in food science, having versatile  
103 functionalities as antimicrobial and bacteriostatic, and able to form a variety of films,  
104 hydrogels or nanoparticles (Qin et al., 2002; Muxika, Etxabide, Uranga, Guerrero, et al.,  
105 2017).

106 Owing to its metal chelation power, chitosan from fungal origin has been  
107 recommended in 2009 by the International Organisation of Vine and Wine (OIV), then  
108 authorized by the EU as an additive in winemaking for removing metals and pollutants,  
109 preventing cloudiness, and for the reduction of *Brettanomyces* spp and other undesirable  
110 wine microbial population (EC Regulation No 53/2011). As a consequence, previous  
111 studies in real wines or model solutions have focused on the efficacy of chitosan in  
112 reducing the tendency to browning (Spagna et al., 1996) and diminishing the oxidative  
113 degradation of one of its chemical determinants, (+)-catechin (Fig. 1) (Chinnici, Natali, &  
114 Riponi, 2014), with no or little impact on polyphenols content and fermentative aromatic  
115 compounds (Filipe-Ribeiro, Cosme & Nunes, 2018; Colangelo, Torchio, De Faveri, &  
116 Lambri, 2018; Picariello, Rinaldi, Blaiotta, Moio, Pirozzi, & Gambuti, 2020). In a recent  
117 EPR work (Castro Marín et al., 2019), a chronology of the antioxidant protection afforded  
118 by dark pretreatment (2 days) with chitosan of a SO<sub>2</sub>-free white wine was proposed, first  
119 involving partial inactivation of the wine catalytic iron pool by complexation, followed by  
120 direct scavenging of HO• formed by thermal Fenton reaction catalyzed by those iron ions  
121 having escaped chelation. In connection with chemical browning, the same authors  
122 (Castro Marín et al., 2019) observed a significant inhibition of acetaldehyde formation  
123 when oxidation of the wine was provoked by UV-Vis illumination.

124 In view of the above, the aim of this study was to decipher some mechanisms  
125 implicated in this latter effect of chitosan on acetaldehyde photoproduction. **Indeed**, storing  
126 bottled white wines under UV-Vis light for long periods is known to accelerate oxidation,  
127 affecting the main oenological attributes and leading to browning. A first mechanism of  
128 photooxidation involves the photochemical cleavage of iron(III) aquacomplex  
129  $\text{Fe}(\text{H}_2\text{O})_5(\text{OH})^{2+}$  (abbreviated as  $\text{Fe}(\text{OH})^{2+}$ ), the major form of aqueous ferric ions at wine  
130 pH, to generate  $\text{HO}\cdot$  in a process termed as 'photo-Fenton' (green pathway in Fig. 1)  
131 (Loures et al., 2013). Another wine relevant pathway has been demonstrated, whereby  
132 photolysis of the [iron(III)-tartrate] complex, the predominant form of Fe(III) found in wine,  
133 regenerates ferrous ions and a transient tartaric acid derived acyloxyl radical, which  
134 subsequently decomposes into glyoxylic acid by a sequence of oxidation/decarboxylation  
135 reactions (red pathway in Fig. 1) **that ultimately provoke** browning (Grant-Preece,  
136 Schmidtke, Barril, & Clark, 2017a, b).

137 In real white wine samples spiked with a relevant Fe(II) concentration and added  
138 allowed doses of chitosan or  $\text{SO}_2$ , there was a similar inhibition of acetaldehyde production  
139 after 6 days illumination with fluorescent light (Castro Marín et al., 2019). **The mechanism**  
140 **of this protection is consistent with an impact of chitosan along the Fenton-driven route of**  
141 **browning that involves ethanol oxidation (Fig. 1). In parallel, chitosan could also**  
142 **encompass the photo-Fenton pathway by inhibiting metal chelators and/or scavenging free**  
143 **radicals, such as  $\text{HO}\cdot$  or 1-HER.** Building on the above, it was reasoned that investigating  
144 the effect of chitosan on Fenton-unrelated 'tartaric acid' route **of** browning (red pathway in  
145 Fig. 1) might bring valuable information on the benefits of using this biopolymer to prevent  
146 light-induced spoilage of wines. To this end, photooxidation was induced in air saturated  
147 sulphite-free white wines and model wine solutions, and high-performance liquid  
148 chromatography with diode array detection (HPLC-DAD) was applied **to evaluate the**  
149 **different mechanisms (shown in blue in Fig. 1) whereby browning development will be**

150 affected by (i) various chitosan or SO<sub>2</sub> treatments and doses, or (ii) target compounds  
151 acetaldehyde, glyoxylic acid, H<sub>2</sub>O<sub>2</sub>, iron, tartaric acid and other wine carboxylic acids, or  
152 (+)-catechin.

153

## 154 2. Experimental

### 155 2.1. Samples, chitosan and chemicals

156 Sulphite-free, 100% Chardonnay wine samples (AOP Côteaux Champenois, vintage  
157 2016) were obtained directly from the winery (Champagne J. de Telmont, Damery,  
158 France). Wines were bottled in 1.5 L green glass bottles, left in darkness at 20 °C, and  
159 conserved in vertical position under N<sub>2</sub> atmosphere after opening. The oenological  
160 parameters, measured using the OIV methods of the 'Compendium of international  
161 methods of analysis of wines and musts' (2018), were: ethanol, 11.35% v/v; pH 3.16;  
162 titratable acidity, 4.60 g/L of sulphuric acid; volatile acidity, 0.45 g/L of sulphuric acid; malic  
163 acid content < 2.0 g/L; free SO<sub>2</sub>, < 5 mg/L, and total SO<sub>2</sub> < 5 mg/L.

164 Chitosan (CAS 9012-76-4; 80–90% deacetylated, average molecular weight, 10–30  
165 kDa) from *Aspergillus niger*, the only source allowed for oenological purposes (OIV/OENO  
166 368/2009), was obtained from KitoZyme (Herstal, Belgium). It will be termed as 'CsG' from  
167 here.

168 Doubly distilled deionized water was used. HPLC grade acetonitrile and all other  
169 solvents and chemicals were of the highest purity from Sigma-Aldrich (Saint Quentin  
170 Fallavier, France), including the standards acetaldehyde and glyoxylic acid monohydrate,  
171 (+)-catechin, (-)-epicatechin, 2,4-dinitrophenylhydrazine (DNPH), H<sub>2</sub>O<sub>2</sub>, ethanol, FeSO<sub>4</sub> (as  
172 ferrous sulphate heptahydrate), FeCl<sub>3</sub> (as ferric chloride hexahydrate), glycine buffer, *N,N'*-  
173 dimethyl-9,9'-biacridinium dinitrate (lucigenin), L-(+)-tartaric acid, L-(–)-malic acid, citric  
174 acid and potassium metabisulphite.

175

## 176 2.2. *Model wine solution*

177 A total of 2 litres of model wine solution was prepared, containing 5 g/L (+)-tartaric acid  
178 and 12% (v/v) ethanol. After adjusting the pH to 3.2 with 5 M NaOH the solution was  
179 stirred overnight at 20 °C in open-air to reach oxygen saturation.

180

## 181 2.3. *Preparation of solutions and suspensions for irradiation*

182 Trials were arranged in triplicate by transferring aliquots (20 mL) of white or model  
183 wine in 50-mL high-clarity polyethylene terephthalate (PET) conical centrifuge tubes  
184 (Corning Inc., Fisher Scientific, Illkirch, France), leaving 30 mL of air in the headspace.

185 PET has a high transmittance of about 90% within the wavelength range of 350–700 nm  
186 and is suitable for transparent applications. All samples were spiked with iron using 250 µL  
187 of freshly prepared aqueous FeSO<sub>4</sub> (1 g/L), yielding a Fe(II) concentration of 2.5 mg/L, an  
188 average value for white wines made with modern stainless-steel equipment.

189 Afterwards, aliquots (100 µL) of water (control samples), SO<sub>2</sub> (as aqueous potassium  
190 metabisulfite, to achieve 25–100 mg/L, final concentration), or weighed CsG (to achieve  
191 0.2–2 g/L, final concentration) were added and each tube was tightly closed and dark pre-  
192 incubated at 20 °C for 48 h. The concentration levels of CsG suspensions were defined  
193 considering both the usual dosages and the maximum admitted addition in wines, equal to  
194 1 g/L. The tubes were then irradiated for varying times (24, 48 and 240 h) by two cool  
195 daylight fluorescent lamps (Sylvania T8Luxline Plus F36 W/840) placed at a distance of 10  
196 cm. The illumination intensity was 2000 lux (Yogokawa, 51,000 series lux meter, Lyon,  
197 France), with emission peaks centered at 313, 365, 405, 436, 546 and 578 nm, and  
198 emission peaks with maxima at 480 and 580 nm. Under these conditions no significant  
199 warming of the samples was noticed during irradiation. All samples were shaken for 2 min  
200 every hour at a 12-h period, in particular to achieve optimal contact in CsG suspensions.



201 Unbound acetaldehyde and glyoxylic acid levels were timely assessed throughout the  
202 entire protocol (see below).

203 For getting closer to the conditions of oenological usage of CsG, it was applied at 0.5–  
204 2 g/L only during dark pretreatment of iron-spiked white wine samples prepared as  
205 described above. In addition, in order to mimic real winemaking conditions, insoluble  
206 chitosan was removed by passing samples on a 0.65- $\mu$ m filter before irradiation for varying  
207 times, at the end of which unbound acetaldehyde and glyoxylic acid levels in the CsG  
208 pretreated samples (termed as 'Pt-CsG') and in the related filtered controls (termed as 'Pt-  
209 controls') were measured.

210 In order to (i) examine the effect of varying iron(II) and (+)-catechin concentrations on  
211 the levels of free acetaldehyde and glyoxylic acid consequent to irradiation and (ii) the  
212 effect of CsG, model and white wine samples were added of iron(II) (1–5 mg/L) and (+)-  
213 catechin (0–200 mg/L), and underwent 48-h dark pretreatment followed by irradiation (for 2  
214 or 10 days). The CsG treated group here consisted of suspensions in white wine  
215 containing 5 mg/L iron, 100 or 200 mg/L (+)-catechin, and 2 g/L CsG. Samples from each  
216 group were kept in darkness for 10 days as dark controls.

217

#### 218 2.4. HPLC-DAD conditions for unbound acetaldehyde and glyoxylic acid analysis

219 Unbound acetaldehyde and glyoxylic acid contents in samples were assayed by  
220 HPLC-DAD as their DNPH derivatives (Stocker et al., 2015). Samples (800  $\mu$ L) were  
221 mixed with 200  $\mu$ L of 10 mM DNPH dissolved in 2.5 M HCl, and dark incubated for 1 h at  
222 45 °C. After cooling at room temperature and centrifugation of the mixture (5 min at  
223 2400g), the DNPH adducts were separated using a 250 mm  $\times$  4.6 mm i.d., 5- $\mu$ m particle  
224 size Nucleodur C18 Htec column (Macherey-Nagel, Düren, Germany) with a flow rate of  
225 0.8 mL/min. The mobile phase was a mixture of acetonitrile (solvent A) and 0.05% (v/v)  
226 phosphoric acid in deionized water (pH 2.7; solvent B) and the elution program with linear

227 gradient was: 0 min, 40% A, 8 min, 85% A, 9 min, 40% A, 13 min, 40% A, with an injection  
228 volume of 20  $\mu$ L. A Merck Hitachi HPLC system consisting of a LaChrom L-7000 interface  
229 module and a L-7455 photodiode array detector coupled to a data processing computer  
230 (EZChrome workstation) was used. Chromatograms were acquired at 220–400 nm and  
231 derivatized compounds were identified by comparing their retention times with those of  
232 standards. Quantification was based on peak area at 360 nm from standard calibration  
233 curves.

234

#### 235 2.5. HPLC determination of catechin and epicatechin

236 Separation and quantitation of catechin and epicatechin isomers were achieved at  
237 room temperature using a Htec RP-18 column (250 mm  $\times$  4 mm; 5  $\mu$ m; Macherey Nagel)  
238 and a gradient with a flow rate of 0.8 mL/min. Gradient was: solvent A (0.05% H<sub>3</sub>PO<sub>4</sub> in  
239 H<sub>2</sub>O, pH 2.6), solvent B (MeOH): 0–45 min, 30–60% B; 45–50 min, 60% B; 50–51 min,  
240 30% B; 51–57 min, 30% B. The wine samples were mixed with mobile phase (1:1 v/v)  
241 before centrifugation for 5 min at 2400g. A 20- $\mu$ L volume of the supernatant was injected  
242 into the HPLC system. UV absorbance was measured at 280 nm, and quantification was  
243 carried out by comparing peak area with that of a (+)-catechin standard.

244

#### 245 2.6. Evaluation of CsG Fe(II) and Fe(III) chelating activities

246 The possibility of biphasic iron(II) chelating action of CsG in wine was assessed. After  
247 48 h dark incubation in 50-mL plastic tubes sealed with stoppers, wine samples (20 mL)  
248 saturated with air were added a mixture of 5 mg/L Fe(II) and varying concentrations of  
249 CsG (0.2–2 g/L). The resulting suspensions (termed as '+CsG') were dark incubated for an  
250 additional 48 h under continuous agitation. In another set of experiments the wine samples  
251 were first preloaded with the same concentration range of CsG and dark incubated for 48

252 h before 5 mg/L Fe(II) was added and the suspensions samples (termed as 'Pi-CsG') dark  
253 incubated for a further 48 h.

254 The iron content of the samples after filtration versus unloaded controls was then  
255 quantitated in triplicate by flame atomic absorption spectrometry according to the current  
256 official OIV method (see Castro Marín et al., 2019).

257 The iron(III) chelating activity of CsG was evaluated in acidified ethanolic solutions  
258 (12% v/v, pH 3.2). Samples (20 mL) loaded with 10 mg/L FeCl<sub>3</sub> were dark incubated for 1  
259 h at 20 °C. Varying concentrations of CsG (1–10 g/L) were added thereafter under  
260 vigorous stirring. After an additional 5 min incubation the suspensions were centrifugated  
261 (2400g) for 5 min, 200 µL of each supernatant was transferred in 96-well microplates and  
262 absorbance was recorded using a microplate reader (Tecan Infinite, Männedorf,  
263 Switzerland). Experiments were made in triplicate.

264

## 265 2.7. Assay of H<sub>2</sub>O<sub>2</sub>

266 The amount of unreacted H<sub>2</sub>O<sub>2</sub> following addition at 20 °C of CsG in H<sub>2</sub>O<sub>2</sub> spiked  
267 model wine was measured by lucigenin chemiluminescence (Maskiewicz, Sogah, &  
268 Bruice, 1979). Samples (20 mL) containing H<sub>2</sub>O<sub>2</sub> (100 or 500 µM) without (controls) or with  
269 added CsG (0.2–2 g/L) were placed in centrifuge plastic tubes and were dark incubated for  
270 10 min at 20 °C. The mixture was filtered (0.45 µm nylon filter) and aliquots (20 µL) were  
271 mixed with 200 µL of a lucigenin solution (0.2 g/L in 0.2 M glycine buffer, pH 10) and dark  
272 incubated for 10 min at 20 °C in 96-well microplates before chemiluminescence was read.  
273 Blank values were measured without H<sub>2</sub>O<sub>2</sub>. The decrease in H<sub>2</sub>O<sub>2</sub> content of CsG-treated  
274 samples was calculated as a percentage of the signal given by the corresponding control  
275 prepared as described above taken as 100%. Concentration of H<sub>2</sub>O<sub>2</sub> was derived from a  
276 calibration curve (10–500 µM range) recorded in another set of lucigenin containing  
277 samples dark incubated for 10 min. Establishing the calibration curve showed that pre-

278 incubation and filtration steps in control samples resulted in a negligible loss of H<sub>2</sub>O<sub>2</sub>. All  
279 measurements were made in triplicate.

280

## 281 2.8. *Browning analysis*

282 The effect of CsG and SO<sub>2</sub> on the susceptibility to browning was assessed in 2.5 mg/L  
283 iron(II)-spiked, dark pre-incubated, then irradiated model wine solution or white wine  
284 samples prepared and processed as described above for acetaldehyde and glyoxylic acid  
285 measurements (see section 2.3), except that model wine samples were also added (+)-  
286 catechin (100 mg/L) before dark pre-incubation. Irradiated samples were timely filtered  
287 (0.45 µm nylon filter) and browning development was monitored at room temperature for  
288 21 days at 420 nm by adapting the protocol of Sioumis et al. (2006) to a microplate reader.  
289 Experiments were made in triplicate.

290

## 291 2.9. *Absorption spectroscopy of CsG-added [Fe(III)-carboxylates] complexes*

292 [Fe(III)-carboxylates] complexes were prepared in triplicate in 50-mL Falcon conical  
293 centrifuge plastic tubes as previously described (Grant-Preece et al., 2017b). Briefly,  
294 freshly prepared mixtures of FeCl<sub>3</sub> (20 mg/L) were stirred for 1 h at 20 °C in the dark with  
295 solutions of either tartaric (5 g/L), malic or citric acids (3 g/L) in acidified ethanolic solutions  
296 (12% v/v, pH 3.2). Afterwards varying concentrations of CsG (1–2 g/L) were added under  
297 stirring and the mixtures were reacted in darkness a few min. Then, the mixtures were  
298 centrifugated for 5 min (2400g), 200 µL of each supernatant was transferred into 96-well  
299 microplates and the absorption was read between 256 and 496 nm at 20 °C.

300

## 301 2.10. *Statistical analysis*

302 Unless otherwise noted all data were analysed and presented as means ± SD for the  
303 indicated number of independent experiments. Intergroup differences were calculated by  
304 one-way analysis of variance (ANOVA) followed by appropriate a posteriori tests. *P*-values

305 < 0.05 were statistically significant (Prism 6 software; GraphPad Software, San Diego,  
306 CA).

307

### 308 **3 Results and discussion**

309

#### 310 *3.1 Development of acetaldehyde and glyoxylic acid in real and synthetic wine during* 311 *light exposure*

312 It is known that submitting iron(II)-supplemented, tartaric acid containing model  
313 solutions or real white wines to fluorescent light results in an increased formation of  
314 glyoxylic acid (Grant-Preece et al., 2017a, b) and acetaldehyde (Castro Marín et al., 2019),  
315 the non-bounded fraction of which actively participating in wine browning phenomena.  
316 Under photooxidative conditions a part of these reactive carbonyls could conceivably have  
317 been formed via the established photo-Fenton mechanism (shown in green in Fig. 1)  
318 through extra HO• formation and faster Fe(III)/Fe(II) recycling (Loures et al., 2013). It was  
319 previously reported that chitosan, suspended in a SO<sub>2</sub> free Chardonnay wine added of 5.5  
320 mg/L Fe(II) and allowed to chelate metal ions for 2 days in darkness, significantly inhibited  
321 acetaldehyde formation upon illumination (Castro Marín et al., 2019).

