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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 SO<sub>2</sub> 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 <sup>1</sup>H NMR profiling used to monitor the  
20 reactivity of SO<sub>2</sub> with acetaldehyde under wine-like oxidation conditions. The  
21 dissociation of acetaldehyde bound SO<sub>2</sub> was evidenced suggesting that released free  
22 SO<sub>2</sub> 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 SO<sub>2</sub>  
26 dissociation provides a completely new representation of the long-term protection  
27 efficiency of SO<sub>2</sub> 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, SO<sub>2</sub> reaches an equilibrium involving free forms, including  
43 molecular SO<sub>2</sub> and the bisulfite anion HSO<sub>3</sub><sup>-</sup>, and bound forms, which result from the  
44 reaction of HSO<sub>3</sub><sup>-</sup> 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 SO<sub>2</sub> 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  $1.5 \times 10^{-6}$  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 SO<sub>2</sub> within 90  
59 minutes, it has been considered that in the presence of 30mg/L of free SO<sub>2</sub>, 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)-O<sub>2</sub>, 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 ( $^1\text{H}$  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 $\Omega$ , 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. Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> 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 D<sub>2</sub>O 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 SO<sub>2</sub> 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 <sup>1</sup>H 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 SO<sub>2</sub>. 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 <sup>1</sup>H  
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 SO<sub>2</sub>.

### 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 D<sub>2</sub>O that contained 0.1% of  
126 TSP. TSP has been used as an internal standard for quantification. <sup>1</sup>H 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 <sup>1</sup>H–<sup>13</sup>C  
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 <sup>1</sup>H–<sup>13</sup>C 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 Na<sub>2</sub>HPO<sub>4</sub> and 14.6 mM citric acid were dissolved in an H<sub>2</sub>O: 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 CO<sub>2</sub> for 15 minutes right before the analysis to ensure the lack of  
152 residual free SO<sub>2</sub> as Pegram *et al* have proposed (2013). After the experimental



153 procedure and letting the samples for four hours in a dark place, EC<sub>20</sub> (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). FeSO<sub>4</sub>·7H<sub>2</sub>O (0.072 mM) and H<sub>2</sub>O<sub>2</sub> (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<sub>max</sub>), the time to reach the maximum amount of radical formed  
176 (T<sub>Max</sub>), 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 $\mu$ 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 $\mu$ 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 $^1\text{H}$ NMR Monitoring of acetaldehyde sulfonation reaction in model wine**

206  $^1\text{H}$  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  $^1\text{H}$  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  $^1\text{H}$  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).  $^1\text{H}$  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 ( $^1\text{H}$ : (-CH) 5.23  
217 ppm, q /  $^{13}\text{C}$ : 88.80 ppm and ( $\text{CH}_3$ )  $^1\text{H}$ : 1.31 ppm, d /  $^{13}\text{C}$ : 23.84 ppm)) was also  
218 produced. After the addition of bisulfite, the quadruplet from the -CH group ( $^1\text{H}$ : 9.66  
219 ppm, q /  $^{13}\text{C}$ : 207.19 ppm) and the doublet of the - $\text{CH}_3$  ( $^1\text{H}$ : 2.23 ppm, d /  $^{13}\text{C}$ : 30.75  
220 ppm) group of the free acetaldehyde are protected and shifted to a higher field region  
221 at 4.54 ppm (q) ( $^{13}\text{C}$ : 80.99 ppm) and 1.46 ppm (d) ( $^{13}\text{C}$ : 17.56 ppm) respectively.

222 The yield of acetaldehyde sulfonation reaction was calculated for the molar ratios 1/1  
223 and 2/1 (Acetaldehyde /  $\text{HSO}_3$ ) (**Figure 2**). The sulfonation reaction of acetaldehyde  
224 was fast and almost complete (yield  $95\% \pm 5\%$ ), 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<sub>max</sub>); the time to reach the maximum amount of radical formed (T<sub>max</sub>); 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 (EC<sub>20</sub>), 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<sub>max</sub>, T<sub>max</sub>, 1st slope and EC<sub>20</sub>, 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 EC<sub>20</sub> value was also estimated  
244 for free SO<sub>2</sub> 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. EC<sub>20</sub> values of free SO<sub>2</sub> 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  $4.17 \times 10^{-4}$  M of free SO<sub>2</sub> which corresponds to 83% of sulfone

250 dissociation after reaction with  $6 \times 10^{-6}$  M of DPPH. Our results are contradictory to  
251 those published by Danilewich *et al.* (2016) where no reactivity of  $\text{SO}_2$  was shown  
252 with DPPH. This difference can be explained by the use of DPPH ( $1.59 \times 10^{-4}$  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  $C_{\text{max}}$ ,  $T_{\text{max}}$  and slope.

263 Acetaldehyde- $\text{HSO}_3$  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 ( $5 \times 10^{-4}$  M,  $10^{-3}$  M,  $5 \times 10^{-3}$  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  $5 \times 10^{-4}$  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-HSO<sub>3</sub> in wines was modulated to  
276 a concentration dependent manner. Going from the lowest (5x10<sup>-4</sup>M) to the highest  
277 (10<sup>-2</sup>M) 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<sup>-2</sup>M) acetaldehyde-HSO<sub>3</sub> inhibited completely the 1-hydroxyethyl  
280 radical formation. The rate of spin adduct degradation may have exceeded speed  
281 adduct formation in 10<sup>-2</sup>M 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 SO<sub>2</sub> 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-HSO<sub>3</sub> in wine samples and its correlation with** 288 **wines antioxidant capacity**

289 Complementary to existing data on the antioxidant activity of SO<sub>2</sub> 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-HSO<sub>3</sub> of 18 wine samples before (control) and after spiking in 50 and  
301 100 mg/L SO<sub>2</sub>. 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-HSO<sub>3</sub> was identified only in 7 out of 18 wine  
304 samples and in lowest intensity compared to both 50 and 100 mg/L SO<sub>2</sub> treatments. It  
305 is important to mention that free SO<sub>2</sub> 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 SO<sub>2</sub>) 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 SO<sub>2</sub>) 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-HSO<sub>3</sub> on  
318 wines global antioxidant capacity, the association between each pair of estimated  
319 variables (EC<sub>20</sub>, C<sub>max</sub>, T<sub>max</sub> 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-HSO<sub>3</sub> with C<sub>max</sub> (*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-HSO<sub>3</sub> intensity and EC<sub>20</sub> 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 EC<sub>20</sub> (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 SO<sub>2</sub> 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  $^1\text{H}$  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  $\text{SO}_2$  (yield of sulfonated acetaldehyde:  
356  $95\% \pm 5\%$ , so  $<10\%$  of  $\text{SO}_2$  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  $\text{SO}_2$ . Thus, the most of the initially bound  $\text{SO}_2$  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  $\text{SO}_2$  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  $\text{SO}_2$  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  $\text{SO}_2$ . This is an  
370 indication that reinforces the belief that acetaldehyde is a strong binder. The next  
371 weeks,  $\text{SO}_2$  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  $\text{SO}_2$  will form three different sulfonated adducts of quinone (Nikolantonaki &

374 Waterhouse, 2012). It is concluded that during slow oxidation, acetaldehyde releases  
375 the bound SO<sub>2</sub> in order to restore the catechol from quinone (Danilewicz & Standing,  
376 2018). These results could explain the decrease of the amount of total SO<sub>2</sub> bound  
377 fraction during wine aging as observed by Sacks *et al* (2020) and indicates that  
378 strongly bound SO<sub>2</sub> is not a waste but it contributes on wines resistance against  
379 oxidation during bottle aging.

#### 380 **4. Conclusions**

381 Combined direct <sup>1</sup>H 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 SO<sub>2</sub> 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 SO<sub>2</sub> management in wine production.

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468

469 **Figure and table captions**

470 **Figure 1:** Assignments of 1D  $^1\text{H}$  NMR signals for quantification of acetaldehyde  
471 sulfonation reaction products after 1h of reaction, in 1/1 and 2/1 (Acetaldehyde/ $\text{HSO}_3$ )  
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).

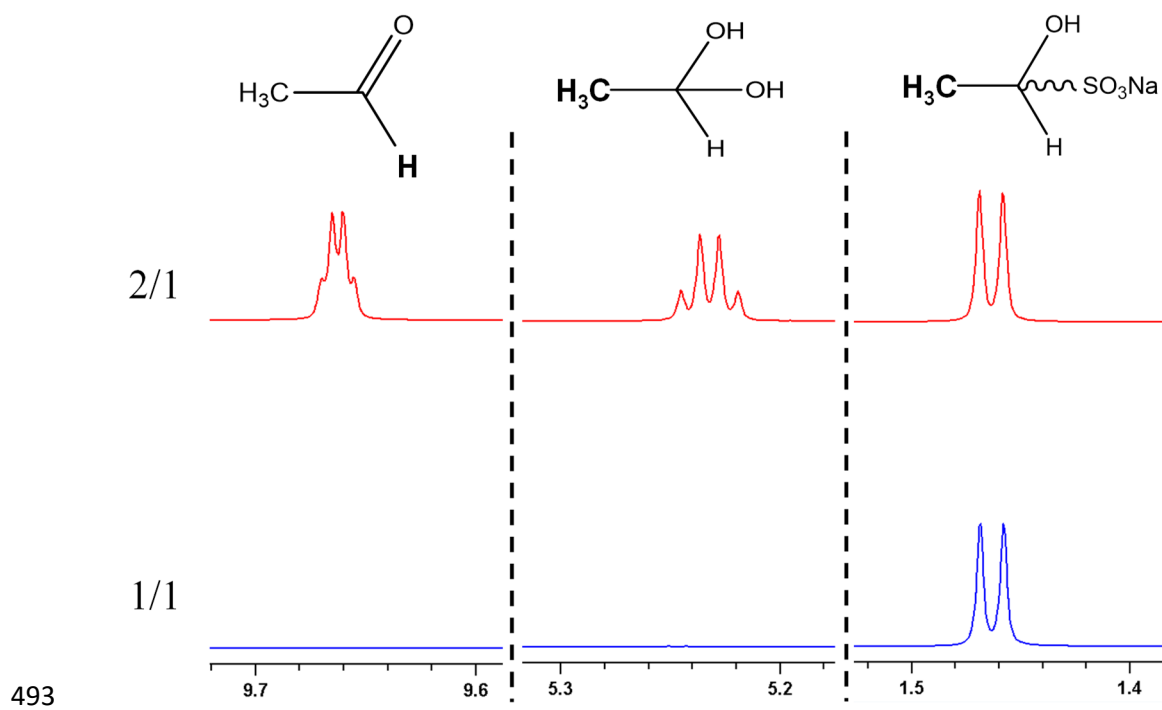
477 **Figure 3:** Acetaldehyde- $\text{HSO}_3$  peak-intensity from LC-MS analysis,  $\text{EC}_{20}$ ,  $\text{C}_{\text{max}}$  and  
478  $\text{T}_{\text{max}}$  relative distributions in 18 chardonnays wines spiked with 50 and 100mg/L  
479  $\text{SO}_2$ . Analysis occurred after 1 week of spiking in order to achieve equilibrium.  
480 Means not sharing a letter are significantly different ( $p < 0.001$ ).

481 **Figure 4:** (A)  $^1\text{H}$  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- $\text{HSO}_3$  (in orange line) and 4-methylcatechol substrate (grey line)  
487 during reaction time at  $20^\circ\text{C}$  in wine like solution.

488 **Table 1:** Optimized DPPH assay and EPR spin trapping evaluation ( $\text{EC}_{20}$ ,  $\text{C}_{\text{max}}$ ,  
489  $\text{T}_{\text{max}}$  and slope) of free, sulfite bound acetaldehyde and catechin, in model wine like  
490 solution and in a white wine at room temperature ( $20^\circ\text{C}$ ).

491

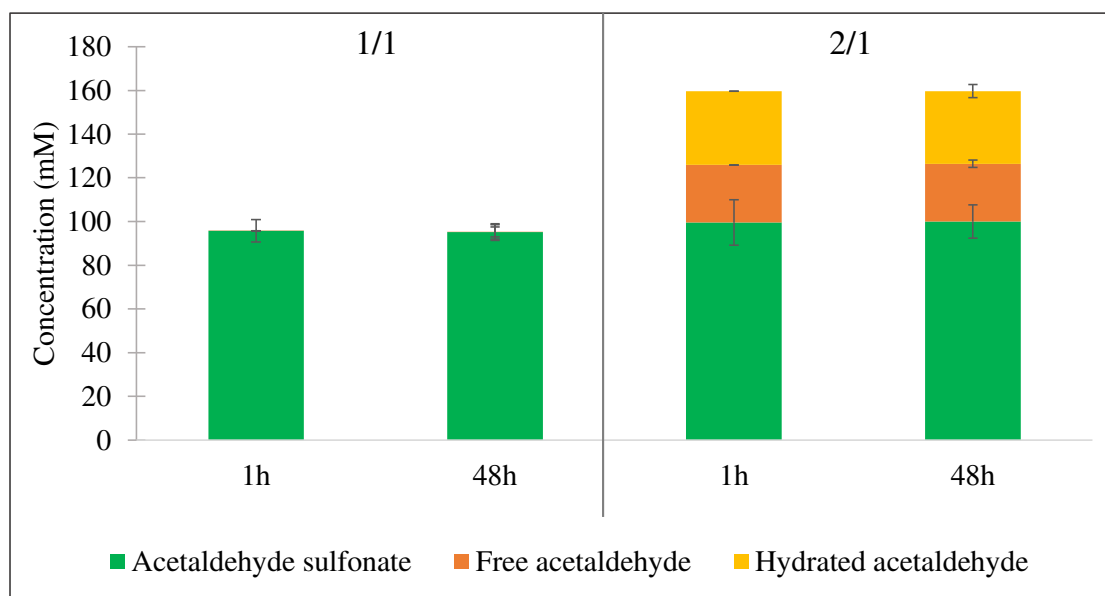
492 **Figure 1**



494



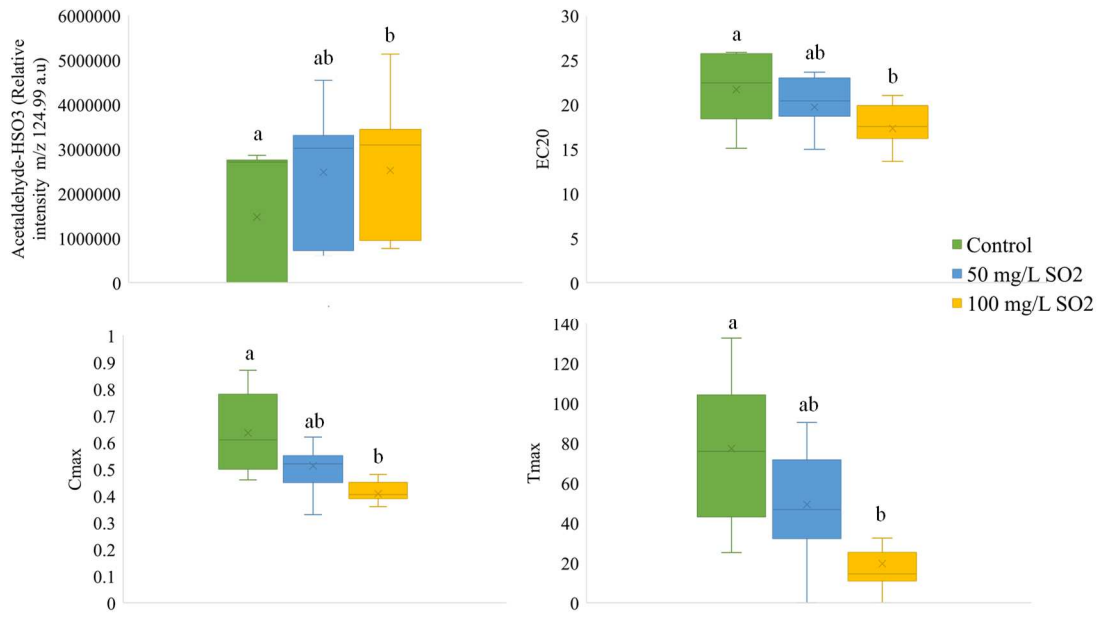
495 **Figure 2**



496

497

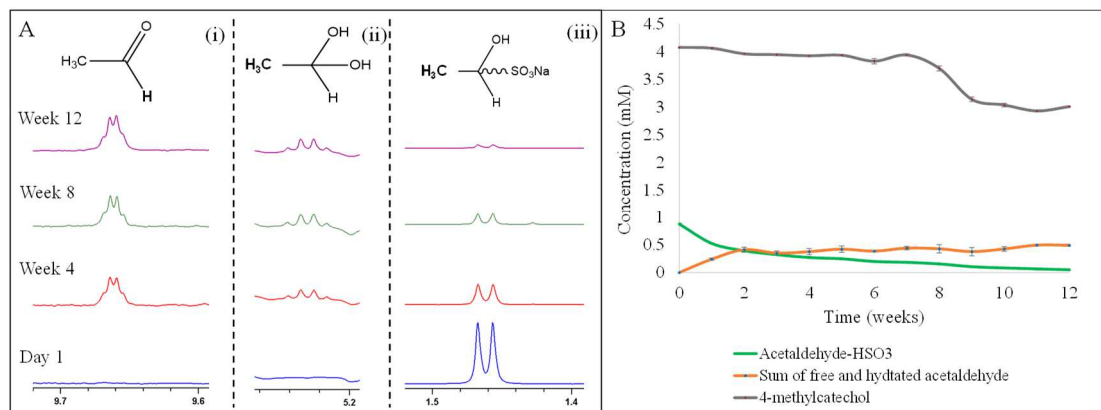
498 **Figure 3**



499

500

501 **Figure 4**



502

503

504 **Table 1**

	<b>EC<sub>20</sub></b>	<b>Cmax</b>	<b>Tmax</b>	<b>1<sup>st</sup> slope</b>
<b>Model wine conditions</b>				
Control a ( <sup>1</sup> 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 <sub>3</sub>	0.15 ± 0.00	64.5 ± 3.2	32.27 ± 1.6	0.0118 ± 0.00
SO <sub>2</sub>	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
<b>White wine</b>				
Control b	17.87 ± 0.16a	59.34 ± 2.9a	107.42 ± 5.3a	0.0063 ± 0.00 a
#AS 5*10 <sup>-4</sup> M	16.32 ± 0.23b	48.34 ± 2.4b	50.28 ± 2.5b	0.0086 ± 0.00 b
AS 10 <sup>-3</sup> M	16.59 ± 0.05b	44.46 ± 2.2c	32.62 ± 1.6b	0.0084 ± 0.00 b
AS 5*10 <sup>-3</sup> M	12.31 ± 0.22c	§T.H. d	T.H. c	T.H. c
AS 10 <sup>-2</sup> M	9.81 ± 0.14d	T.H. d	T.H. c	T.H. c

505 \*N.R.: No response; <sup>1</sup>MW: model wine; #AS: acetaldehyde-HSO<sub>3</sub>; §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.