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1 **Direct NMR evidence for the dissociation of sulfur-dioxide-bound acetaldehyde**
2 **under acidic conditions: Impact on wines oxidative stability**

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16 **Abstract**

17 SO₂ reaction with electrophilic species present in wine, including in particular
18 carbonyl compounds, is responsible for the reduction of its protective effect during
19 wine aging. In the present study, direct ¹H NMR profiling used to monitor the
20 reactivity of SO₂ with acetaldehyde under wine-like oxidation conditions. The
21 dissociation of acetaldehyde bound SO₂ was evidenced suggesting that released free
22 SO₂ can further act as an antioxidant. EPR and DPPH assays showed an increasing
23 antioxidant capacity of wine with the increase in the concentration of acetaldehyde
24 sulfonate. The presence of acetaldehyde sulfonate in wines was correlated with the
25 overall antioxidant activity of wines. The first evidence of acetaldehyde bound SO₂
26 dissociation provides a completely new representation of the long-term protection
27 efficiency of SO₂ during bottle aging.

28 **KEYWORDS:** antioxidant activity, white wine, oxidation, chardonnay, aldehydes

29

30 **1. Introduction**

31 Sulfite is usually the most common preservative that is widely used in wine
32 production due to its antioxidant, antimicrobial, and anti-enzymatic properties (Li,
33 Guo, et al., 2008). However, due to existing health concerns, there is a current trend to
34 limit its use and to look for alternatives (Castro Marín, Culcasi, et al., 2019;
35 Kontaxakis, Trantas, et al., 2020). Yet, in situations where the microbial stability is
36 achieved through various chemical and/or physical processes, the main issue about
37 shelf-life matter remains the chemical stability against chemical oxidation. This is
38 particularly true for wines, where the high added value relies on their aging potential.
39 In a context of restrictive regulations about additives in food and wine, a deeper
40 understanding of the chemistry of the widely used sulfites is required, in order to be
41 able to reduce reliably their use and find efficient alternatives.

42 When added to wine, SO₂ reaches an equilibrium involving free forms, including
43 molecular SO₂ and the bisulfite anion HSO₃⁻, and bound forms, which result from the
44 reaction of HSO₃⁻ with a diversity of wine components, including aldehydes, ketonic
45 acids, sugars, sulfur-containing compounds, acids (Arapitsas, Guella, et al., 2018;
46 Jackowetz & Mira de Orduña, 2013; Roullier-Gall, Hemmler, et al., 2017). Enology
47 had acknowledged for decades that free bisulfite and weakly bound forms are actually
48 assuming protection against oxidation, whereas strongly bound SO₂ consider to be
49 lost for wine preservation (Ribereau-Gayon, Doneche, et al., 2006). Among the
50 various binding compounds, acetaldehyde is considered as the strongest binder,
51 through the electrophilic character of its carbonyl group, with a dissociation
52 equilibrium constant for its sulfonate as low as 1.5×10^{-6} at pH=3.3 (Burroughs &
53 Sparks, 1973). Acetaldehyde has been extensively studied in wine since it is a major
54 carbonyl compound that is formed upon alcoholic fermentation, but which can further

55 result from chemical oxidation of ethanol promoted by oxygen exposure during aging.
56 Acetaldehyde concentrations are usually higher in white wines than in red wines, with
57 values of the order of 80 mg/L and 30 mg/L, respectively (Jackowetz & Mira de
58 Orduña, 2013; Liu & Pilone, 2000). As 98% of acetaldehyde will bind SO₂ within 90
59 minutes, it has been considered that in the presence of 30mg/L of free SO₂, only
60 0.04% of acetaldehyde will remain in the free form (Coetzee, Buica, et al., 2018).

61 The first step of oxidation involves the oxidation of an Fe (II)- tartrate complex to Fe
62 (III)-O₂, which then reacts with o-diphenols. The final product of oxidation are the
63 quinones, form polymerization products (brown pigments) that cause the browning of
64 wine (Li, et al., 2008). Danilewicz and Standing (2018) have proposed a mechanism
65 by which sulfites will react with quinones to restore catechol and to protect the wine
66 from oxidation. Consequently, an accurate evaluation of the antioxidant activity of
67 wines as well as the identification of the wine components that contribute to their
68 oxidative stability, is necessary. Numerous methods have been developed for the
69 evaluation of the antioxidant activity of wines, especially spectrophotometric ones.
70 DPPH (2,2-Diphenyl-1-picrylhydrazyl) is widely used as well, for the rapid and easy
71 determination of the radical scavenging properties of compounds and wines. This
72 colored radical is stable and when it reacts, the purple colour changes to yellow and
73 the absorbance can be measured at 525nm. A recent study from our research group
74 has shown the complementary results that DPPH assay and Electron Paramagnetic
75 Resonance spectroscopy (EPR) provide, for the better understanding of the correlation
76 between the antioxidant capacity of wines and their chemical composition by
77 targeting essentially sulfur containing compounds (R. Romanet, Sarhane, et al., 2021).
78 EPR spin trapping approach has been successfully used for the analysis of wines
79 oxidative mechanisms (Elias, Andersen, et al., 2009). Nikolantonaki *et al* (2019)

80 proposed a more effective way to use EPR spectroscopy for the determination of the
81 antioxidant activity of wines and to classify them according to their resistance to
82 oxidation by modeling the kinetic of 1-hydroxyethyl radical formation after Fenton
83 based initiation of the chemical oxidation.

84 In this study, the antioxidant capacity associated with acetaldehyde sulfonate in model
85 and real white wines had been investigated by DPPH and EPR measurements,
86 combined with high-resolution LC-MS-Q-ToF molecular profiling. Direct Nuclear
87 Paramagnetic Resonance (^1H NMR) analysis has been used for quantifying the rate of
88 production of the sulfonate and for deciphering the possible mechanisms responsible
89 for the antioxidant capacity of acetaldehyde sulfonate.

90 **2. Materials and methods**

91 **2.1 Chemicals**

92 The water used in this study was ultrapure water (18.2 M Ω , Millipore, Germany).
93 Ethanol and methanol were purchased from Honeywell (United states) and were used
94 for the preparation of DPPH solution. Acetaldehyde, ethanol MS grade (>99.8%),
95 tartaric acid, citric acid, sodium phosphate, alpha-(4-pyridyl-1-oxide)-N-tert-
96 butylnitrone (POBN), 2,2-Diphenyl 1-picrylhydrazyl (DPPH), trimethylsilyl
97 propionic acid sodium salt (TSP) and 4-methylcatechol have been purchased from
98 Sigma-Aldrich (St. Louis, MO, USA). Fe (II) sulfate heptahydrate was from Carlo
99 Erba (Milan, Italy) reagents. Na₂S₂O₅ was from Merck (Darmstadt, Germany).
100 Sodium hydroxide and hydrogen peroxide were from ChemLab, methanol (MS grade)
101 and acetonitrile (MS grade) were purchased from Biosolve Chimie (Dieuze, France)
102 and D₂O from euriso-top (Saarbrücken, Germany).

103 **2.2 Synthesis of acetaldehyde sulfonate**

104 A model wine solution was prepared freshly by adding 12% (v/v) of ethanol and 5g/L
105 of tartaric acid in ultrapure water. The pH has been adjusted to 3.5, with drops of 10%
106 w/v NaOH solution. For the synthesis of acetaldehyde sulfonate, acetaldehyde (1mM)
107 and SO₂ have been added in model wine solution in a molar ratio of 1/1 (**solution a**)
108 and 2/1 (**solution a'**). The yield of each reaction has been monitored by ¹H NMR
109 spectroscopy.

110 **2.3 Wine samples**

111 18 freshly made Chardonnay wines from the region of Burgundy, France, were used
112 for the spike addition experiments with 50 and 100mg/L of SO₂. Wines were stored in
113 dark place at room temperature for a week in order to achieve equilibrium before EPR
114 spin trapping and DPPH analysis.

115 **2.4 Solutions for simulation of wines chemical oxidation conditions in presence of** 116 **acetaldehyde sulfonate**

117 4-methylcatechol (4mM), acetaldehyde sulfonate (1 mM) (**solution a**) and Fe (II)
118 (0.44mM) have been added to a model wine solution in excess of oxygen (7.68mg/L).
119 The device for measuring the oxygen was NOMASense O2 P6000 and the sensor type
120 was SP-PSt3-NAU-D5-CAF. The yield of the reaction has been monitored by ¹H
121 NMR spectroscopy. There was no control in this experiment, as it is already known
122 that 4-methylcatechol would be oxidized in the absence of a source of SO₂.

123 **2.5 Acquisition of NMR spectra and apparatus**

124 NMR experiments were performed on a Bruker 600 MHz spectrometer using a
125 cryoprobe at 298 K. Samples were mixed with 100μL of D₂O that contained 0.1% of
126 TSP. TSP has been used as an internal standard for quantification. ¹H NMR spectra
127 were obtained by using WET multiple solvent suppression experiment for

128 simultaneous suppression of water and ethanol proton peaks. 2D NMR spectra have
129 been performed for the identification of all forms of acetaldehyde (free, hydrated and
130 bound). The 2D acquisition parameters for the COSY experiment were as follows:
131 The standard cosygpprqf Bruker pulse sequence was used, with a spectral width of
132 9014.4 Hz (F2) and 9003.4 Hz (F1), the acquisition time was 0.11 sec, 2K(t2) × 256
133 (t1) data points, 64 scans and 16 dummy scans. Acquisition parameters for the ¹H–¹³C
134 HSQC experiment were as follows: The hsqcedetgpcisp2.3 Bruker pulse sequence,
135 which also incorporates DEPT editing of the signals based on carbon multiplicity
136 (methine, methylene, methyl) was used, with a spectral width of 9615.3 Hz in the F2
137 dimension and 24904.9 Hz in the F1 dimension. The acquisition time was 0.106 sec,
138 2K (t2) × 256 (t1) data points, 96 scans and 16 dummy scans. Acquisition parameters
139 for the ¹H–¹³C HMBC experiment were as follows: The hmbcgpplndqf Bruker pulse
140 sequence was used, with a spectral width 7812.5Hz in the F2 dimension and 33207.4
141 Hz in the F1 dimension. Acquisition time was 0.262 sec, 4K (t2) × 128 (t1) data
142 points, 128 scans and 16 dummy scans. All NMR data were processed using Topspin
143 4.0.6 NMR (Bruker) software.

144 **2.6 Optimized DPPH assay**

145 Optimized DPPH assay for sulfur containing compounds was carried out based on the
146 protocol proposed by Romanet *et al* (Remy Romanet, christian, et al., 2019). 10 mL
147 of DPPH (0.63 mM in methanol) and 90 mL of citrate/phosphate buffer (12.5 mM
148 Na₂HPO₄ and 14.6 mM citric acid were dissolved in an H₂O: Methanol (4:5 v/v)
149 solution) were added in a 100 mL amber volumetric flask. The ultrapure water was
150 degassed for 2 minutes using argon (Ar) gas. In the case of wine samples, they were
151 degassed with CO₂ for 15 minutes right before the analysis to ensure the lack of
152 residual free SO₂ as Pegram *et al* have proposed (2013). After the experimental

153 procedure and letting the samples for four hours in a dark place, EC₂₀ (the quantity of
154 the antioxidant needed to scavenge 20% of DPPH radical) has been calculated
155 spectrophotometrically at 525nm using 10 mm QS absorption cells. The control used
156 in model wine was the model wine without addition of any compound (Control a),
157 while the control in the wine experiments was the white wine before the addition of
158 acetaldehyde sulfonate (Control b).

159 **2.7 Acquisition of EPR spectra and apparatus**

160 EPR analysis was carried out based on the protocol proposed by Romanet et al.
161 (2021). FeSO₄·7H₂O (0.072 mM) and H₂O₂ (0.215 mM) were used as a source of
162 hydroxyl radicals and 29.9 mM of POBN solution as a spin trap. Finally, each sample
163 was added to achieve a final volume of 1mL. EPR measurements were performed
164 using an EMXnano EPR spectrometer (Bruker, Germany). The parameters used for
165 the experiments were as follows: modulation frequency 100 kHz, modulation
166 amplitude 1.500 G, time constant 1.28 ms, conversion time 15 ms, attenuation 10 dB,
167 and receiver gain 40. Spectra were recorded at room temperature (298 K). Serial 2-
168 min EPR acquisitions were performed by using the function 2D FieldDelay. SpinFit
169 has been used for the quantification of the EPR signal. The SpinFit parameters that
170 have been used were diameter 0.8 mm, center 125 mm, and length 25 mm. Model
171 wines were analyzed daily to ensure repeatability of the experimental conditions and
172 the radical oxidation. To increase repeatability day-to-day, the intensity of POBN-1-
173 HER adducts in model wine and Chardonnay wine samples was normalized by the
174 maximal intensity obtained from model wine. For data analysis, the maximum amount
175 of radical produced (C_{max}), the time to reach the maximum amount of radical formed
176 (T_{Max}), and the initial slope of the kinetic curve (1st Slope) were used as parameters
177 to evaluate the antioxidant capacity. The control used in model wine was the model

178 wine without addition of any compound (Control a), while the control in the wine
179 experiments was the white wine before the addition of acetaldehyde sulfonate
180 (Control b).

181 **2.8 UPLC-MS-Q-ToF analysis**

182 MS targeted analysis of acetaldehyde sulfonate in white wine samples has been
183 performed using an ultra-high-pressure liquid chromatography (Dionex Ultimate
184 3000, ThermoFischer) coupled to a MaXis plus MQ ESI-Q-TOF mass spectrometer
185 (Bruker, Bremen, Germany). The column used was an Acquity BEH C18 1.7 μ m, 100
186 x 2.1 mm by Waters (Guyancourt, France) in reverse phase to analyze non-polar
187 compounds. The mobile phase was (A) acidified water with 5% of acetonitrile (0.1%
188 v/v of formic acid) and (B) acidified acetonitrile (0.1% v/v of formic acid). The
189 temperature of elution was 40°C using the gradient: 0-1.10 min 5% (v/v) of B and
190 95% (v/v) of B at 6.40 min. The flow was 400 μ L/min. The negative ionization takes
191 place in electrospray. End plate offset (500V) and capillary voltage (4500V) permit
192 the ions transfer. Before each analysis batch, the mass spectrometer was calibrated
193 using Na Formate in enhanced quadratic mode. The mass range was between 100 and
194 600 m/z in negative ionization mode. Quality controls were analyzed before and
195 throughout each batch, to verify the stability of the LC-MS system. All samples were
196 analyzed randomly. Detection was carried out in negative ionization mode with the
197 following parameters: Nebulizer pressure = 3.0 bar, dry gas flow = 10.0 l/min, dry gas
198 temperature = 200 °C, capillary voltage = 4500 V, end plate off set= 500 V.

199 **2.9 Data analysis**

200 All experiments were performed at least in triplicate and if not specified, results were
201 expressed as average \pm standard deviation for the triplicate. Non parametric t-student

202 rank sum test and spearman test were used for median comparison and correlation
203 estimation, respectively.

204 **3. Results and Discussion**

205 **3.1 ¹H NMR Monitoring of acetaldehyde sulfonation reaction in model wine**

206 ¹H NMR methodology was used for directly investigating acetaldehyde interaction
207 with sulfur dioxide under wine like acidic conditions. The presence of 12% of ethanol
208 as well as the excess of water limited the sensitivity of 1D ¹H NMR to low
209 concentrations substances as the intensities of both water and ethanol signals obscured
210 some other signals. In that respect WET experiment was applied, which allowed their
211 suppression. The ¹H NMR method was used to determine the yield of sulfonation
212 reaction of acetaldehyde in different ratios under wine-like acidic conditions at room
213 temperature (298 K). ¹H NMR spectra for both ratios are presented in **Figure 1**, while
214 2D NMR spectra of acetaldehyde sulfonate in white wine are presented in
215 supplementary information (**Figures S2, S3 and S4**).

216 When acetaldehyde was dissolved in model wine, the hydrated form (¹H: (-CH) 5.23
217 ppm, q / ¹³C: 88.80 ppm and (CH₃) ¹H: 1.31 ppm, d / ¹³C: 23.84 ppm)) was also
218 produced. After the addition of bisulfite, the quadruplet from the -CH group (¹H: 9.66
219 ppm, q / ¹³C: 207.19 ppm) and the doublet of the -CH₃ (¹H: 2.23 ppm, d / ¹³C: 30.75
220 ppm) group of the free acetaldehyde are protected and shifted to a higher field region
221 at 4.54 ppm (q) (¹³C: 80.99 ppm) and 1.46 ppm (d) (¹³C: 17.56 ppm) respectively.

222 The yield of acetaldehyde sulfonation reaction was calculated for the molar ratios 1/1
223 and 2/1 (Acetaldehyde / HSO₃) (**Figure 2**). The sulfonation reaction of acetaldehyde
224 was fast and almost complete (yield 95% ± 5%), in concordance with already reported
225 results (Cassino, Tsolakis, et al., 2021; Nikolantonaki, Magiatis, et al., 2015). Since

226 the reaction is almost complete, in the ratio 1/1 we observe no and minimal signal of
227 free and hydrated acetaldehyde respectively.

228 **3.2 Global evaluation of acetaldehyde sulfonate importance on model and real** 229 **white wine antioxidant activity**

230 The antioxidant activity of free and sulfonated acetaldehyde has been evaluated by
231 combined optimized DPPH assay and EPR spin trapping spectroscopy first in model
232 wine and second in white wine matrices. According to Romanet *et al* (2021) these
233 methods show good complementarity and affinity with sulfur containing compounds.
234 Based on the same study, the maximum amount of POBN-1-HER radical produced
235 (C_{max}); the time to reach the maximum amount of radical formed (T_{max}); the initial
236 slope of the kinetic curve (1st Slope) and the concentration of the compound that is
237 required to scavenge 20% of DPPH initial concentration (EC₂₀), derived analytical
238 parameters from EPR spin trapping and optimized DPPH assay, respectively, were
239 used to compare samples. According to our analytical approach, the lower the values
240 of C_{max}, T_{max}, 1st slope and EC₂₀, the higher the antioxidant activity.

241 **Table 1** shows the antioxidant capacity results of free and sulfonated acetaldehyde
242 compared to catechin which was used as a reference compound presenting high
243 antioxidant capacity (Remy Romanet, et al., 2019). The EC₂₀ value was also estimated
244 for free SO₂ in order to use it as a negative control. DPPH results put in evidence for
245 the first time that sulfonated acetaldehyde has an antioxidant capacity compared to
246 free acetaldehyde which showed no response. EC₂₀ values of free SO₂ and bound to
247 acetaldehyde were estimated at 0.18 and 0.15, respectively. These results after
248 conversion to the concentration of each substrate reacted with DPPH radical, assume
249 the release of 4.17×10^{-4} M of free SO₂ which corresponds to 83% of sulfone

250 dissociation after reaction with 6×10^{-6} M of DPPH. Our results are contradictory to
251 those published by Danilewich *et al.* (2016) where no reactivity of SO_2 was shown
252 with DPPH. This difference can be explained by the use of DPPH (1.59×10^{-4} M) in
253 concentration 300 times higher compared to our experimental conditions, which lead
254 to the no-linear decrease of DPPH during the time of reaction. In comparison with a
255 very good antioxidant compound as catechin, sulfonated acetaldehyde appeared not to
256 be a waste while presenting five times lower antioxidant capacity of that reported for
257 catechin (Remy Romanet, et al., 2019). The use of EPR spin trapping analysis
258 confirmed the overmentioned result and put in light that sulfonated products have
259 greater hydroxyl radical scavenging activity than polyphenols (due to the fact that
260 catechin generates radicals in presence of ROS (reactive oxygen species) and acts
261 both as antioxidant and prooxidant (Caro, Davis, et al., 2019), resulting in lower
262 responses in C_{max} , T_{max} and slope.

263 Acetaldehyde- HSO_3 was added to a white wine in order to test the dependence of
264 DPPH decrease and 1-hydroxyethyl radical production on its concentration. When
265 acetaldehyde sulfonate was spiked in white wine using increasing concentrations
266 (5×10^{-4} M, 10^{-3} M, 5×10^{-3} M and 10^{-2} M), DPPH and EPR spin trapping analyses at
267 room temperature confirmed its antioxidant activity observed in model wine
268 solutions. DPPH results were subjected to a hierarchical linear model in order to
269 detect significant differences among compounds considering repeated measurements.
270 The student test (t-test) for all the wine samples has shown that there is a difference
271 between samples with different concentrations of acetaldehyde sulfonate (p -values
272 below 0.02), while the two wine samples with concentration of acetaldehyde sulfonate
273 5×10^{-4} M and 10^{-3} M have shown that there is no difference between them, since p -
274 value = 0.28.

275 In conclusion, the antioxidant effect of acetaldehyde-HSO₃ in wines was modulated to
276 a concentration dependent manner. Going from the lowest (5x10⁻⁴M) to the highest
277 (10⁻²M) concentration, the antioxidant capacity of the wines increases in a significant
278 manner (t-student, p<0.01). Moreover, EPR spin trapping results showed that at high
279 concentration (10⁻²M) acetaldehyde-HSO₃ inhibited completely the 1-hydroxyethyl
280 radical formation. The rate of spin adduct degradation may have exceeded speed
281 adduct formation in 10⁻²M treatments, however, which may explain this result. These
282 results lead us to the conclusion that acetaldehyde sulfonate has a major role on wine
283 oxidative stability indirectly by the release of SO₂ under wine oxidation conditions as
284 proved in section 3.4. Moreover, in a context of restrictive regulations about sulfite
285 additions in wine, these results could enable to reduce reliably sulfite use for wines
286 oxidative stability.

287 **3.3 Identification of acetaldehyde-HSO₃ in wine samples and its correlation with** 288 **wines antioxidant capacity**

289 Complementary to existing data on the antioxidant activity of SO₂ released from
290 acetaldehyde sulfonate, the radical scavenging properties as well as the molecular
291 profile of eighteen Chardonnay samples, have been combined in a spearman
292 correlation analysis. DPPH and EPR analyses give information about the antioxidant
293 activity of wines, while LC-MS analysis provides us instant wines molecular
294 fingerprints. Targeted analysis of the data has been acquired and the area of the peak
295 of acetaldehyde sulfonate has been calculated. The spectrum is presented in **Figure**
296 **S1**, where the retention time is 0.72 min and the exact $m/z = 124.9917$. The
297 identification was confirmed after comparison with the standard compound and the
298 isotopic profile.

299 **Figure 3** shows the antioxidant capacity results as well as the content of
300 acetaldehyde-HSO₃ of 18 wine samples before (control) and after spiking in 50 and
301 100 mg/L SO₂. These results were subjected to a statistical analysis in order to detect
302 significant differences among the treatments considering repeated measurements and
303 biological duplicates. Acetaldehyde-HSO₃ was identified only in 7 out of 18 wine
304 samples and in lowest intensity compared to both 50 and 100 mg/L SO₂ treatments. It
305 is important to mention that free SO₂ is removed before sampling according to the
306 applied protocols (Nikolantonaki, et al., 2019; Remy Romanet, et al., 2019) in order
307 to avoid artefacts. In that respect, considering the complex chemical environment of
308 wine matrixes, the sulfonation reaction of acetaldehyde occurred rapidly after the
309 addition of sulfites for the two considered concentrations. Wine's antioxidant capacity
310 was positively related to sulfonation reactions for both treatments. However,
311 significant differences among treatments (Control, 50 and 100 mg/L SO₂) were
312 observed only when EPR spin trapping method was used. Optimised DPPH assay was
313 not significantly discriminant for the low (50 mg/L SO₂) concentration of sulfites.
314 These results agree with those obtained in model wine and put in evidence the
315 importance of using complementary analytical methods in order to study the
316 antioxidant capacity of complex matrices.

317 In order to investigate the direct impact of the presence of acetaldehyde-HSO₃ on
318 wines global antioxidant capacity, the association between each pair of estimated
319 variables (EC₂₀, C_{max}, T_{max} and slope) with the relative intensity of the detected
320 sulfonate was analysed using Spearman's rank-order correlation coefficients (*r*). This
321 revealed significant anti-correlation of acetaldehyde-HSO₃ with C_{max} (*r* = -0.542,
322 *p*<0.001) and the slope (*r* = -0.872, *p*<0.0001) indicating a great contribution of
323 acetaldehyde sulfonate on wines general antioxidant capacity monitored by EPR spin

324 trapping analysis. On the other hand, in the present study, no significant correlation
325 was established between the acetaldehyde-HSO₃ intensity and EC₂₀ and Tmax.
326 However, Cmax was positively correlated with Tmax (r = 0.874, p<0.001) and slope
327 (r = 0.528, p<0.000), while Tmax was positively correlated with EC₂₀ (r = 0.468,
328 p=0.002). These results agree with the observations made by Romanet et al. (2021)
329 who proposed for the first time the modelization of white wines antioxidant capacity
330 by coupling optimized DPPH assay and EPR spin trapping analysis, based on the
331 different antioxidant mechanisms measured in each. Based on the same study, among
332 the 365 identified molecular markers of wines with high antioxidant capacity, 32%
333 were CHO compounds including phenolics and sugar compounds, 20% were CHOS
334 compounds and 36% were CHONS compounds. This diversity is due to the different
335 antioxidant mechanisms studied with hydrogen atom or electron transfer identified
336 through DPPH and phenolic regeneration, nucleophilic properties and 1-HER trapping
337 properties identified through EPR analysis. We understand so that sulfonation
338 reaction occurring during winemaking and aging (Arapitsas, et al., 2018) is a
339 mechanism related to wines antioxidant capacity, and the present study is an addition
340 to the current knowledge on sulfonation reactions and a direct proof of the
341 dissociation of bound SO₂ to acetaldehyde.

342 **3.4 Mechanistic understanding of acetaldehyde sulfonate suggested antioxidant** 343 **activity**

344 In order to provide a mechanistic approach of the antioxidant activity of acetaldehyde
345 sulfonate, we simulated wine's oxidation conditions by monitoring a Fenton-like
346 reaction (excess of oxygen and Fe (II)), in the presence of 4-methylcatechol and
347 acetaldehyde sulfonate, expecting the observation of the decrease concentration of
348 acetaldehyde sulfonate and the presence of free acetaldehyde. The reaction has been

349 monitored by ^1H NMR spectroscopy. The table of peak assignments is presented in
350 **Table S1**. As it is observed in **Figure 4**, the first week of reaction, there is a slight
351 appearance of free acetaldehyde and the concentration of 4-methylcatechol remains
352 stable. Additionally, in the first two weeks, the concentration of 4-methylcatechol
353 remains quite stable, while acetaldehyde sulfonate's concentration has decreased more
354 than a half. These observations lead to the conclusion that at the first step of
355 oxidation, quinone consumes the available free SO_2 (yield of sulfonated acetaldehyde:
356 $95\% \pm 5\%$, so $<10\%$ of SO_2 remains in free form) and then, the equilibrium between
357 free and bound acetaldehyde should be re-established by the dissociation of the bound
358 form and the release of SO_2 . Thus, the most of the initially bound SO_2 is used in order
359 to restore catechol from the quinone. As Nikolantonaki and Waterhouse (2012) have
360 shown, 89% of quinone is reduced back to catechol, while 11% forms three quinone-
361 SO_2 adducts in ratio 5:1:2. Danilewicz had previously observed that sulfites or other
362 good nucleophiles are necessary to have rapid oxygen consumption (Danilewicz,
363 2011). This could be explained by the fact that the oxidation of o-diphenols to
364 quinone is thermodynamically unfavorable, and nucleophiles are needed to drive the
365 reaction to completion (Danilewicz, 2013). The current study appears to show that the
366 small amount of free SO_2 formed from acetaldehyde sulfonate, facilitates this
367 pathway.

368 From week 2 until week 4, free and bound acetaldehyde remain stable, due to the
369 competition between acetaldehyde and quinone for the free/available SO_2 . This is an
370 indication that reinforces the belief that acetaldehyde is a strong binder. The next
371 weeks, SO_2 release is slower than at the beginning of the reaction. Thus, the
372 concentration of 4-methylcatechol starts to decrease, due to oxidation, while a part of
373 SO_2 will form three different sulfonated adducts of quinone (Nikolantonaki &

374 Waterhouse, 2012). It is concluded that during slow oxidation, acetaldehyde releases
375 the bound SO₂ in order to restore the catechol from quinone (Danilewicz & Standing,
376 2018). These results could explain the decrease of the amount of total SO₂ bound
377 fraction during wine aging as observed by Sacks *et al* (2020) and indicates that
378 strongly bound SO₂ is not a waste but it contributes on wines resistance against
379 oxidation during bottle aging.

380 **4. Conclusions**

381 Combined direct ¹H NMR kinetic monitoring, DPPH assay and EPR spectroscopy
382 under wine oxidation conditions supported the more recent studies on the dissociation
383 of acetaldehyde sulfonate and showed that this adduct has antioxidant properties in
384 contrast to previous studies claiming the opposite (Ribereau-Gayon, et al., 2006). We
385 have provided a direct proof that acetaldehyde sulfonate can be dissociated under
386 wine oxidation conditions, with the concomitant release of free SO₂ which can act as
387 antioxidant by restoring 4-methylcatechol. These results could possibly enhance the
388 knowledge on the compounds contributing to wine's oxidative stability and initiate
389 the reconsideration of SO₂ management in wine production.

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396

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468

469 **Figure and table captions**

470 **Figure 1:** Assignments of 1D ^1H NMR signals for quantification of acetaldehyde
471 sulfonation reaction products after 1h of reaction, in 1/1 and 2/1 (Acetaldehyde/ HSO_3)
472 molar ratios, in model wine solution at pH 3.5 and at room temperature (298 K).
473 Protons used for quantification are indicated in bold for each compound.

474 **Figure 2:** Average free, hydrated, and sulfonated acetaldehyde concentrations during
475 the sulfonation reaction between acetaldehyde and sulfur dioxide, in 1/1 and 2/1
476 molar ratios, in model wine solution at pH 3.5 and at room temperature (298 K).

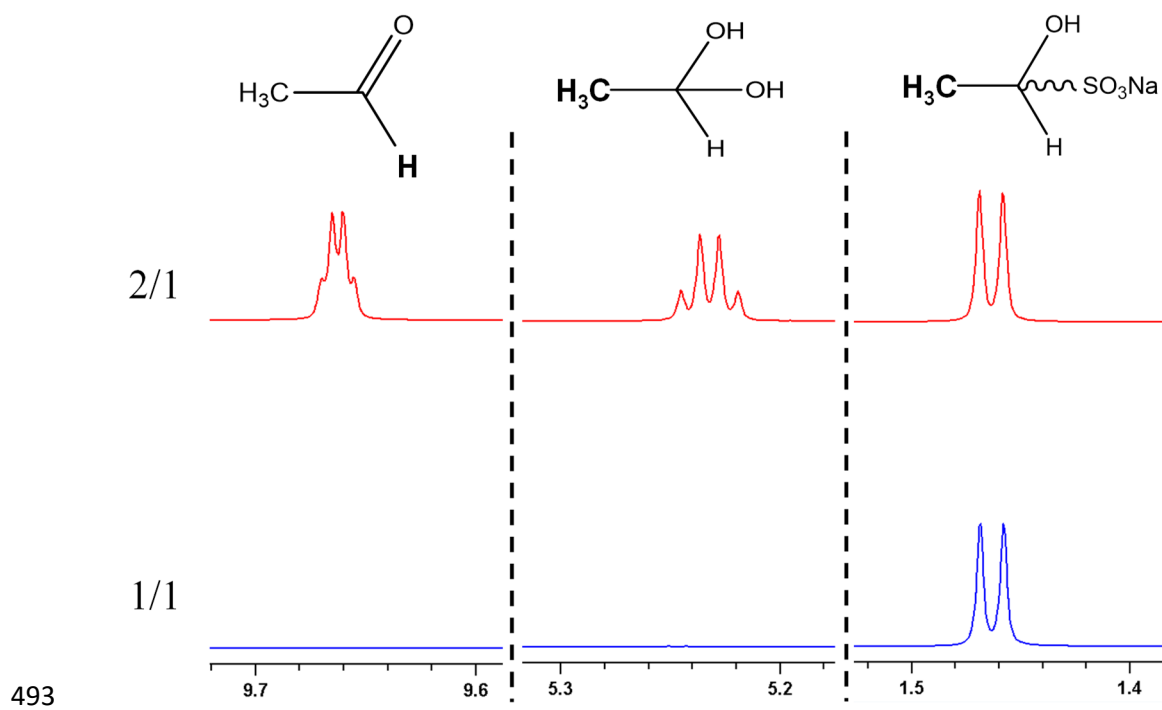
477 **Figure 3:** Acetaldehyde- HSO_3 peak-intensity from LC-MS analysis, EC_{20} , C_{max} and
478 T_{max} relative distributions in 18 chardonnays wines spiked with 50 and 100mg/L
479 SO_2 . Analysis occurred after 1 week of spiking in order to achieve equilibrium.
480 Means not sharing a letter are significantly different ($p < 0.001$).

481 **Figure 4:** (A) ^1H NMR monitoring of acetaldehyde sulfonation reaction products: (i)
482 free acetaldehyde (9.66 ppm), (ii) hydrated acetaldehyde (5.23 ppm) and (iii)
483 acetaldehyde sulfonate (1.46 ppm); under wine oxidative conditions (presence of 4-
484 methylcatechol, sulfur dioxide, dissolved oxygen, and Fe (II)) during time. (B)
485 Kinetic profile of free acetaldehyde (summary of free and hydrated form in green
486 line), Acetaldehyde- HSO_3 (in orange line) and 4-methylcatechol substrate (grey line)
487 during reaction time at 20°C in wine like solution.

488 **Table 1:** Optimized DPPH assay and EPR spin trapping evaluation (EC_{20} , C_{max} ,
489 T_{max} and slope) of free, sulfite bound acetaldehyde and catechin, in model wine like
490 solution and in a white wine at room temperature (20°C).

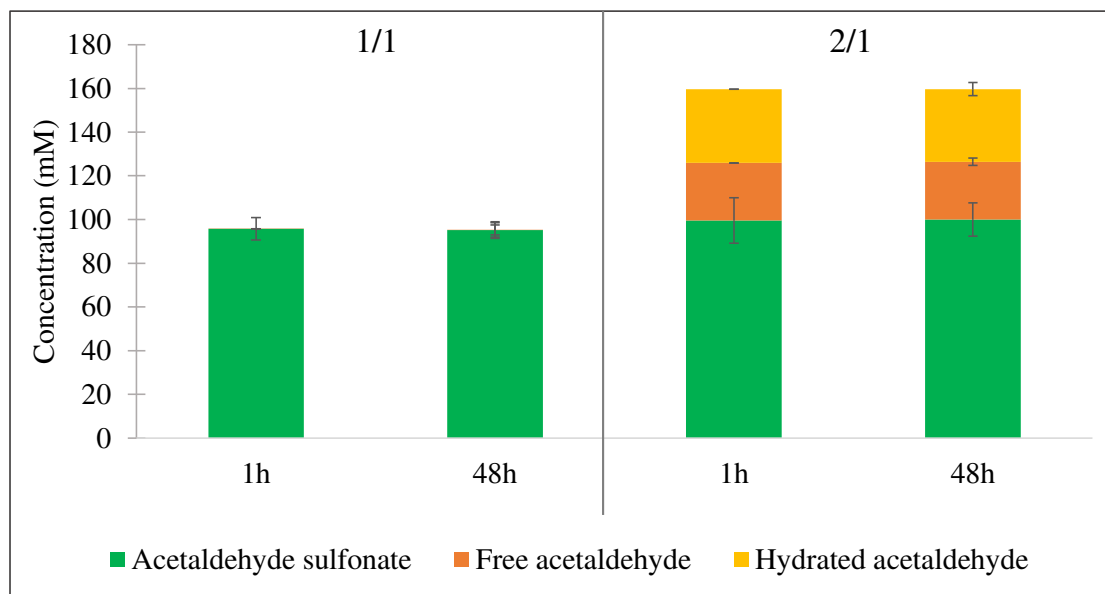
491

492 **Figure 1**



494

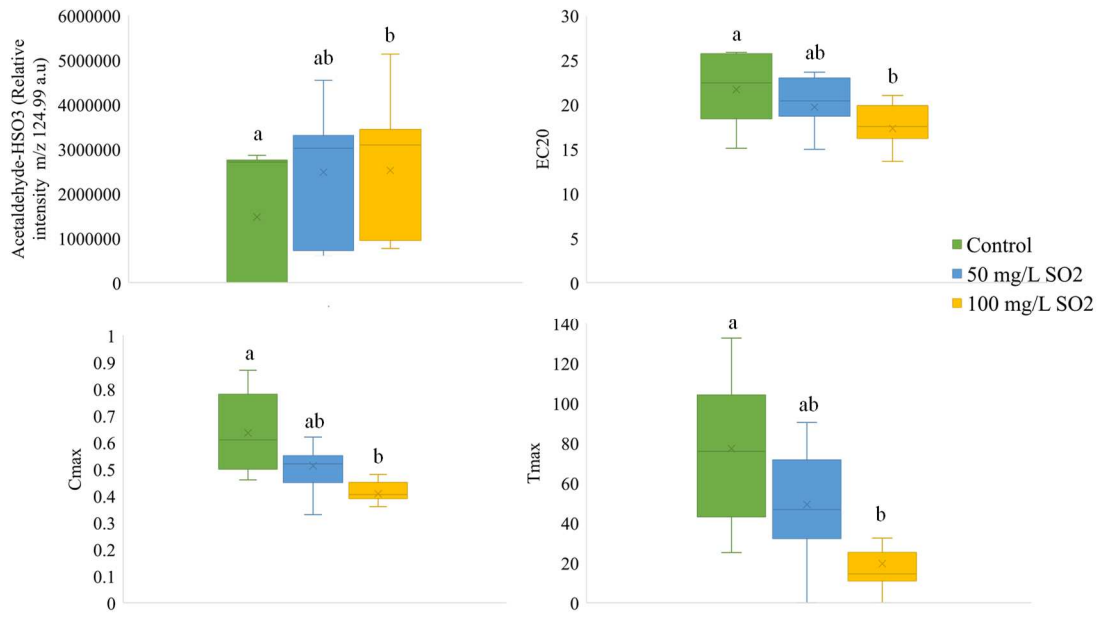
495 **Figure 2**



496

497

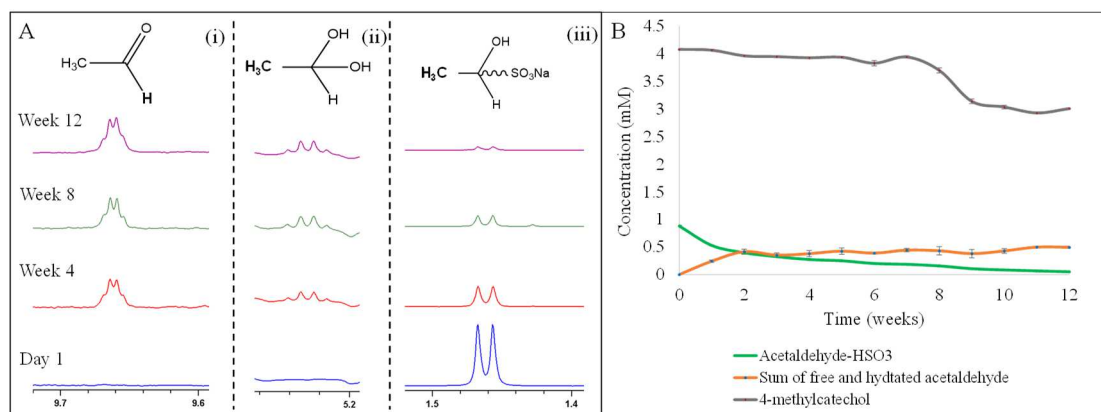
498 **Figure 3**



499

500

501 **Figure 4**



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503

504 **Table 1**

	EC₂₀	Cmax	Tmax	1st slope
Model wine conditions				
Control a (¹ MW)	*N.R.	100	69.43 ± 3.4	0.01 ± 0.00
Acetaldehyde	N.R.	94.4 ± 4.7	86.82 ± 4.3	0.0093 ± 0.00
Acetald-HSO ₃	0.15 ± 0.00	64.5 ± 3.2	32.27 ± 1.6	0.0118 ± 0.00
SO ₂	0.18 ± 0.00	7.26 ± 0.36	52.4 ± 2.6	1.3525 ± 0.07
Catechin	0.029 ± 0.00	80.7 ± 4.0	57.42 ± 2.8	1.052 ± 0.05
White wine				
Control b	17.87 ± 0.16a	59.34 ± 2.9a	107.42 ± 5.3a	0.0063 ± 0.00 a
#AS 5*10 ⁻⁴ M	16.32 ± 0.23b	48.34 ± 2.4b	50.28 ± 2.5b	0.0086 ± 0.00 b
AS 10 ⁻³ M	16.59 ± 0.05b	44.46 ± 2.2c	32.62 ± 1.6b	0.0084 ± 0.00 b
AS 5*10 ⁻³ M	12.31 ± 0.22c	§T.H. d	T.H. c	T.H. c
AS 10 ⁻² M	9.81 ± 0.14d	T.H. d	T.H. c	T.H. c

505 *N.R.: No response; ¹MW: model wine; #AS: acetaldehyde-HSO₃; §T.H: total
506 inhibition; different letters indicating significant statistical differences among samples
507 from the same column (p<0.01). Control a: model wine, Control b: wine without the
508 addition of acetaldehyde sulfonate.