322 Here a first objective was to widen these above data to photooxidation produced  
323 glyoxylic acid in tartaric acid-based model matrix, and a sulphite-free white wine. After 48 h  
324 of timely agitation at 20 °C in darkness with 1.5 times air volume headspace, no significant  
325 acetaldehyde or glyoxylic acid formation was evidenced by HPLC-DAD in model wine  
326 samples containing 2.5 mg/L iron(II) (not shown). Further lightning of these samples for 24  
327 h stimulated the generation of 14.1 ± 0.7 mg/L of acetaldehyde and 108 ± 1 mg/L of  
328 glyoxylic acid in the controls (Fig. 2A), consistent with the findings of Clark et al. (2007)  
329 who detected glyoxylic acid only after direct sunlight exposure of model wines containing  
330 tartaric acid. This production of oxidation carbonyls was directly related to illumination

331 time, since after 48 h the content of glyoxylic acid doubled while acetaldehyde level  
332 increased by 30% (Fig. 2A). When the experimental Chardonnay wine was stored in  
333 darkness with iron, the mean acetaldehyde concentrations of  $6.1 \pm 0.4$  mg/L at opening  
334 remained nearly stable at  $7.1 \pm 0.3$  mg/L after 2 days (red bar in Fig. 2B) and slightly  
335 increased up to  $8.9 \pm 0.3$  mg/L after 10 days. At the same experimental times glyoxylic  
336 acid levels were found under the detection limits, i.e.,  $< 2$  mg/L. When storage under light  
337 exposure was applied to this wine, formation of both carbonyls was remarkably reduced  
338 with respect to model wine, especially in the case of glyoxylic acid (Fig. 2B). This could  
339 have been somewhat expected since wine macromolecules, including proteins,  
340 polysaccharides, tannins, as well as monomeric species such as phenolic acids or  
341 flavanols, may have quenched a portion of oxidizing species, particularly free radicals such  
342 as HO• (Oliveira et al., 2011), thereby decreasing the extent of ethanol or tartaric acid  
343 oxidation. After 10 days illumination,  $118 \pm 1$  mg/L of glyoxylic acid and  $18.1 \pm 0.1$  mg/L of  
344 acetaldehyde were measured in real wine (Fig. 2B), the latter value being close to those  
345 obtained in model solution after only 48 h (Fig. 2A).

346 For all the suspensions of CsG added up to 1 g/L, the level of free acetaldehyde and  
347 glyoxylic acid was dose-dependently and significantly reduced at any time of light  
348 exposure when compared to the untreated controls (Fig. 2A and B). For model wine  
349 containing 2 g/L CsG this decrease was strong for both glyoxylic acid ( $22.8 \pm 0.2$  mg/L,  
350 i.e., 79% decrease) and acetaldehyde ( $3.7 \pm 0.7$  mg/L, i.e., 77% decrease) contents after 1  
351 day of irradiation, but illuminating the mixtures for one additional day led to a better  
352 protection for the glyoxylic acid (78% decrease) versus acetaldehyde (50% decrease)  
353 levels. A different pattern was observed in illuminated, SO<sub>2</sub>-added model wine samples,  
354 where a stronger inhibition of both free glyoxylic acid (ranging 91–94%) and acetaldehyde  
355 (ranging 84–89%) levels was observed at 24 h and 48 h at any dose of the additive (Fig.  
356 2A). In those samples, these reductions were considerable even at the lowest SO<sub>2</sub> dosage

357 likely due to its ability to both scavenge H<sub>2</sub>O<sub>2</sub> and strongly bind aldehydes (Oliveira et al.,  
358 2011). Indeed, the apparent equilibrium constants for sulphite-aldehyde adducts were  
359 estimated to be  $3.7 \times 10^{-6}$  and  $1.5 \times 10^{-6}$  for glyoxylic acid and acetaldehyde, respectively  
360 (Sonni et al., 2011b), suggesting that a noticeable portion of those aldehydes may have  
361 been present in the bound form (Grant-Preece et al., 2017a).

362 Next, the progressive inhibitions of fluorescent lighting-induced build-up of  
363 acetaldehyde and glyoxylic acid seen above upon adding CsG or SO<sub>2</sub> were confirmed in  
364 2.5 mg/L iron(II) added sulphite-free Chardonnay wine, again with a better effect on  
365 glyoxylic acid versus acetaldehyde levels. For example, a dose of 2 g/L CsG in suspension  
366 significantly lowered glyoxylic acid formation from  $55 \pm 2$  mg/L to  $17.6 \pm 0.3$  mg/L after 48  
367 h irradiation, and such protection was even significantly better than that provided by  
368 adding SO<sub>2</sub> up to 100 mg/L instead of CsG (Fig. 2B). Interestingly, after 10 days of lighting  
369 the glyoxylic acid content markedly increased in all wine samples but 0.5 g/L CsG, a  
370 concentration below the **maximum authorized dose** for winemaking, guaranteed a  
371 significantly better control of this glyoxylic acid generation when compared to any dose of  
372 SO<sub>2</sub> (Fig. 2B). When wine samples were kept in contact with 1–2 g/L CsG over the 10-  
373 days irradiation time, glyoxylic acid content was decreased by up to 65% compared to  
374 controls or SO<sub>2</sub>-treated samples. **This dramatic increase might be due to an early and**  
375 **almost complete consumption of sulphur dioxide, e.g., by oxidation with H<sub>2</sub>O<sub>2</sub> generated by**  
376 **thermal Fenton pathway (Fig. 1), making insufficient its potential to bind glyoxylic acid at**  
377 **day 10.** Figure 2B also shows an opposite situation was observed for acetaldehyde levels  
378 which were significantly best inhibited by SO<sub>2</sub> (maximum value of 60% at day 10) than by  
379 CsG (only 23% inhibition at 2 g/L), and this acetaldehyde versus glyoxylic acid difference  
380 may be the reflection of the better binder behaviour of the former toward SO<sub>2</sub> (as their  
381 above cited equilibrium constants suggest).

382 Fungal chitosan at 1 g/L has been found poorly soluble in red wine (i.e., < 0.9%; Filipe-  
383 Ribeiro, Cosme & Nunes, 2018) and practically insoluble in white wine after 12 h of contact  
384 under gentle stirring (Colangelo et al., 2018). Therefore, its presence in bottled products  
385 could be prevented by racking, membrane filtration or centrifugation (Spagna et al., 1996).  
386 Recent studies have reported a good acetaldehyde control by CsG in white wine after  
387 several days of light exposure following a dark pretreatment / filtration sequence (Castro  
388 Marín et al., 2019). Given the positive results of Fig. 2B showing a marked inhibition of  
389 glyoxylic acid formation in the unfiltered protocol after only 24 h of irradiation, it seemed  
390 interesting to investigate the behaviour of CsG suspensions submitted to membrane  
391 filtration before being irradiated for short times. For this purpose, the experimental 2.5  
392 mg/L iron(II) spiked, non-sulphited Chardonnay wine was loaded for 48 h in darkness with  
393 different doses of CsG and the suspensions passed through a 0.65- $\mu$ m filter prior to 24 or  
394 48 h light exposure (Pt-CsG samples).

395 After filtration the carbonyls levels of non-irradiated, unsupplemented wine samples  
396 (dark controls, red bars in Fig. 2C) remained relatively low, close to that of unfiltered  
397 samples (Fig. 2B). As expected, forced aeration of wine samples during filtration notably  
398 increased acetaldehyde and glyoxylic acid concentrations upon irradiation in  
399 unsupplemented controls (Fig. 2C) compared to unfiltered samples (Fig. 2B). All Pt-CsG  
400 samples exhibited significantly lower levels of carbonyls compared to Pt-Controls, i.e., they  
401 decreased by 47% for glyoxylic acid and 35% for acetaldehyde after 48 h of irradiation  
402 following dark pretreatment with 2 g/L CsG. From Fig. 2B and C it was clearly seen that,  
403 compared to the unsupplemented controls, pre-incubating the wine with a CsG dose as  
404 low as 0.5 g/L followed by 1 day illumination resulted in a better relative inhibition of  
405 glyoxylic acid formation in the filtered (-37%) than in the unfiltered (-16%) samples whilst  
406 acetaldehyde development was not affected. Nevertheless, at the highest tested CsG  
407 doses, the contents of both oxidation carbonyls were always best inhibited when CsG was



408 present in the wine during irradiation. For example, at 2 g/L CsG and after 48 h irradiation,  
409 the inhibitions in the CsG versus the Pt-CsG samples were of 34% and 21%, respectively  
410 for acetaldehyde, and 68% and 49%, respectively for glyoxylic acid. Besides confirming  
411 the enhanced trend for chitosan to slower glyoxylic acid versus acetaldehyde formation  
412 seen above, marked differences for low versus high CsG doses seem consistent with EPR  
413 data showing a biphasic interfacial antioxidant action initiated by metal chelation followed,  
414 if enough uncomplexed CsG is still available, by direct scavenging of radical species  
415 intermediates of the thermal and photo-Fenton mechanisms of Fig. 1 (Castro Marín et al.,  
416 2019). This biphasic action would therefore be relevant in the case of extended contact of  
417 chitosan with wine (e.g., during storage in tank) while in filtered and bottled wines the  
418 antioxidant activity coming from the chelating properties of CsG will probably prevail.

419

### 420 3.2 Iron chelating capacity

421 Direct inactivation of metal catalysts, especially Fe(II)/Fe(III) or copper, at any stage of  
422 the thermal Fenton reaction leading to acetaldehyde and glyoxylic acid (Fig. 1), is the  
423 major complementary mechanism conceivable for chitosan as an alternative or  
424 complement to SO<sub>2</sub> in the control of wine oxidation and reduction of browning (Bornet &  
425 Teissedre, 2008; Chinnici et al., 2014; Colangelo et al., 2018, Castro Marín et al., 2019).  
426 First, two CsG addition protocols were compared for their chelating efficiency in  
427 experimental sulfite free Chardonnay wine added 5 mg/L Fe(II). Flame atomic absorption  
428 spectrometry indicated an average content of 0.39 mg/L for total iron at opening,  
429 remaining almost stable at 0.42 mg/L after 48 h aerial dark incubation with stirring.  
430 Addition of a mixture of 5 mg/L Fe(II) + CsG (0.2–2 g/L) to the wine (+CsG samples)  
431 resulted in a dose-dependent decrease of total iron content after an additional 48 h  
432 incubation in darkness (Fig. 3A, white bars). Under the same concentration conditions a  
433 significant better iron removal was observed when the wine was added of CsG during pre-

434 incubation, followed by iron for the remaining 48 h incubation (Pi-CsG samples). Hence,  
435 for the same 0.5 g/L CsG input that already provided a good protection against Gly  
436 development during 24 h irradiation of the iron(II) added wine (Fig. 2C), only 24% of initial  
437 iron was removed in +CsG samples while a higher loss of about 48% was found in Pi-CsG  
438 samples (Fig. 3A, black bars). A ~40% iron reduction has been reported when a white  
439 wine naturally containing a threefold higher iron content was put in contact with 1 g/L  
440 chitosan for 12 h (Colangelo et al., 2018).

441 The interaction of iron with chitosan is an intricate molecular process comprising  
442 chelation, ion-exchange, and surface adsorption (Bornet & Teissedre, 2008; Gylliené,  
443 Binkiené, Baranauskas, Mordas et al., 2014) involving the lone pairs of amino groups of  
444 the polysaccharide (Fig. 1). In fact, no chitosan-complexes with, e.g., free Fe(III) can  
445 directly form at wine pH because of the preferred protonation of amino into ammonium  
446 groups and the ensuing repulsive electrostatic interactions establishing with metal cations.  
447 For this reason, ternary complexes between chitosan and anionic forms, such as [Fe(III)-  
448 tartrate] complex schematically represented in Fig. 1, may form in wine (Rocha, Ferreira,  
449 Coimbra, & Nunes, 2020).

450 It can be inferred from the green pathway in Fig. 1 that decreasing the catalytic activity  
451 of iron(III) in wine will participate in limiting the photoproduction of acetaldehyde. This  
452 property of CsG was demonstrated by UV-Vis spectrophotometry in a simplified solution  
453 consisting of ferric chloride (10 mg/L) dissolved in 12% v/v ethanol (pH 3.2). After 1 h dark  
454 incubation the control absorption spectrum exhibited a small shoulder at 297 nm,  
455 characteristic of Fe(OH)<sup>2+</sup> (Loures et al., 2013). Figure 3B shows that the whole spectrum  
456 (256–496 nm) was dose-dependently inhibited by incremental additions of CsG (1-10 g/L).

457

### 458 3.3 Scavenging of H<sub>2</sub>O<sub>2</sub>

459 Maybe the most efficient intervention to prevent photooxidation of wines is to limit or  
460 suppress the continuous formation of H<sub>2</sub>O<sub>2</sub> which occurs at several steps of the thermal  
461 and photo-Fenton processes (Fig. 1). In this regard and beyond increasing consumers  
462 mistrust, sulphites are still considered essential additives for wine quality. Among other  
463 antioxidant effects (Oliveira et al., 2011), SO<sub>2</sub> is prone to interrupt thermal Fenton  
464 reactions by directly scavenging H<sub>2</sub>O<sub>2</sub> to yield sulfate (Danilewicz, 2007, 2012;  
465 Waterhouse & Laurie, 2006). The mechanism of the reaction of H<sub>2</sub>O<sub>2</sub> with chitosan in  
466 homogeneous solution has been investigated in detail (Chang, Tai, & Cheng, 2001; Qin et  
467 al., 2002) but information is scarce for what concerns its behaviour at pH and for addition  
468 modes (e.g., in suspensions) relevant to wine.

469 To gain insights on this matter, CsG suspensions (0.2–2 g/L) in H<sub>2</sub>O<sub>2</sub> added model  
470 wine solution were stirred for 10 min at ambient temperature in darkness, and residual  
471 hydrogen peroxide was assayed by the lucigenin assay after removal of chitosan by  
472 filtration. Despite the very high H<sub>2</sub>O<sub>2</sub> concentrations of 100 and 500 μM tested (see Castro  
473 Marín et al., 2019), a strong concentration-dependent inhibition was observed over a  
474 winemaking range of CsG (Fig. 3C), thus, at the maximum tested concentration of 2 g/L  
475 CsG removed 76 and 102 μM of the initial 100 and 500 μM of H<sub>2</sub>O<sub>2</sub> in the samples,  
476 respectively. Based on EPR data on model wine solutions estimating that total H<sub>2</sub>O<sub>2</sub> levels  
477 produced by the thermal Fenton reaction (i.e., in darkness) catalyzed by wine-like iron are  
478 within the micromolar range (Castro Marín et al., 2019), the data herein suggest that H<sub>2</sub>O<sub>2</sub>  
479 scavenging may play a further antioxidant role in wine oxidation. This includes photo-  
480 Fenton conditions where accumulation of hydrogen peroxide has been demonstrated in  
481 tartaric acid containing model wine solutions (Fig. 1; Clark et al., 2007). Mechanistically, it  
482 has been proposed that peroxide scavenging by chitosan could result in its  
483 depolymerization via a metal-sensitive oxidative breakdown of the 1.4-β-glycosidic

484 linkages of the polysaccharide backbone, leading to decrease of apparent molecular  
485 weight of the polymer (Chang et al., 2001).

486

#### 487 3.4 *Browning development*

488 Having assessed the mechanisms linked to Fenton chemistry by which chitosan, in  
489 suspension at winemaking doses, interfered with the photoproduction of acetaldehyde and  
490 glyoxylic acid, the next step of the study was to measure its anti-browning effect compared  
491 to SO<sub>2</sub> under the conditions of Fig. 2B. According to the mechanism of formation of (+)-  
492 catechin derived xanthylium cation pigments of Fig. 1, aerial oxidations of tartaric based  
493 model solutions containing 100 mg/L of this flavan-3-ol, and sulphite-free Chardonnay  
494 wine were initiated by adding 2.5 mg/L iron(II) during dark incubation of the samples, and  
495 optical density at 420 nm ( $A_{420}$ ) was monitored in filtered samples throughout ensuing light  
496 exposure.

497 Figure 4A depicts the kinetics of  $A_{420}$  increase in model wine solutions. All solutions  
498 remained colorless after dark pretreatment and a lag time of 2 days was observed before  
499 brown color developed in all illuminated samples. **No significant change in absorption**  
500 **values was seen in additional samples kept in the dark (dark controls) up to 21 days.**  
501 Compared to the controls (day 0) all treated solutions had their browning development  
502 significantly decreased, with SO<sub>2</sub> at any dose being most effective up to day 7, before a  
503 steady increase of brown nuances appeared from day 10 up to the final recording day 21.  
504 At day 7 and onward, a significantly better dose-dependent anti-browning efficacy versus  
505 all sulphited samples was found in CsG suspensions, whose  $A_{420}$  values exhibited a  
506 plateau. After 21 days lighting, mean  $A_{420}$  values in CsG samples (combined doses)  
507 decreased by 77% and 57% compared to controls and samples added 100 mg/L SO<sub>2</sub>,  
508 respectively. Chinnici et al. (2014) observed a lower browning tendency of model wines  
509 treated with 1 g/L chitosan, suggesting that up to 70% of the xanthylium yellow pigments

510 generated during (+)-catechin oxidation and relevant percentages of intermediate dimers  
511 may adsorb onto the polysaccharide surface (see below). Consistently, the observations  
512 herein on CsG solutions are probably the result of an initially faster oxidative process  
513 (mediated by acetaldehyde and glyoxylic acid, and generating carboxymethine dimers and  
514 brown pigments) followed by a delayed phase where the progressive accumulation of  
515 dimeric intermediates and pigments is counterbalanced by their adsorption on CsG. In  
516 contrast the smaller and more diffusible SO<sub>2</sub> will interact faster at any relevant step of the  
517 browning pathways, e.g., by reducing H<sub>2</sub>O<sub>2</sub>, including that formed by catechin autoxidation,  
518 (not represented in Fig.1 for clarity), or binding acetaldehyde and glyoxylic acid (Grant-  
519 Preece et al., 2017a, b), but would stop after complete consumption of sulphites, as  
520 already observed for the inhibition of 1-HER formation (Castro Marin et al., 2019).

521 Figure 4B shows the oxidative browning in experimental white wine which, at opening,  
522 contained 2.9 mg/L total catechins and 1.2 mg/L total epicatechins. Right after 48 h dark  
523 incubation, both SO<sub>2</sub> and CsG exhibited discolouring properties, having significantly  
524 decreased initial absorption value up to 29% for wines incubated with 2 g/L CsG, likely by  
525 an adsorption effect of phenolics on the chitosan chain and/or the lack in generation of  
526 brown pigments in this latter case (Spagna et al., 1996; Chinnici et al., 2014). **Again, no**  
527 **significant change in color was seen in dark controls at day 21 versus day 0.** Throughout  
528 photolysis of the wines the discolouring effect of CsG was found constantly and  
529 significantly higher than in SO<sub>2</sub> samples, with no clear dose dependency for both  
530 treatments. After 21 days lighting, mean A<sub>420</sub> values in CsG samples decreased by 63%  
531 and 43% compared to controls and samples added 100 mg/L SO<sub>2</sub>, respectively. It is  
532 possible that this relatively poor effect of SO<sub>2</sub> was due to the presence in the wine of  
533 quinones and carbonyls that may quench or oxidize sulphites, diminishing their anti-  
534 browning efficacy. Last, it is also believed that extending the time of contact with CsG,  
535 rather than increasing the dose, could had strongly improved the anti-browning effect seen

536 here since a recent study reported only a limited effect (as measured by OIV methods) in  
537 non-sulphited white wines stirred with 1 g/L chitosan for only 12 h (Colangelo et al., 2018).  
538 Despite catechin and epicatechin contents were found relatively low in the studied white  
539 wine (see above), it is possible that the comparable  $A_{420}$  levels shown in Fig 4B versus  
540 those of (+)-catechin added model wine (Fig. 4A) are due to the presence in the real wine  
541 of other compounds abundant in the grape juice, such as *trans*-caftaric and *trans*-coutaric  
542 acids, which, as *ortho*-diphenols, can undergo an oxidative cascade toward pigments  
543 absorbing at the same wavelength. A more obvious explanation could be related to the  
544 presence of (-)-epicatechin in the white wine, which has been consistently found to  
545 produce faster and deeper browning as compared to (+)-catechin when reacting with  
546 glyoxylic acid (Labrouche, Clark, Prenzler, & Scollary, 2005).

547

### 548 3.5 Effect of catechin and CsG on photoproduction of reactive carbonyls in the presence 549 of iron

550 As schematized in Fig. 1 and suggested from the results of Fig. 4B, a complementary  
551 effect of CsG in reducing photo-assisted browning would rely on its known ability to adsorb  
552 on its surface either (+)-catechin, its colorless dimeric intermediates, or yellowish  
553 xanthylium cations (Spagna et al, 1996; Chinnici et al., 2014). The (+)-catechin model for  
554 such equivalent proanthocyanidin reactions was used to investigate the photoproduction  
555 (for two or ten days irradiation) of acetaldehyde and glyoxylic acid in model solution and  
556 (+)-catechin added Chardonnay wine dark pre-incubated (for 2 days) with varying (+)-  
557 catechin and iron concentrations.

558 As expected from literature data (Danilewicz, 2007), stimulating thermal and photo-  
559 Fenton oxidation routes by increasing iron concentration (1–5 mg/L) resulted in a dose-  
560 dependent increase of acetaldehyde and glyoxylic acid photoproduction (for 2 days) in all  
561 (+)-catechin free samples (Fig. 5). In real wine samples kept in darkness for 10 days (dark

562 controls) carbonyls remained close to background values, ranging 8 – 12 mg/L for  
563 acetaldehyde and 1– 2 mg/L for glyoxylic acid. The significant illumination-dependent  
564 increase of unbound carbonyls seen in Fig. 5 was dose-dependently reversed by  
565 incremental addition of (+)-catechin, suggesting that these oxidation products became  
566 involved in condensation reactions, leading to the formation of 8-vinyl-(+)-catechin and  
567 xanthylium cations (Fig. 1; Drinkine et al., 2005). Strikingly, this (+)-catechin dependent  
568 inhibitory effect on acetaldehyde and glyoxylic acid was almost independent from the  
569 amount of added iron, being more marked in model wine solutions. For 100 and 200 mg/L  
570 added (+)-catechin, 2 days-photoproduction for both carbonyls was inhibited by ~30% and  
571 ~50%, respectively in model solutions, and by only ~20% and ~40%, respectively in  
572 experimental wine. Extending the irradiation of the samples up to 10 days confirmed this  
573 lack of impact of iron concentration, with mean inhibitions of ~44% for glyoxylic acid and  
574 ~30% for acetaldehyde in model solutions, and only of ~20% for glyoxylic acid and ~11%  
575 for acetaldehyde in real wine, when 100 mg/L (+)-catechin was added to the samples.  
576 Actually (+)-catechin concentrations available for reacting with free acetaldehyde and  
577 glyoxylic acid in real wine were found lower than in model solutions, likely because of  
578 competition reactions for the flavanol with a variety of other wine components such as  
579 phenols or proteins (Waterhouse & Laurie, 2006). On the other hand, glyoxylic acid rather  
580 than acetaldehyde levels were found best reduced by (+)-catechin at both irradiation times  
581 (not shown at 10 days), consistent with Drinkine and co-workers (2005) who proposed a  
582 higher reactivity of (+)-catechin towards glyoxylic acid due to structural differences (e.g.,  
583 functional groups and polarizability).

584 In order to evaluate the antioxidant contribution of CsG in inhibiting the carbonyls  
585 production seen above, it was added in suspension to the experimental wine at a dose of 2  
586 g/L throughout the experiment. Fig. 5B shows that, in samples unsupplemented with (+)-  
587 catechin and oxidized by 5 mg/L iron(II), acetaldehyde and glyoxylic acid productions after

588 2 days photolysis decreased to concentrations similar or even lower than those obtained  
589 with lower iron contents of 2 and 1 mg/L, respectively. Of the possible mechanisms  
590 responsible for this early effect, metal chelation during pre-incubation could be privileged  
591 (Fig. 3A), although the presence of the polysaccharide during lighting does not rule out the  
592 possibility of direct radical scavenging or adsorption effects such as those implicated in  
593 Fig. 4B. In connection, it was reported that putting a similar real white wine added 5.5 mg/L  
594 ferrous ions in contact with 2 g/L chitosan decreased the metal content by 68% after 2  
595 days in darkness (Castro Marín et al., 2019). Also, for the two (+)-catechin doses tested  
596 here and for 5 mg/L added iron, inhibition by 2 g/L CsG of carbonyls photoproduction was  
597 near constant in the case of glyoxylic acid (about 64%), while, for acetaldehyde, CsG  
598 inhibitory efficacy was higher after the addition of 200 mg/L of the flavanol (Fig. 5B),  
599 suggesting that under these conditions some adsorption of (+)-catechin onto CsG has  
600 begun to occur to protect acetaldehyde levels.

601 To substantiate this hypothesis, residual (+)-catechin contents following addition of  
602 100 mg/L of the flavanol  $\pm$  2 g/L CsG were determined by HPLC in those model and real  
603 wine samples after irradiation for 48 h. As anticipated from Fig. 1, when added iron  
604 increased from 1 to 5 mg/L, (+)-catechin consumption in untreated samples increased from  
605  $12.8 \pm 0.7$  to  $24.8 \pm 1.6\%$  in model solution and from  $9.8 \pm 0.9$  to  $19.9 \pm 2\%$  in real wine.  
606 When the experimental wine was oxidized by 5 m/L of iron, treatment with 2 g/L of CsG  
607 significantly limited this (+)-catechin consumption to only 13% ( $P < 0.05$ ).

608

### 609 3.6 *Effect of CsG on absorption spectra of tartaric, malic and citric acid wine bases in* 610 *presence of iron (III)*

611 Derived from the grapes, the  $\alpha$ -hydroxy acids tartaric, malic and citric acids are among  
612 the main contributors of wine titratable acidity (the two former being the predominant acids  
613 in wine), forming stable  $\alpha$ -carboxylate complexes with Fe(III) at wine pH. However, under



614 fluorescent light irradiation these ferric ion complexes decompose to acyloxyl free radicals,  
615 subsequently releasing ferrous ions to carry on the Fenton wine oxidation. Tartaric acid  
616 derived acyloxyl radicals, but not the parent species from malic and succinic acids, have  
617 been proposed (Grant-Preece et al., 2017a) as unique precursors of glyoxylic acid in the  
618 browning development, and therefore any wine additive preventing the formation of the  
619 [Fe(III)-tartrate] complex would be inhibitory (Fig. 1).

620 To understand if CsG fulfils this role it was reacted for a few minutes at varying  
621 concentrations with dark, pre-formed [Fe(III)-carboxylates] complexes in acidic 12%  
622 ethanolic solutions, and the mixtures were centrifugated before UV-Vis spectrophotometry  
623 was carried out. To ensure for complete reactions over the CsG concentration range used  
624 throughout, ferric ions (20 mg/L), tartaric (5 g/L), malic and citric acid (both at 3 g/L)  
625 concentrations studied here were set in large excess with respect to typical wine-like  
626 values, which are 5 mg/L, 2.7, 2.4, and 0.5 g/L, respectively. Figure 6 shows that, under  
627 these conditions, [Fe(III)-carboxylates] complexes exhibited more or less defined  
628 absorption maxima at 338 (dimer form; Danilewicz, 2014), 330, and 350 nm for tartrate  
629 (blue arrow), malate, and citrate, respectively (Grant-Preece et al., 2017b). These  
630 absorptions were distinct from that given by  $\text{Fe}(\text{OH})^{2+}$  at 297 nm (red arrow in Fig. 6; see  
631 also Fig. 3B), or by CsG alone and each individual acid. CsG (1 or 2 g/L) dose  
632 dependently reduced the optical densities near to baseline (Fig. 6), suggesting that, under  
633 the methodology used, chitosan showed strong inhibitory properties toward the tested iron  
634 complexes. However, it is not clear whether this could be related to a displacement  
635 mechanism of the carboxylates from their iron complexes since no clear UV-Vis evidence  
636 for the formation [Fe(III)-chitosan] complexes as depicted in Fig. 3B was obtained.  
637 Regarding tartrate the discovery of this property of CsG is of obvious importance since it  
638 virtually rules out the red pathway of Fig. 1 for forming glyoxylic acid-derived xanthylum  
639 cations under fluorescent lighting.

640

### 641 **3 Conclusion**

642 In conclusion, this work demonstrates that chitosan decreases the amount of free  
643 aldehydic intermediates related to wine photooxidation of oxygen saturated samples. In a  
644 sulphite-free Chardonnay wine inhibition as high as 80% was found for glyoxylic acid,  
645 while acetaldehyde was somewhat less affected. Particularly at doses > 0.5 g/L, the  
646 presence of chitosan significantly reduced the oxidative browning of wines to a  
647 comparable or even higher extent than did SO<sub>2</sub> at 100 mg/L, especially in extended  
648 oxidative conditions, because of the progressive consumption of sulphites. Notably, the  
649 antioxidant and anti-browning action of chitosan in wine samples submitted to fluorescent  
650 lighting partially persisted after its removal. Apart from aldehyde reduction, this study  
651 gained evidence that, in addition to the phenolic adsorption established in previous works  
652 (Spagna et al., 1996), these effects can be attributed to other complementary mechanisms  
653 such as iron chelation and scavenging of H<sub>2</sub>O<sub>2</sub>. Further, this study demonstrates for the  
654 first time the ability of chitosan to block the regeneration of Fe(II) from tartaric acid by  
655 interacting directly with the [Fe(III)-tartrate] complex intermediate.

656

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661

### 662 **Author contributions**

663 A. Castro Marín and P. Stocker contributed equally to this work.

664

665 **Conflict of interest**

666 The authors declare no competing financial interest.

667

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674

675 **Figure Captions**

676

677 **Figure 1.** General structure of chitosan and inhibitory mechanisms (blue arrows) against  
678 oxidation processes mediated by hydroxyl radical formed by (i) 'thermal' Fenton (pathway  
679 in black), (ii) photo-Fenton (pathway in green), or (iii) upon fluorescent lighting (pathway in  
680 red) in a sulphite-free wine. Key wine constituents and oxidation products or mediators  
681 involved in browning investigated in the study are marked in bold.

682

683 **Figure 2.** Effect of chitosan (CsG) or SO<sub>2</sub> on free acetaldehyde and glyoxylic acid  
684 formation (determined by HPLC-DAD) during fluorescent light irradiation (24–240 h) of 2.5  
685 mg/L Fe(II)-spiked (A) model wine, and (B, C) non-sulphited Chardonnay wine. Samples  
686 were dark pre-incubated for 48 h in the absence (controls) or presence of treatments.  
687 Compounds were assayed in unfiltered samples (A, B) or in samples filtered before  
688 irradiation (C; 'Pt' samples). Red bars refer to dark pre-incubated controls maintained in  
689 darkness for: (B) 0 h, (C) 24 h (full) or 48 h (empty). One-way ANOVA followed by Duncan

690 test: \* $P < 0.01$  vs. corresponding dark control, \* $P < 0.02$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs.  
691 control samples at the same time of light exposure; # $P < 0.01$  and ## $P < 0.001$  vs. SO<sub>2</sub> (all  
692 at any doses and at the same time of light exposure). Values are means  $\pm$  SD ( $n = 3-5$ ).

693

694 **Figure 3.** Antioxidant properties of chitosan (CsG) related to some determinants of the  
695 thermal Fenton mechanism of wine oxidation in darkness. (A) Decrease of iron content in  
696 SO<sub>2</sub>-free Chardonnay wine spiked with 5 mg/L Fe(II). Pi-CsG samples: wine was pre-  
697 incubated with CsG for 48 h, then iron was added and analysis occurred after 48 h  
698 additional incubation. +CsG samples: wine was incubated alone for 48 h, then CsG and  
699 iron were added simultaneously and analysis occurred after 48 h. Data are percent of the  
700 corresponding CsG free controls; (B) Absorbance spectra recorded in filtered samples 10  
701 min after addition of CsG to 12% ethanolic solutions (pH 3.2) pre-incubated with FeCl<sub>3</sub> (10  
702 mg/L) for 1 h. Arrow marks Fe(OH)<sup>2+</sup> absorption at 297 nm; (C) H<sub>2</sub>O<sub>2</sub> inhibition by CsG (by  
703 lucigenin chemiluminescence) in model wine solutions spiked with H<sub>2</sub>O<sub>2</sub>. Percent inhibition  
704 refers to corresponding initial H<sub>2</sub>O<sub>2</sub> concentrations given on top. One-way ANOVA followed  
705 by Duncan test: \*\* $P < 0.01$  vs. CsG at the same concentration. Values are means  $\pm$  SD ( $n$   
706 = 3-5).

707

708 **Figure 4.** Development of browning (absorbance at 420 nm) during fluorescent light  
709 irradiation at 20 °C of model and non-sulphited wines spiked with 2.5 mg/L Fe(II) and  
710 effect of treatments. (A) Model wine containing 100 mg/L (+)-catechin. (B) SO<sub>2</sub>-free  
711 Chardonnay wine. Samples were pre-incubated for 2 days in darkness and treatments  
712 were applied throughout the experiment. One-way ANOVA followed by Duncan test. \* $P <$   
713 0.05, \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. untreated control wines at the same time of light  
714 exposure; # $P < 0.01$  and ## $P < 0.001$  vs. SO<sub>2</sub> (all at any doses and at the same time of light

715 exposure);  $^{\S}P < 0.001$  vs.  $\text{SO}_2$  at doses 25 and 50 mg/L. Values are means  $\pm$  SD ( $n = 3$ –  
716 5).

717

718 **Figure 5.** Effect of varying (+)-catechin and iron(II) concentrations on photooxidation (48  
719 h) induced free acetaldehyde and glyoxylic acid levels in (A) model wine solutions, and (B)  
720  $\text{SO}_2$ -free Chardonnay wine. Solutions were dark pre-incubated for 48 h before illumination.  
721 Treatment with 2 g/L CsG in panel B was applied in 5 mg/L iron-spiked Chardonnay wine.  
722 One-way ANOVA followed by Duncan test:  $^*P < 0.05$ ,  $^{**}P < 0.01$  vs. (+)-catechin  
723 unsupplemented controls at the same dose of iron;  $^{\#}P < 0.01$  vs. iron(II) at 5 mg/L at the  
724 corresponding dose of (+)-catechin. Values are means  $\pm$  SD ( $n = 3$ –5).

725

726 **Figure 6.** UV-Vis absorption spectra showing interaction of chitosan (CsG) with [Fe(III)-  
727 carboxylates] complexes from (A) tartaric acid (5 g/L), (B) malic acid (3 g/L), and (C)  
728 succinic acid (3 g/L). Complexes were formed in acidified 12% ethanolic solution (pH 3.2)  
729 by stirring acids with  $\text{FeCl}_3$  (20 mg/L) for 1 h in darkness at 20 °C. In inhibition experiments  
730 spectra were recorded from centrifugated suspensions sampled a few min after addition of  
731 varying CsG doses to the mixtures. Arrows indicate typical maximum absorptions.

732

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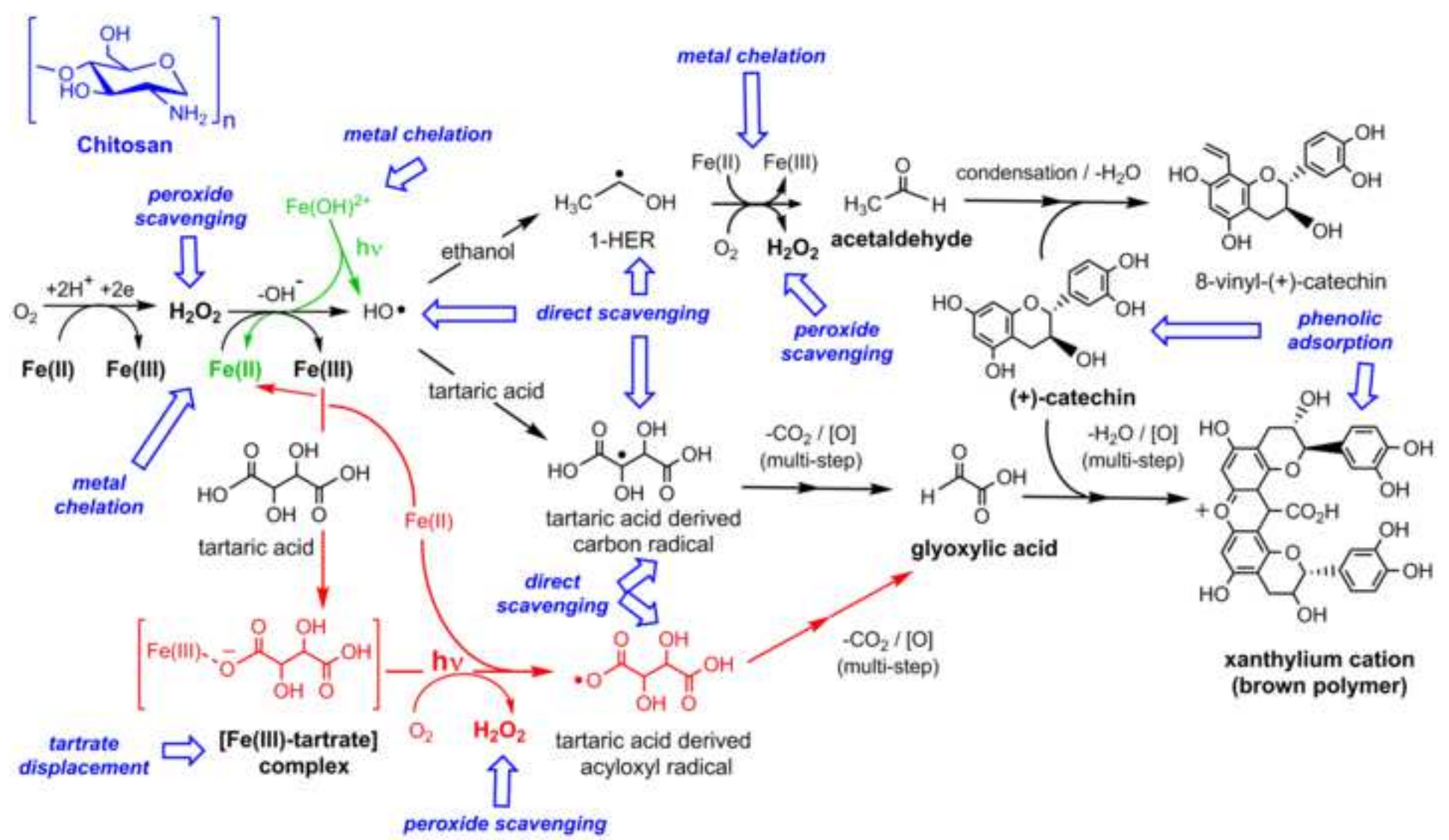


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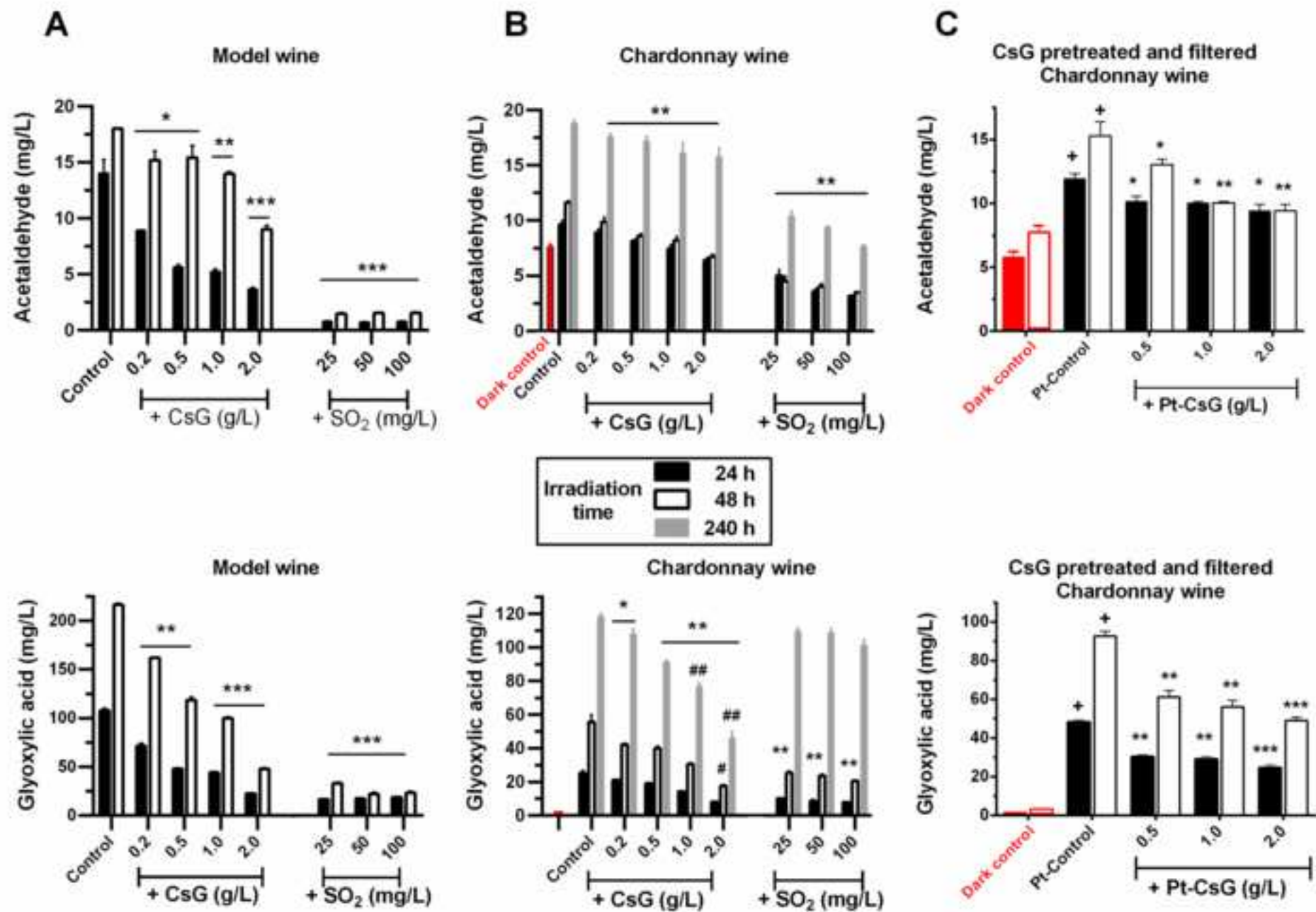


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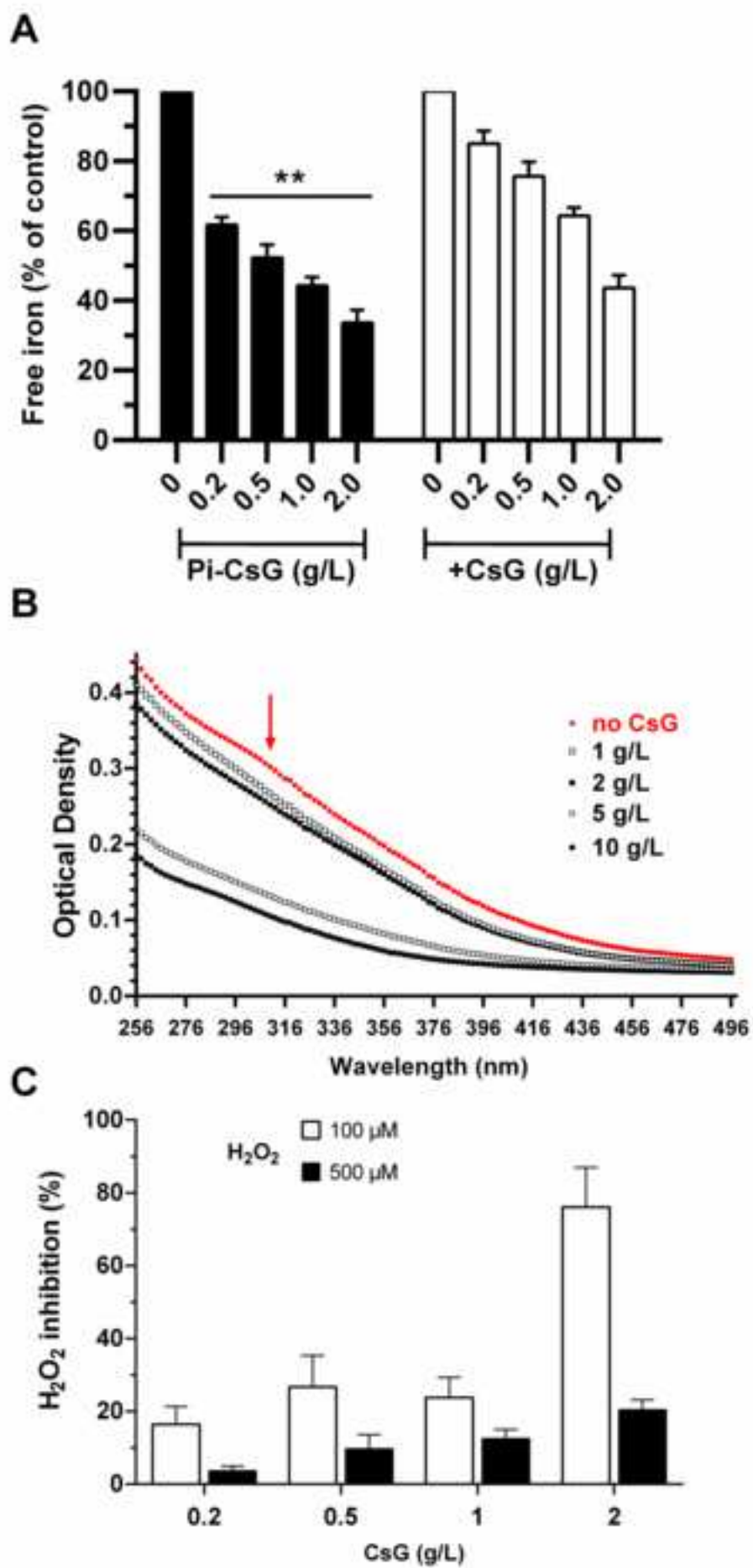




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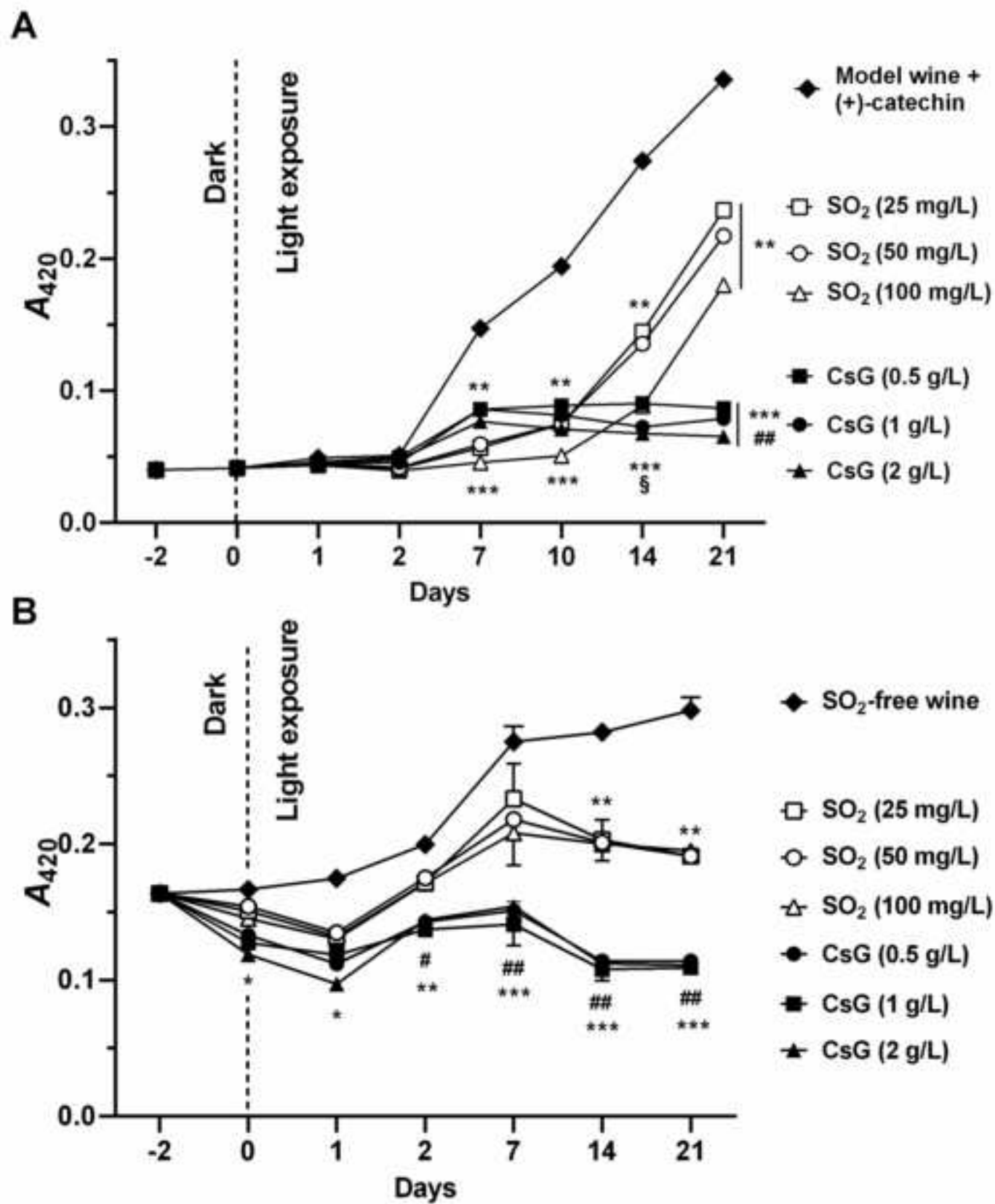


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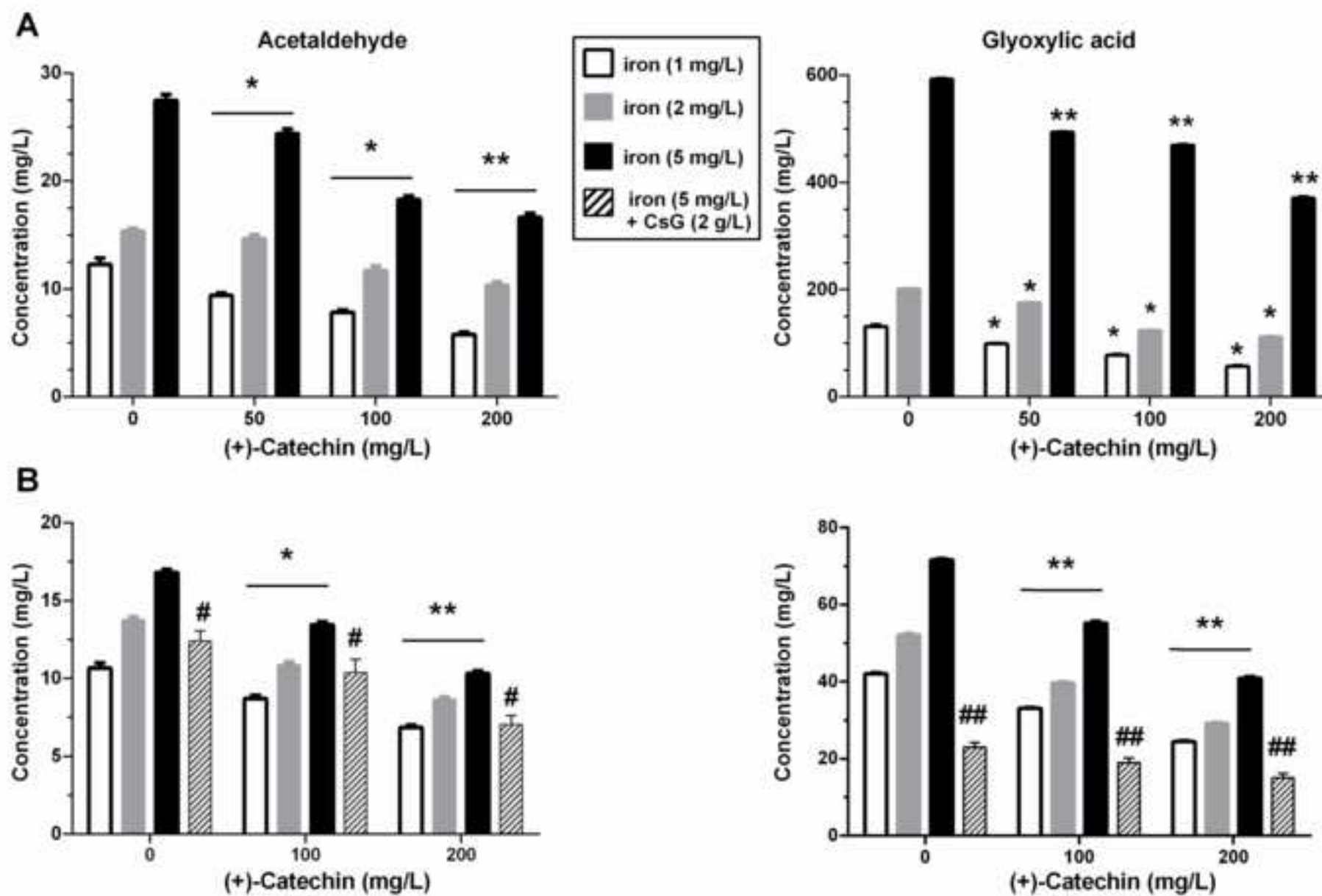
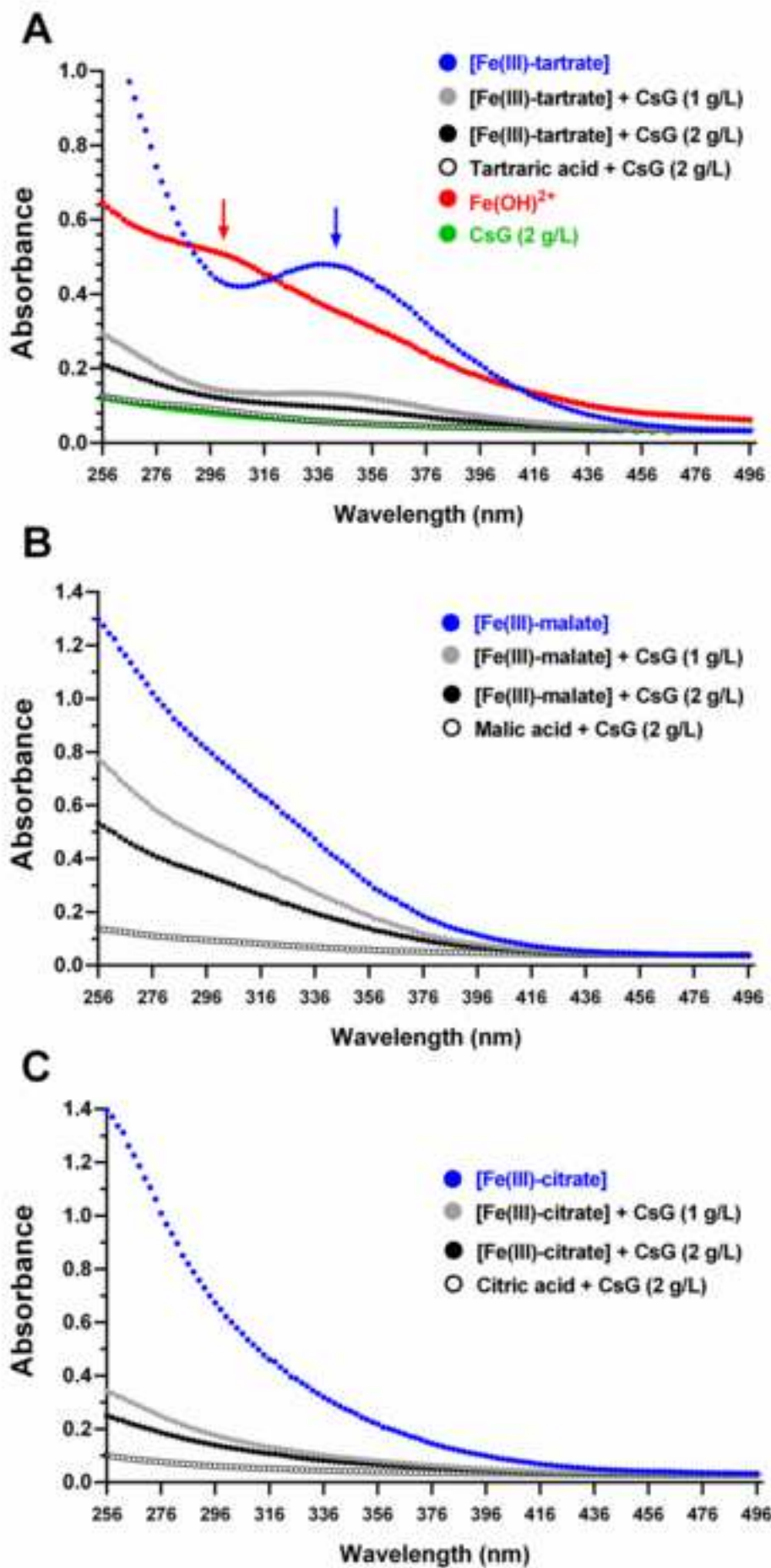


Figure 6

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1. Fungoid chitosan reduces the generation of aldehydes during wine photo-oxidation
2. Chitosan reduces iron amounts in solution by adsorbing [carboxylates-Fe(III)] complexes
3. In extended oxidative conditions SO<sub>2</sub> is a poorer wine anti-browning agent than chitosan
4. Sulphites better control free acetaldehyde but not glyoxylic acid amounts
5. In wines chitosan can mitigate the browning while preserving catechin amounts

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: