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

Antonio Castro Marin^{a,b}, Pierre Stocker^a, Fabio Chinnici^b, Mathieu Cassien^{a,c},
Sophie Thétiot-Laurent^a, Claudio Riponi^b, Bertrand Robillard^d, Marcel Culcasi^{a*}
and Sylvia Pietri^a

Addresses:

^aAix Marseille Univ, CNRS, ICR, Marseille, France.

^bDepartment of Agricultural and Food Sciences, University of Bologna, Bologna, Italy.

^cYelen Analytics, Ensues-la-Redonne, France.

^dInstitut Œnologique de Champagne, Epernay, France.

Corresponding author: Marcel Culcasi, PhD

Institut de Chimie Radicalaire, Sondes Moléculaires en Biologie et Stress Oxydant,

Aix Marseille Université, CNRS UMR 7273

Centre Scientifique de Saint-Jérôme, Avenue Escadrille Normandie Niemen, Service 522

13397 Marseille cedex 13, France

Tel: 00 33 (0)4 91 28 90 25

email: marcel.culcasi@univ-amu.fr

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 SO₂ 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 must 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 ($HO\bullet$) are formed (Waterhouse & Laurie, 2006;
64 Oliveira, Ferreira, De Freitas, & Silva, 2011; Danilewicz, 2012). The most conclusive proof
65 of $HO\bullet$ 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 $HO\bullet$ 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 xanthylum 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 (SO₂) is widely used in oenology for its global antioxidant
90 power, acting upstream (by scavenging H₂O₂ 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 SO₂ 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 SO₂-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 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 SO₂ treatments and doses, or (ii) target compounds
151 acetaldehyde, glyoxylic acid, H₂O₂, 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 N₂ 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 SO₂, < 5 mg/L, and total SO₂ < 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), H₂O₂, ethanol, FeSO₄ (as
172 ferrous sulphate heptahydrate), FeCl₃ (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 FeSO₄ (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), SO₂ (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- μ 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 μ L) were
221 mixed with 200 μ 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- μ 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 μ 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 μ m; Macherey Nagel)
238 and a gradient with a flow rate of 0.8 mL/min. Gradient was: solvent A (0.05% H_3PO_4 in
239 H_2O , 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- μ 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

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

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

The iron(III) chelating activity of CsG was evaluated in acidified ethanolic solutions (12% v/v, pH 3.2). Samples (20 mL) loaded with 10 mg/L FeCl₃ were dark incubated for 1 h at 20 °C. Varying concentrations of CsG (1–10 g/L) were added thereafter under vigorous stirring. After an additional 5 min incubation the suspensions were centrifuged (2400g) for 5 min, 200 µL of each supernatant was transferred in 96-well microplates and absorbance was recorded using a microplate reader (Tecan Infinite, Männedorf, Switzerland). Experiments were made in triplicate.

264

2.7. Assay of H₂O₂

The amount of unreacted H₂O₂ following addition at 20 °C of CsG in H₂O₂ spiked model wine was measured by lucigenin chemiluminescence (Maskiewicz, Sogah, & Bruice, 1979). Samples (20 mL) containing H₂O₂ (100 or 500 µM) without (controls) or with added CsG (0.2–2 g/L) were placed in centrifuge plastic tubes and were dark incubated for 10 min at 20 °C. The mixture was filtered (0.45 µm nylon filter) and aliquots (20 µL) were mixed with 200 µL of a lucigenin solution (0.2 g/L in 0.2 M glycine buffer, pH 10) and dark incubated for 10 min at 20 °C in 96-well microplates before chemiluminescence was read. Blank values were measured without H₂O₂. The decrease in H₂O₂ content of CsG-treated samples was calculated as a percentage of the signal given by the corresponding control prepared as described above taken as 100%. Concentration of H₂O₂ was derived from a calibration curve (10–500 µM range) recorded in another set of lucigenin containing samples dark incubated for 10 min. Establishing the calibration curve showed that pre-

incubation and filtration steps in control samples resulted in a negligible loss of H₂O₂. All measurements were made in triplicate.

2.8. Browning analysis

The effect of CsG and SO₂ on the susceptibility to browning was assessed in 2.5 mg/L iron(II)-spiked, dark pre-incubated, then irradiated model wine solution or white wine samples prepared and processed as described above for acetaldehyde and glyoxylic acid measurements (see section 2.3), except that model wine samples were also added (+)-catechin (100 mg/L) before dark pre-incubation. Irradiated samples were timely filtered (0.45 µm nylon filter) and browning development was monitored at room temperature for 21 days at 420 nm by adapting the protocol of Sioumis et al. (2006) to a microplate reader. Experiments were made in triplicate.

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

[Fe(III)-carboxylates] complexes were prepared in triplicate in 50-mL Falcon conical centrifuge plastic tubes as previously described (Grant-Preece et al., 2017b). Briefly, freshly prepared mixtures of FeCl₃ (20 mg/L) were stirred for 1 h at 20 °C in the dark with solutions of either tartaric (5 g/L), malic or citric acids (3 g/L) in acidified ethanolic solutions (12% v/v, pH 3.2). Afterwards varying concentrations of CsG (1–2 g/L) were added under stirring and the mixtures were reacted in darkness a few min. Then, the mixtures were centrifugated for 5 min (2400g), 200 µL of each supernatant was transferred into 96-well microplates and the absorption was read between 256 and 496 nm at 20 °C.

2.10. Statistical analysis

Unless otherwise noted all data were analysed and presented as means ± SD for the indicated number of independent experiments. Intergroup differences were calculated by 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 SO₂ 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

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

For all the suspensions of CsG added up to 1 g/L, the level of free acetaldehyde and glyoxylic acid was dose-dependently and significantly reduced at any time of light exposure when compared to the untreated controls (Fig. 2A and B). For model wine containing 2 g/L CsG this decrease was strong for both glyoxylic acid (22.8 ± 0.2 mg/L, i.e., 79% decrease) and acetaldehyde (3.7 ± 0.7 mg/L, i.e., 77% decrease) contents after 1 day of irradiation, but illuminating the mixtures for one additional day led to a better protection for the glyoxylic acid (78% decrease) versus acetaldehyde (50% decrease) levels. A different pattern was observed in illuminated, SO_2 -added model wine samples, where a stronger inhibition of both free glyoxylic acid (ranging 91–94%) and acetaldehyde (ranging 84–89%) levels was observed at 24 h and 48 h at any dose of the additive (Fig. 2A). In those samples, these reductions were considerable even at the lowest SO_2 dosage

likely due to its ability to both scavenge H_2O_2 and strongly bind aldehydes (Oliveira et al., 2011). Indeed, the apparent equilibrium constants for sulphite-aldehyde adducts were estimated to be 3.7×10^{-6} and 1.5×10^{-6} for glyoxylic acid and acetaldehyde, respectively (Sonni et al., 2011b), suggesting that a noticeable portion of those aldehydes may have been present in the bound form (Grant-Preece et al., 2017a).

Next, the progressive inhibitions of fluorescent lighting-induced build-up of acetaldehyde and glyoxylic acid seen above upon adding CsG or SO_2 were confirmed in 2.5 mg/L iron(II) added sulphite-free Chardonnay wine, again with a better effect on glyoxylic acid versus acetaldehyde levels. For example, a dose of 2 g/L CsG in suspension significantly lowered glyoxylic acid formation from 55 ± 2 mg/L to 17.6 ± 0.3 mg/L after 48 h irradiation, and such protection was even significantly better than that provided by adding SO_2 up to 100 mg/L instead of CsG (Fig. 2B). Interestingly, after 10 days of lighting the glyoxylic acid content markedly increased in all wine samples but 0.5 g/L CsG, a concentration below the **maximum authorized dose** for winemaking, guaranteed a significantly better control of this glyoxylic acid generation when compared to any dose of SO_2 (Fig. 2B). When wine samples were kept in contact with 1–2 g/L CsG over the 10-days irradiation time, glyoxylic acid content was decreased by up to 65% compared to controls or SO_2 -treated samples. **This dramatic increase might be due to an early and almost complete consumption of sulphur dioxide, e.g., by oxidation with H_2O_2 generated by thermal Fenton pathway (Fig. 1), making insufficient its potential to bind glyoxylic acid at day 10.** Figure 2B also shows an opposite situation was observed for acetaldehyde levels which were significantly best inhibited by SO_2 (maximum value of 60% at day 10) than by CsG (only 23% inhibition at 2 g/L), and this acetaldehyde versus glyoxylic acid difference may be the reflection of the better binder behaviour of the former toward SO_2 (as their 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- μ 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 SO₂ 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; Gyliéné,
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 $\text{Fe}(\text{OH})^{2+}$ (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_2O_2

459 Maybe the most efficient intervention to prevent photooxidation of wines is to limit or
460 suppress the continuous formation of H_2O_2 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_2 is prone to interrupt thermal Fenton
464 reactions by directly scavenging H_2O_2 to yield sulfate (Danilewicz, 2007, 2012;
465 Waterhouse & Laurie, 2006). The mechanism of the reaction of H_2O_2 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_2O_2 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_2O_2 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_2O_2 in the samples,
476 respectively. Based on EPR data on model wine solutions estimating that total H_2O_2 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_2O_2
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 SO₂ under the conditions of Fig. 2B. According to the mechanism of formation of (+)-
492 catechin derived xanthylum 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 SO₂ 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 SO₂,
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 xanthylum 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 SO₂ will interact faster at any relevant step of the
517 browning pathways, e.g., by reducing H₂O₂, 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 SO₂ 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 SO₂ samples, with no clear dose dependency for both
530 treatments. After 21 days lighting, mean A₄₂₀ values in CsG samples decreased by 63%
531 and 43% compared to controls and samples added 100 mg/L SO₂, respectively. It is
532 possible that this relatively poor effect of SO₂ 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 have 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 (Labrousche, 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

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

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

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

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

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

Derived from the grapes, the α -hydroxy acids tartaric, malic and citric acids are among the main contributors of wine titratable acidity (the two former being the predominant acids in wine), forming stable α -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 SO₂ 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 H₂O₂. 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 SO₂ 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_2 (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_2 -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_3 (10
702 mg/L) for 1 h. Arrow marks $\text{Fe}(\text{OH})^{2+}$ absorption at 297 nm; (C) H_2O_2 inhibition by CsG (by
703 lucigenin chemiluminescence) in model wine solutions spiked with H_2O_2 . Percent inhibition
704 refers to corresponding initial H_2O_2 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_2 -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_2 (all at any doses and at the same time of light

715 exposure); ^s*P* < 0.001 vs. SO₂ at doses 25 and 50 mg/L. Values are means ± 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 SO₂-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 ± 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 FeCl₃ (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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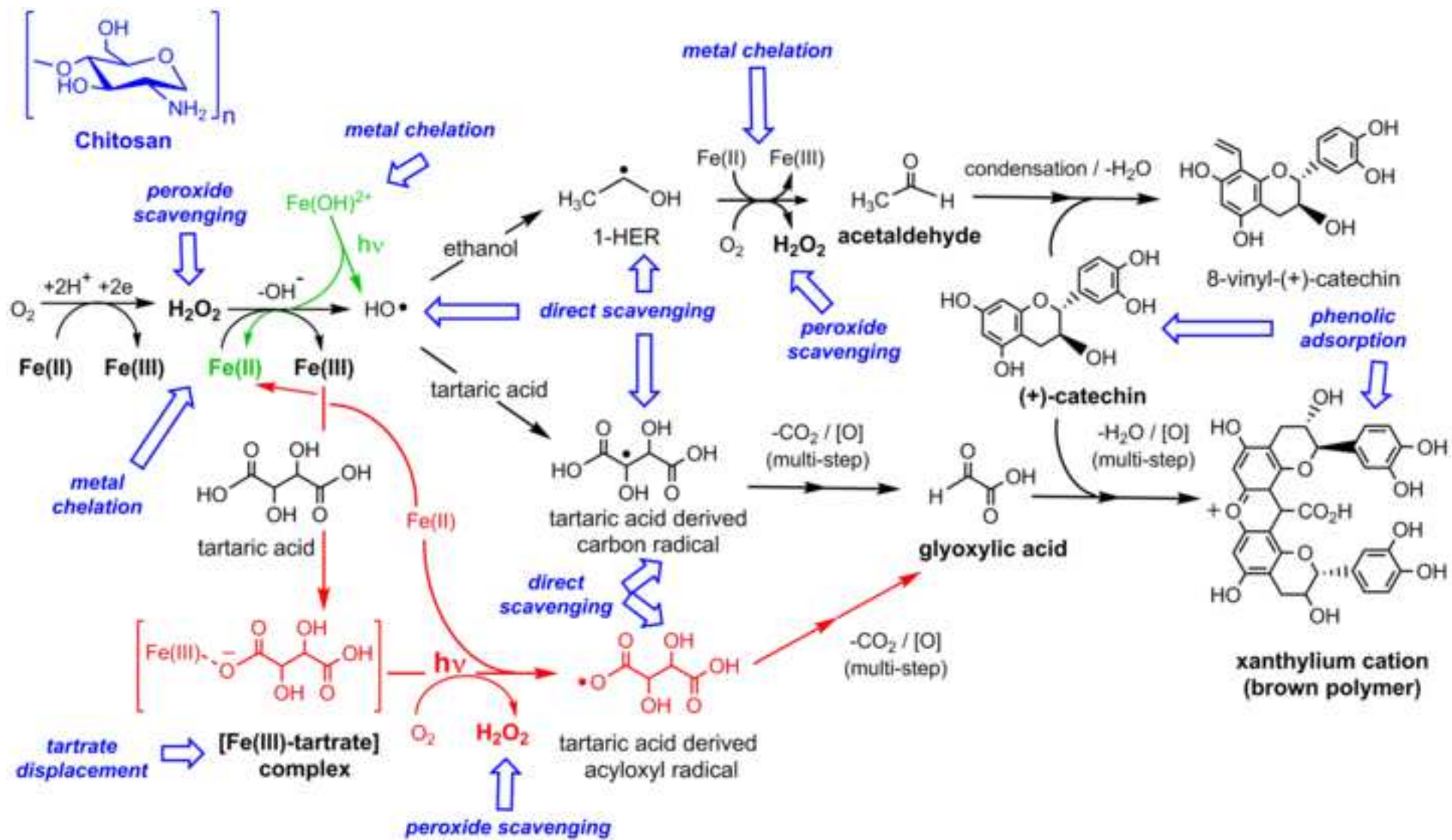


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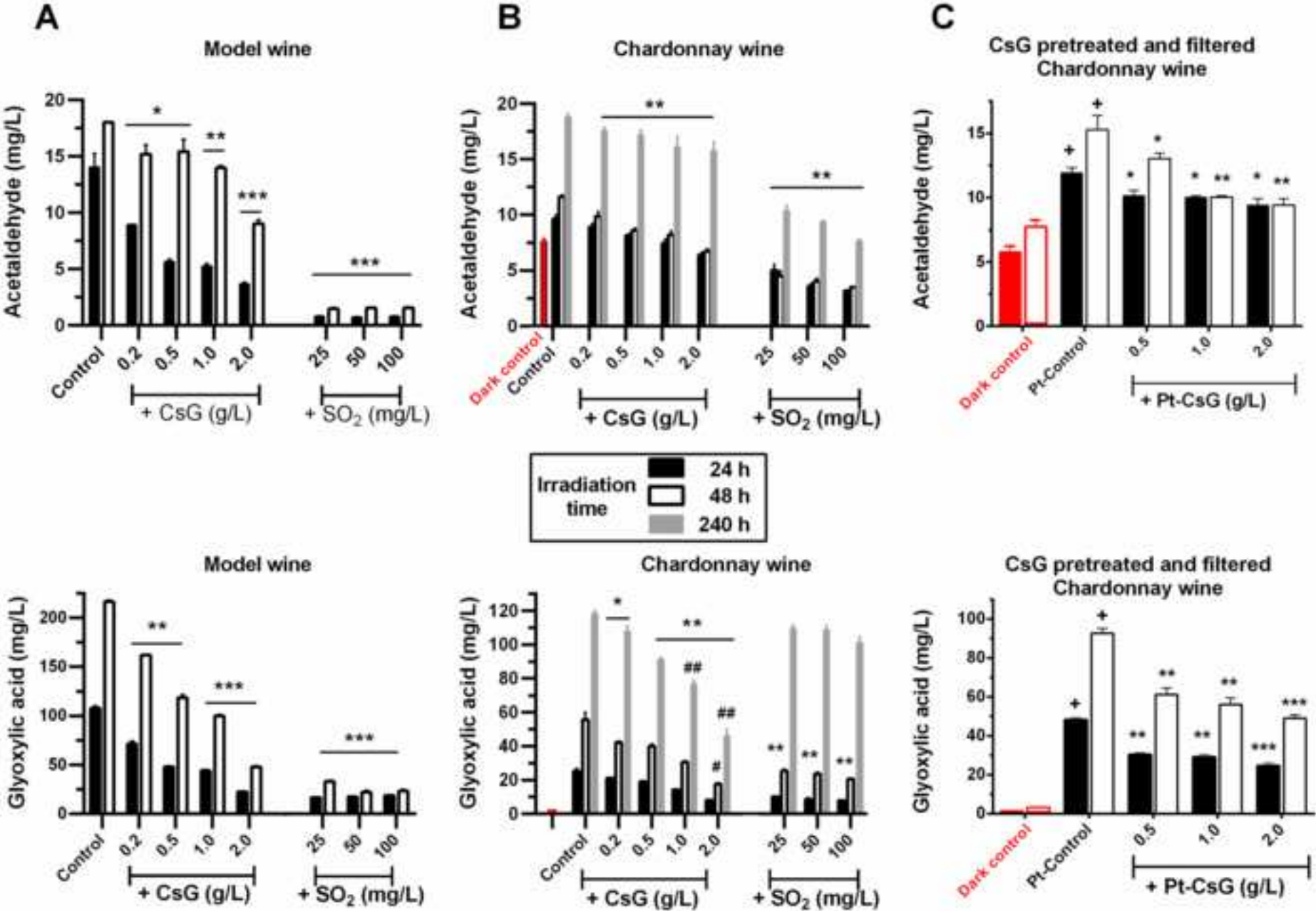


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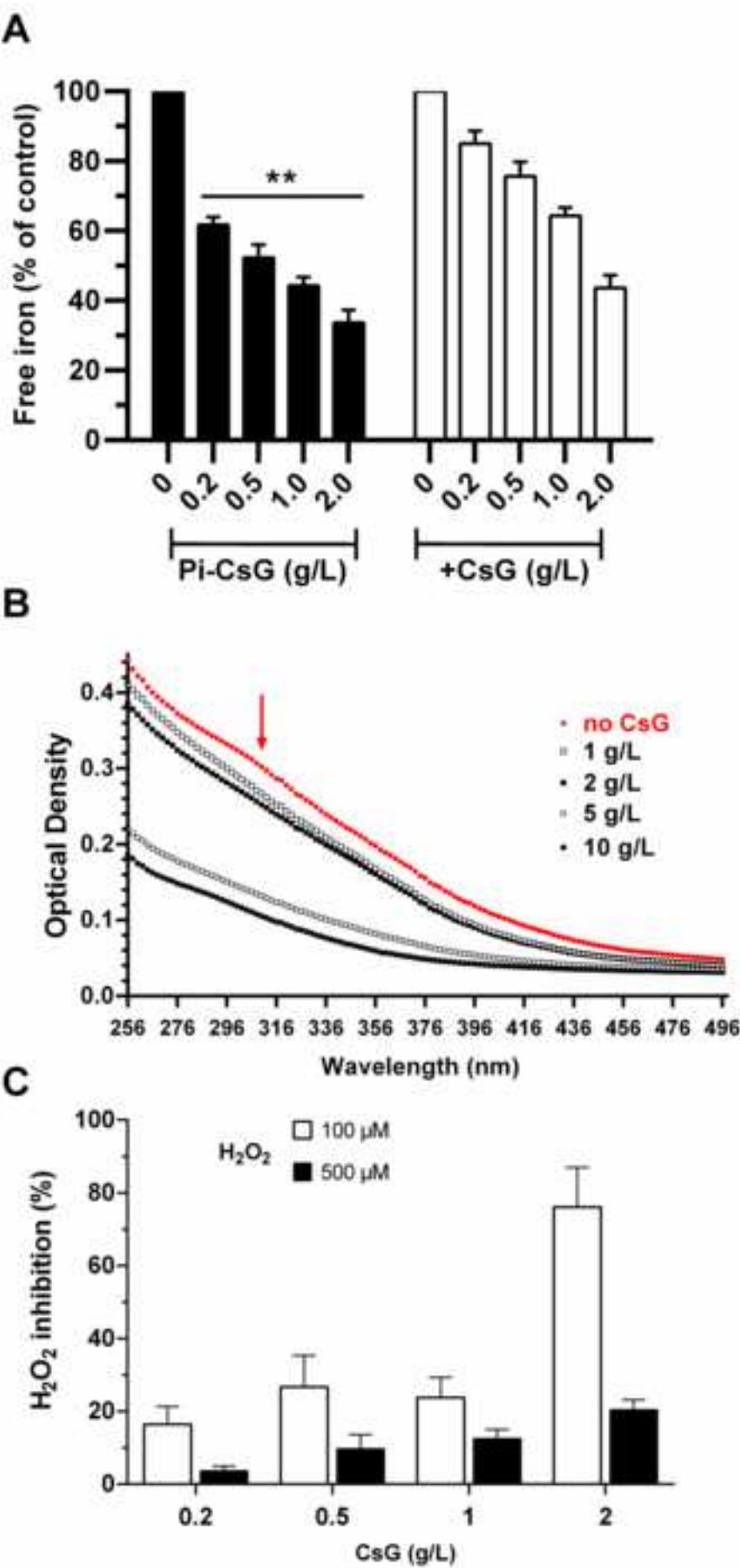


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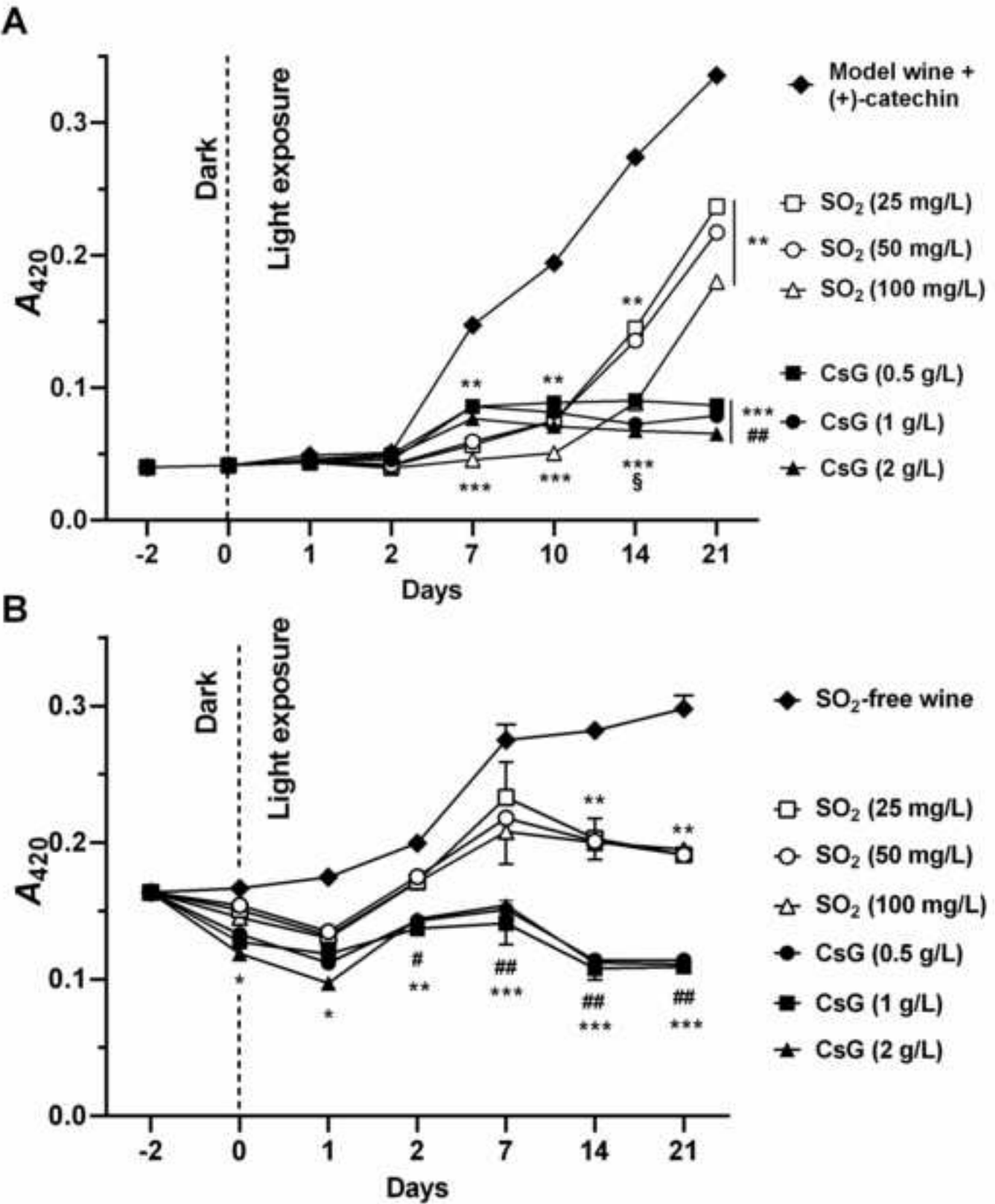


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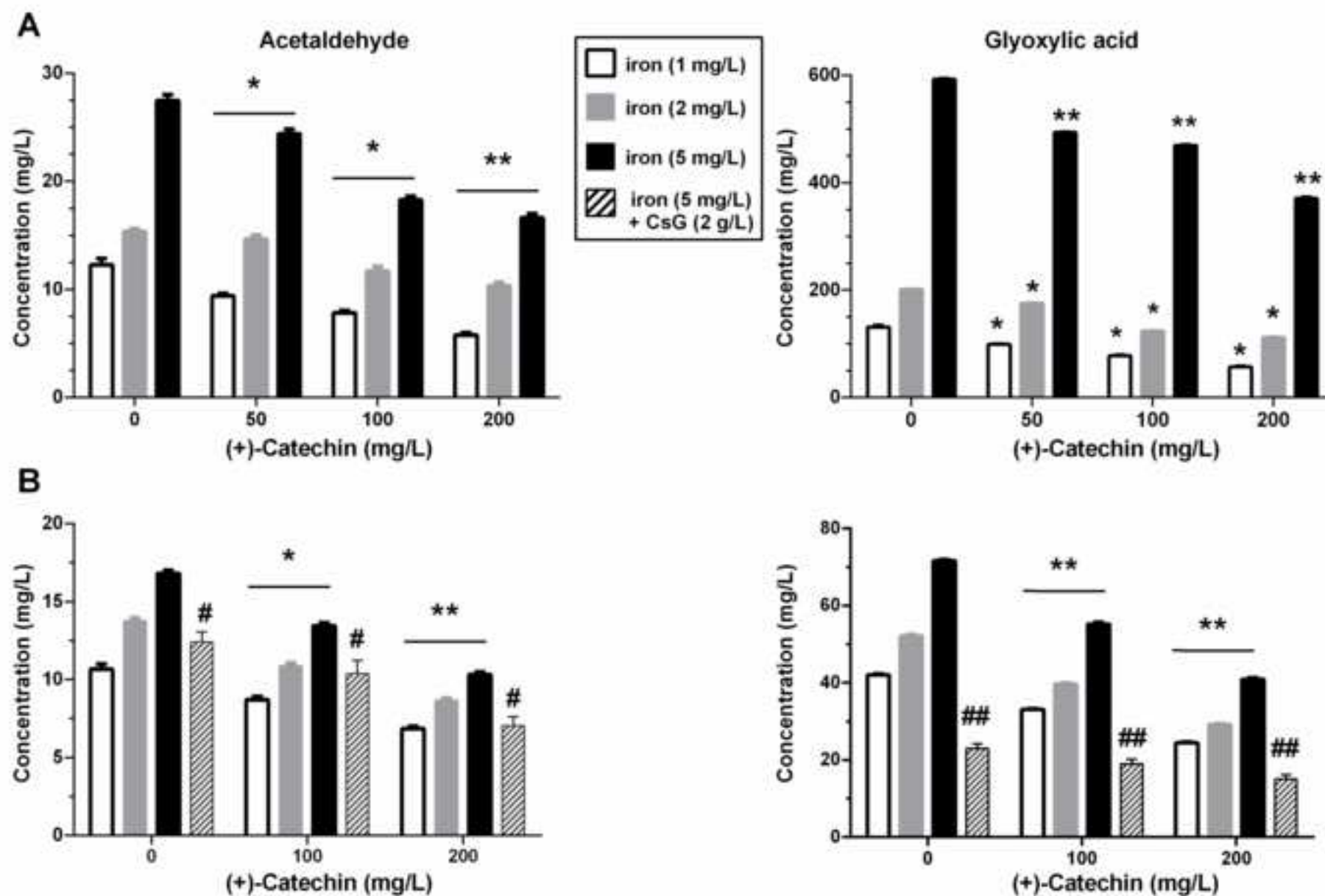
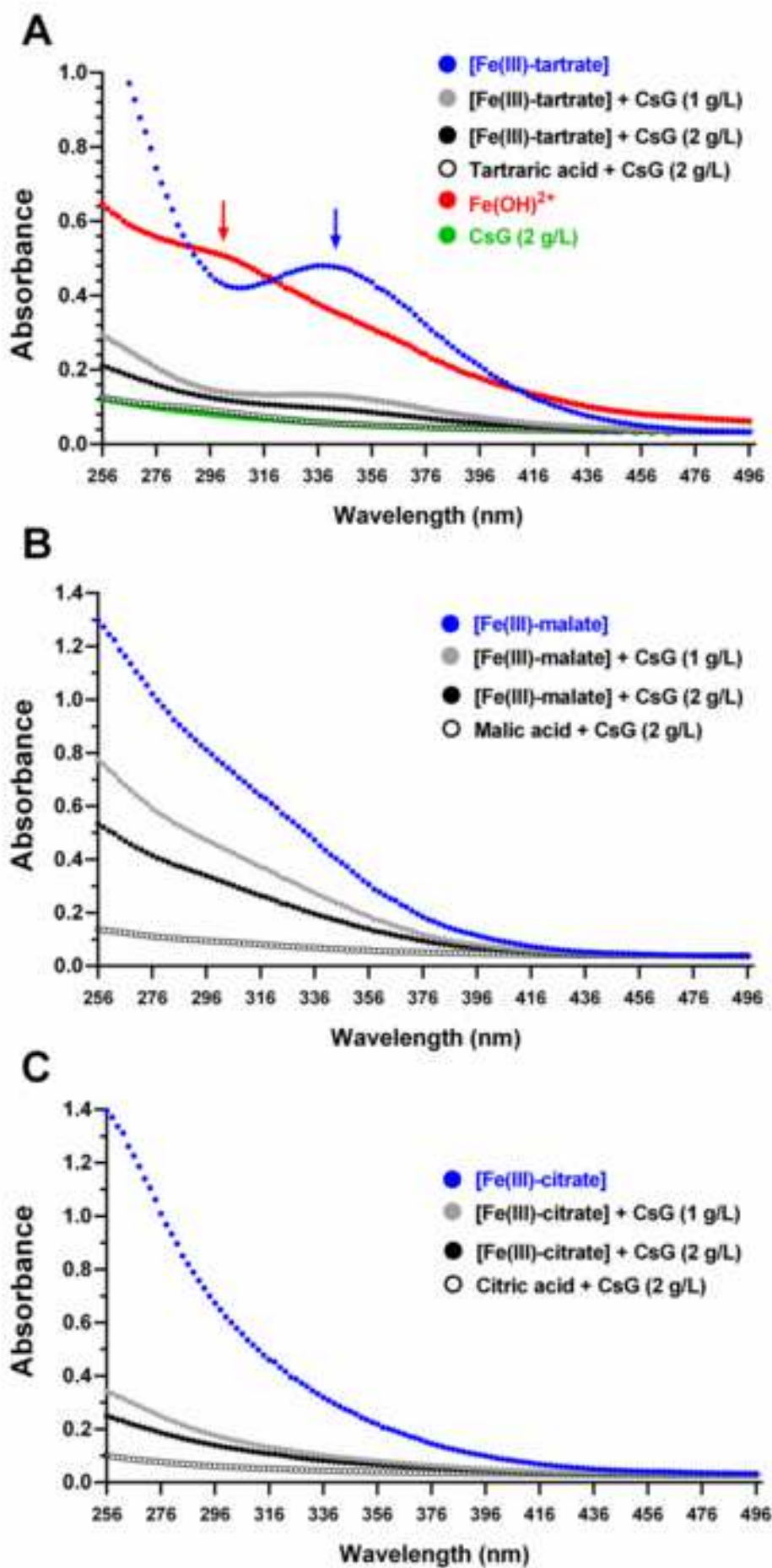


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1. Fungoid chitosan reduces the generation of aldehydes during wine photo-oxidation
2. Chitosan reduces iron amounts in solution by adsorbing [carboxylates-Fe(III)] complexes
3. In extended oxidative conditions SO₂ is a poorer wine anti-browning agent 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: