The effects of copper fining on the wine content in sulfur off-odors and on their evolution during accelerated anoxic storage

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Abstract

Three different red wines with reductive character have been treated with two different doses of copper sulfate (0.06 and 0.5 mg/L) and with a commercial copper-containing product at the recommended dose (0.6 mg/L). Wines were in contact with copper one week, centrifuged and stored at 50°C in strict anoxia for 2 weeks (up to 7 in one case). Brine-releasable (BR-) and free fractions of Volatile Sulfur Compounds were determined throughout the process. Relevant increases of BR-H_2S suggest that those wines contained other H_2S precursors non-detectable by the brine dilution method. Copper treatments had two major effects: 1) immediate decrease the levels of free H_2S and methanethiol (MeSH); 2) slow the rate at which free H_2S (not MeSH) increases during anoxic storage. After 7 weeks of anoxia levels of free H_2S and MeSH were high and similar regardless of the copper treatment. Higher copper doses could induce the accumulation of BR-H_2S.

Keywords: reductive off-odors, sulfide, copper fining, reduction, sulfide-metal complexes, sulfide precursors

1. Introduction

Reductive or sulfur-like off-odors are responsible for an important proportion of faulty wines with potentially large economic losses (Goode, 2014). Such a problem is mostly caused by the development of low molecular weight Volatile Sulfur Compounds (VSCs) of which H_2S is the most frequently found above its odor threshold followed by MeSH (Franco-Luesma & Ferreira, 2016b; Siebert, Solomon, Pollnitz, & Jeffery, 2010; Ugliano, Kolouchova, & Henschke, 2011). Dimethyl sulfide (DMS) is also frequently included within the group of reductive problems, although it strongly differs from H_2S and MeSH

both in sensory effects (Escudero, Campo, Farina, Cacho, & Ferreira, 2007; Franco-Luesma et al., 2016; Lytra et al., 2014; Segurel, Razungles, Riou, Salles, & Baumes, 2004) and in chemical origin (Loscos et al., 2008).

The major source of H_2S seems to be alcoholic fermentation, since this molecule can be directly formed by yeast from elemental sulfur (Schutz & Kunkee, 1977), sulfate or sulfite (Jiranek, Langridge, & Henschke, 1995). Factors affecting the levels of H_2S are the yeast sulfite reductase activity (Linderholm, Dietzel, Hirst, & Bisson, 2010), level of oxygen during fermentation (Bekker, Day, Holt, Wilkes, & Smith, 2016) and yeast assimilable nitrogen (Jiranek et al., 1995). Both H_2S and MeSH production seems to be also related to methionine (Barbosa, Mendes-Faia, & Mendes-Ferreira, 2012; Spiropoulos, Tanaka, Flerianos, & Bisson, 2000) and cysteine (Moreira et al., 2002) metabolism. The most immediate intervention is the addition of ammonium salts to the must (Jiranek et al., 1995; Ugliano, Fedrizzi, et al., 2009; Ugliano, Kolouchova, et al., 2011). In the event of a large production of these unpleasant sulfur containing compounds, winemakers try to decrease their levels by copper finning, aeration or addition of lees (Clark, Grant-Preece, Cleghorn, & Scollary, 2015; Ugliano, Kwiatkowski, et al., 2009; Viviers, Smith, Wilkes, & Smith, 2013).

Copper finning is a relatively common winemaking practice supported by international organisations (O.I.V., 2013) whose underlying chemical mechanism are, however, not well understood. While some traditional enology text books assumed that given the low solubility product of copper sulfide ($10^{-36.1}$), the precipitation of H_2S as the solid salt CuS(s) would be quantitative (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2000), there is much evidence that such precipitation does not occur. First, as noted by Clark et al. (Clark, Grant-Preece, et al., 2015) there is a strong discrepancy between the levels of copper

theoretically required to reduce wine H_2S levels below the threshold and the levels really required as determined by bench sensory trials, which suggests that other ligands may compete for copper. Second, other sources have noted that precipitation is not always observed, suggesting small particle size (Boulton, Singleton, Bisson, & Kunkee, 1999). Most recently, Clark et al. have demonstrated that copper sulfide cannot be easily removed from wine, and that in fact most of it remains in the wine after racking and filtration (Clark, Grant-Preece, et al., 2015). By using model solutions, they have identified tartaric acid as a major contributor to the formation of small copper sulfide particles. In a very recent report, the size of those particles has been found to depend on pH and on the molar ratios between Cu(II) and H_2S (Bekker, Mierczynska-Vasilev, Smith, & Wilkes, 2016).

In addition, recent reports demonstrate that the interaction between copper and H_2S and wine mercaptans is more complex than a simple precipitation equilibrium. Apparently, sulfide and mercaptans form complexes with copper in which there is a charge transfer so that copper is reduced to Cu(I) and S(-II) is oxidized to S(-I) (Kreitman, Danilewicz, Jeffery, & Elias, 2016a). These species can further coordinate, release disulfides and form large clusters some of which could remain in solution. Upon oxidation in the presence of iron, most H_2S and mercaptans would end as polysulfides and other complex species (Kreitman, Danilewicz, Jeffery, & Elias, 2016b). Nevertheless, some of the complexes formed between Cu(II) and other metal cations with H_2S and MeSH have been found to be reversible (Chen, Jastrzembski, & Sacks, 2017; Franco-Luesma & Ferreira, 2014), so that a simple dilution of wine in brine makes it possible to recover the volatile species. Dilution in general favors dissociation of complex forms, the increase of ionic strength reduces solubility of neutral forms and hence promotes volatility and the presence of chloride ions may further complex Cu(I) and even Zn(II). In any case, such reversibility implies that the

different metal-H_2S and metal-thiol complexes present in wine constitute a reservoir of these powerful odorants. Furthermore, recent reports confirm that during anoxic storage of wines this bound fraction spontaneously decreases while levels of free forms increase (Franco-Luesma & Ferreira, 2016a, 2016b), which suggests that the observed increases of H_2S and mercaptans are in fact, at least in part, the result of release of free forms from bound forms. With this evidence, if the different species formed between copper and H_2S and mercaptans are not removed during copper finning, the efficiency of the process could be compromised.

On the other hand, several reports have suggested that added copper may be active in detrimental reactions in wine. In fact, the relationship between the presence of residual copper and H_2S levels has been suggested by several researchers (Bekker, Mierczynska-Vasilev, et al., 2016; Franco-Luesma & Ferreira, 2016b; Ugliano, Kwiatkowski, et al., 2011; Viviers et al., 2013). Residual copper may be active in the catalytic desulfuration of cysteine and methionine, which would contribute to increased levels of H_2S and MeSH (Bekker, Mierczynska-Vasilev, et al., 2016) through complex synergic effects with other wine metals (Viviers et al., 2013).

In spite of all this evidence, copper finning is still carried out by winemakers and the wine industry, and many companies offer different products containing copper as a solution for reductive problems. In this context, the present work aims to assess the efficiency of a standard copper finning strategy on the removal of wine VSCs and on their evolution during accelerated anoxic storage.

2. Material and methods

110 2.1. Solvents and Chemical Standards

111 Ethanol and methanol were purchased from Merck (Darmstadt, Germany). Water with
112 resistance of 18.2 MΩ·cm at 25 °C was purified in a Milli-Q system from Millipore
113 (Bedford, Germany), $CuSO_4 \cdot 5H_2O$ was purchased from Sigma-Aldrich (St. Louis, MO,
114 USA).

115 Pure standards (>95%) for VSCs calibration: H_2S, MeSH and ethanethiol (EtSH) were
116 produced by addition of a water solution of Na_2S, CH_3SNa and CH_3CH_2SNa (supplied by
117 Sigma–Aldrich, St. Louis, MO, USA) at pH 9.6. This solution was daily prepared and was
118 kept in the anoxic chamber. The Na_2S standard is stored under Ar in a desiccator to avoid
119 hydration. Dimethylsufide (DMS) was from Merck (Darmstadt, Germany),
120 ethylmethylsulfide (EMS), 1-propanethiol (PrSH) and thiophene were provided by Sigma-
121 Aldrich (Steinheim, Germany). Stock solutions of DMS, EMS, PrSH and thiophene (ca. 2
122 g/L) were prepared in iso-octane in amber vials and were stored at -25ºC. These solutions
123 were controlled by direct injection in the gas chromatography with pulsed flame
124 photometric detection (GC-pFPD) system.

125 Intermediate methanolic solutions were stored at -25ºC in amber vials with Mini-inert
126 valves (Supelco, CA, USA). All these solutions were manipulated in the anoxic chamber.

127 Brine containing 350 g/L of NaCl (Panreac, Barcelona, Spain) in Milli-Q water. Synthetic
128 wine was a pure water solution containing 5 g/L of tartaric acid, 12% v/v ethanol and pH
129 3.4 adjusted with diluted NaOH (0.1 M).

130

131 2.2. Wines

132 Wine R1 was a bottled red wine from 2010 made with a blend of Garnacha, Cariñena and
133 Cabernet-Sauvignon in Fuendejalón (Zaragoza, Spain) and contained 5 mg/L of free and 45

mg/L of total SO_2, as well as 0.061, 1.93, 1.15 and 0.42 mg/L of Cu, Fe, Mn and Zn, respectively. Wines R2 and R3 were made from Tempranillo in Alfaro (La Rioja, Spain). R2 was from 2013 vintage and contained 11 mg/L of free and 62 mg/L of total SO_2, as well as 0.011, 2.20, 0.63 and 0.46 mg/L of Cu, Fe, Mn and Zn, respectively. R3 was from 2014 and contained 20 mg/L of free and 68 mg/L of total SO_2, as well as 0.008, 2.44, 0.71 and 0.50 mg/L of Cu, Fe, Mn and Zn, respectively. These last two wines had not been previously filtered, although R2 had spent several months (>12) in the bottle before the experiment, while R3 spent just several weeks in the bottle. Both wines had been micro-oxygenated. The three wines were selected because of their tendency to develop sulfur off-odors.

2.3. Treatments

~~Essay~~ Assay 1; Three bottles of each wine (R1, R2 and R3) were introduced into the anoxic chamber. The bottles from the same wine were mixed in a large beaker to ensure complete homogeneity. Samples for the initial analysis of free and BR-VSCs were then taken as described below. The liquid was then divided into eight-250 mL centrifuge bottles. Two of the bottles were left as control, two were treated with 60 µg/~~l~~ L of copper (as copper sulfate), two with 500 µg/L and the third pair with Reduless (Lallemand, France) at the recommended dose of 0.15 g/L. Reduless is a mixture of copper-infused inactivated yeast and bentonite. By analysis it was determined that the recommend dose released 0.596 mg/L of copper. After copper addition, the bottles were vigorously shaken to ensure homogeneity and were then left to stand still for 1 week within the chamber and protected from light. After this time, the bottles were taken out of the anoxic chamber to be centrifuged at 4500

r.p.m. for 15 min (Allegra X-22R Beckman Coulter) and reintroduced into the chamber. Additional samples for the analysis of free and BR-VSCs were taken from the supernatant. Sixty mL of each one of the supernatants were then transferred into glass tubes with tight screw capped closures supplied by WIT (Blanquefort, France). The tubes were hermetically closed and were further bagged with two layers of thermos-sealed plastic bags with certified oxygen permeability and containing between the layers an activated charcoal containing an oxygen-scavenger (AnaeroGen^{TM} from Thermo Scientific Waltham, Massachusetts, United States). The bagged tubes were taken out of the chamber and were incubated in the dark at 50ºC for 2 weeks. After this time, the tubes were reintroduced into the chamber, opened and sample was taken for the analysis of free and BR-VSCs. In this ~~essay~~assay, a single measurement was carried out in each analysis.

~~Essay~~ Assay 2. This ~~essay~~ assay was carried out exclusively on wine R3. Four bottles of the wine were introduced in the anoxic chamber and mixed in a large beaker as previously described. Sample was taken for the determination of the initial levels of free and BR-VSCs. The homogenized wine was distributed into twelve equivalent 250 mL centrifuge bottles to carry out five (4 + control) different treatments in an unbalanced design. Three bottles were prepared for the controls, and the low and intermediate-high doses of copper (60 and 500 µg/L of copper as copper sulfate, respectively). Two bottles were further treated with Reduless at 0.15 g/L as recommended and the last bottle was treated with an intermediate-low dose of copper (180 µg/L of copper as copper sulfate). The 12 bottles were shaken and then left to stand still in the chamber and protected from light for 1 week. After this time, the bottles were taken out of the chamber, centrifuged and taken into the chamber again. In the three treatments in triplicate, only samples from the two first bottles were taken for the analysis of free and BR-VSCs. The supernatants of the three bottles of

each treatment were then pooled together and mixed in a beaker. The homogenized liquid was then distributed into 10 glass 60-mL WIT tubes, which were closed and bagged as indicated previously (see scheme 1). The two bottles from the Reduless treatment, were independently sampled for the analysis of free and BR-VSCs, the supernatants of the bottles were mixed in a beaker and the liquid was further distributed into seven 60-mL WIT tubes as indicated in the scheme. Finally, the 180 mg/L treatment was sampled and distributed into 2x60 mL WIT tubes as indicated in the scheme. Bagged tubes were then taken out of the chamber and were incubated at 50ºC for the times indicated in the scheme 1. After the corresponding incubation times, tubes were introduced into the chamber, opened and sampled for the analysis of free or of free and BR-VSCs as indicated in the scheme. In this experiment, two analytical replicates were carried out in all the samples.

2.4. Analysis of Sulfur dioxide

Free and total sulfur dioxide were measured in accordance with the protocols issued by the Office International de la Vigne et du Vin (O.I.V., 2014).

2.5. Analysis of VSCs

The methods and instruments for the analysis of free and BR-VSCs are derived from those described in references (Franco-Luesma & Ferreira, 2014) and (López, Lapena, Cacho, & Ferreira, 2007). Samples for the analysis of free VSCs were directly taken in a 20 mL standard headspace vial, which was filled with 12 mL of supernatant and with 40 µL of the internal standard solution (EMS, PrSH and thiophene at 2 mg/L each in methanol). Vials

were sealed with a crimper and were taken out of the chamber for their immediate analysis. The time elapsed from the opening of the bottle or tube with the treatment and the analysis was never longer than 20 min. The time elapsed from the time at which samples were taken out of the chamber and analysis was never longer than 5 min. The analysis consists of a direct headspace injection in the gas chromatography with pulsed flame photometric detection system.

For the analysis of BR-VSCs, a brine (350 g/L NaCl) was prepared out of the chamber and was purged with a strong stream of nitrogen (>100 mL/min) for at least 10 min and it was further introduced into the anoxic chamber at least 24 hours before the analyses. Then 9.6 mL of brine were transferred into 20 mL standard headspace vials and were sealed with the cramp. At the time of analysis, 0.4 mL of the supernatant of the sample are injected through the septum followed by 40 µL of the internal standard solution (EMS, PrSH and thiophene, 20 µg/L each in methanol). The vial is then taken out of the chamber, shaken and immediately analyzed by headspace SPME and GC with pFPD.

In both determinations, the areas are normalized to those of the internal standard (PrSH for H_2S, MeSH and EtSH and EMS for DMS) and interpolated in calibration graphs built by the analysis of calibration standards prepared in synthetic wine.

2.6. Analysis of metals

Trace metal analysis was carried out based on a protocol published by Grindlay et al. (Grindlay, Mora, de Loos-Vollebregt, & Vanhaecke, 2014) based on inductively coupled plasma mass spectrometry and adapted for this work. The instrument used was a Perkin Elmer (Waltham, USA) NexION 300X. The nuclides monitored were ^{63}Cu and ^{65}Cu for copper; ^{56}Fe and ^{57}Fe for iron; ^{55}Mn for manganese; and ^{66}Zn and ^{68}Zn for zinc. The

227 collision/reaction cell was filled with He (3 mL/min) to minimize potential overlaps.
228 Wine samples were diluted 1:4 with ultrapure water prior to analysis and the internal
229 standard (Rh) was added to then. Calibration was carried out with aqueous standards to
230 which, in addition to the internal standard, 2.5% ethanol was also added for matrix
231 matching. In all cases, multi-isotope analytes provide very similar results with both
232 nuclides monitored.
233

3. Results

3.1. Effects of the copper treatments on the levels of free forms of VSCs

The effects of the treatments with copper sulfate and with a popular commercial copper containing product on the levels of free H_2S, MeSH and DMS of three different wines can be seen in Figure 1. Effects noted immediately after the treatments are marked as –OR and after 2 weeks of reductive accelerated aging as –AR-.

Figure 1a reveals that the three treatments exerted a dramatic effect on the immediate levels of free H_2S, which in all cases dropped below 0.35 µg/L. As this value is close to the detection limit of the analytical method, standard deviations are high and not much can be said about the relative efficiencies of the different treatments. Residual remaining levels suggest that the three treatments were able to remove 85-100% of the free H_2S initially present in the wines. After 2 weeks of anoxic storage (AR samples), the levels of free H_2S of the three wines had strongly increased regardless of the copper treatment, although levels of free H_2S in the aged samples were inversely proportional to the amount of copper added to the wine. The proportionality factor was, however, wine-dependent. In R1, the levels of free H_2S were reduced by 20, 66 and 81% by the three doses, respectively. In R2, reductions were just 5, 29 and 49%, while in R3 reductions were 23, 53 and 66% the levels found in the controls.

In the case of MeSH, shown in Figure 1b, the effects of the treatments on the immediate levels of free MeSH (OR samples) were also clear and the levels of free MeSH of all wines were significantly reduced in all cases. Reduction was not as effective as in the case of H_2S, since the proportion removed was between 70 and 82% for R1, between 76 and 92% for R2 and between 80 and 85% for R3. In the case of wine R1 remaining levels of MeSH were

above 1 µg/L in one of the treatments. Two-way ANOVA revealed that effects were not significantly related to the dose. A look to the figure shows that in R2 the dose had some effect, but certainly not in R1. After 2 weeks of anoxic storage (samples AR), levels of MeSH had strongly increased in all samples, particularly in sample 1, and the differences linked to the treatment had diminished. In the case of R1, the treatments reduced the level of free MeSH by 7 (non-significant), 19 and 22%, in the case of R2 the treatments did not exert any significant effect, while in R3, for which the levels in the control were already very low, the treatments reduced the levels of free MeSH by 13, 19 and 24%.

In the case of DMS, shown in Figure 1c, the treatments had also a significant effect on the immediate levels of this molecule in the headspace (samples OR), but as expected, the effects were of little magnitude. The effect of the dose did not reach the significance level, although it is apparent from the figure that the smallest doses in all cases removed smaller levels. The treatments removed between 20 and 30% of the levels of this molecule. After two weeks of accelerated aging (samples AR) the effects of the treatments were significant but weak. In R1 reductions induced by the treatments versus the control were 13, 19 and 16%; in R2, 8, 13 and 10% and in R3, 3.6, 8 and 14.5%.

3.2. Effects of the treatments on brine-releasable (BR-) levels of H_2S and MeSH

Brine-releasable forms were in previous papers named as “total” forms and include free forms and reversible cation complexed forms of H_2S and MeSH. The reasons why the name has been changed are explained in the discussion. The effects of the treatments on the immediate levels of BR-H_2S are shown in Figure 2a (samples OR). Results show that the copper treatments did not have any consistent effect on the levels of brine-releasable forms.

In wine R1, results were quite irreproducible and the high variability made that differences were not significant, but in any case it is clear that the treatments did not reduce the levels of BR-H_2S. In wine R2, the surprising result is that the treatment at low doses seemed to be extremely efficient at removing BR-H_2S, while treatments at higher doses did not show any effect on the levels of this molecule. Finally, in wine R3, all treatments were able to significantly reduce the levels of BR-H_2S, the lower copper dose again being most effective. After 2 weeks of accelerated reductive storage (samples AR), levels of brine-releasable forms had in all cases strongly increased. In the untreated controls the increases were from 40 to 72 µg/L in R1, from 29 to 81 in R2 and from 11 to 51 µg/L in R3. Increases of this magnitude have not been previously observed. Additionally, the treatments did not reduce the levels of BR-H_2S. On the contrary, levels found in the R1 wine treated with the commercial product were significantly higher than those of the control, and effects were not significant in R2 and R3.

In the case of BR-MeSH, shown in Figure 2b, the immediate effects of the treatments (samples OR) were again wine-dependent. In wines R1 and R3 the treatments had no effect, while in R2 all the treatments seemed to be equally effective reducing brine-releasable levels of this molecule to around a 55-60% of its initial content. After 2 weeks of accelerated reductive aging (samples AR) levels of BR-MeSH were again higher than those found in the original wines, but increases were in this case expected (Franco-Luesma & Ferreira, 2016b). Treatments did not have any significant effect on the levels of BR-MeSH. Remarkably, levels of BR-MeSH in the independent R1 replicated treatments turned out to be very different which caused a high variability.

Crossing results from free and brine-releasable forms, it is possible to estimate the proportion of H_2S present as free forms and see the change of this parameter with the

304 copper treatment and with the anoxic storage. This information is summarized in Figure 3.
305 The figure shows that the reductive storage induced a strong increase in the fraction of free
306 forms in all cases. The second observation is that the fraction of H_2S under free forms is in
307 all cases approximately and inversely related to the level of copper of the sample.
308

309 3.3. Detailed study of copper finning with wine R3

310 Wine R3 was subjected to a more complete study in terms of replicates and sampling
311 points. In this case metals were also determined in the treated samples. Results (data not
312 shown) revealed that the copper content of the treated samples corresponded closely to the
313 amount of copper introduced by the treatment, suggesting that there was no formation of
314 any solid phase.
315 The evolution of the levels of free H_2S of the control and treated wines during the whole
316 experiment can be seen in Figure 4a. The figure reveals that the levels of free H_2S of this
317 wine, independently of the treatment applied, strongly increase during the anoxic storage
318 and tend to stabilize around 35-40 µg/L. The major difference introduced by the treatments
319 is the rate at which free H_2S levels increase during the anoxic storage. The rates of increase
320 of free forms are inversely related to the amount of total copper contained in the sample.
321 The control, containing just 8 µg/L of copper releases H_2S very fast and in fact 85% of the
322 maximum level is already obtained after just 1 week of reductive storage. It should be noted
323 that in this sample copper levels are so low that H_2S and MeSH have to be bound
324 preferably to different metals, such as Fe(II) or Zn(II), which are at much higher levels (2.4
325 and 0.50 mg/L, respectively). The sample treated with the lowest doses (with a measured
326 total copper content of 77 µg/L) needs two weeks to release 20 µg/L, 50% of its maximum

amount, while that treated with 500 µg/L (471 µg/L measured) needs around 3 weeks to reach the same 20 µg/L of H_2S. Finally, the sample treated with the commercial product (containing 604 µg/L of copper) needed 7 weeks to release 36 µg/L. After 7 weeks of anoxic storage differences in the levels of free H_2S between treatments were not significant.

Results for MeSH are shown in Figure 4b. The figure confirms the much limited effectivity of copper treatments to avoid the accumulation of this molecule during the anoxic storage. It can be seen that during the first week of storage, levels of free MeSH increased in all the samples although at rates slightly slower the higher the level of copper of the sample. Therefore, after 1 week of storage, the sample treated with 60 µg/L of copper contained levels significantly higher than those of the samples treated with 500 and 600 µg/L of copper. In the second week of anoxic storage, there is a slight decrease in the control and slight increases in the treated samples, so that differences are diminished. From this point on, there are continuous increases and differences between the control and treated samples disappear.

Results for DMS are shown in Figure 4c. In this case it can be seen how the treatments evolve in parallel and the sample treated with the commercial product in all cases had slightly but significantly smaller levels of this volatile compound.

Results regarding the evolution of BR-H_2S, for which only one additional sampling point after 7 weeks of accelerated aging was added, are given in Figure 5a. Results reveal that in this case, levels of the control remained constant between 2 and 7 weeks of reductive storage, those of the sample treated with a low dose of copper increased slightly to equal those of the control, while those of the samples containing higher levels of copper significantly increased in the last sampling points reaching levels of BR-H_2S above 80 µg/L.

In the case of MeSH, results are presented in Figure 5b, and show that levels of BR-MeSH increase in all cases between 2 and 7 weeks of anoxic storage but the increase is stronger in samples containing higher levels of copper, so that final levels of BR-MeSH become equivalent in all samples after 7 weeks of storage.

Finally, the evolution of the fractions of H_2S and MeSH under free forms is summarized in Figures 6a and 6b. Both figures confirm that there is a continuous increase in the proportion of both molecules present as free forms. The figures also reveal that the effects of the copper treatments are very strong for H_2S, while for MeSH they are barely noticeable. In the case of H_2S it is evident that the magnitude of the fraction under free forms at each sampling point is inversely related to the copper levels of the sample. In the case of MeSH, in the first sampling point the control contains significantly a higher proportion in free forms, in the second only the 500 µg/L treatment contained a significantly smaller fraction, and after 7 weeks, differences were not significant.

4. Discussion

4.1. About the increases of free forms

Results presented in this work confirm previous observations on the strong increases in the levels of free forms of VSCs during the anoxic storage of wines (Franco-Luesma & Ferreira, 2016b). In fact, increases of these VSCs during wine aging have been repeatedly reported, even if the wine is stored in containers not completely anoxic (Lopes et al., 2009; Ugliano et al., 2012; Ugliano, Kwiatkowski, et al., 2011). As shown in Figures 1 and 4, increases depend on the wine, on the level of copper and on the time of anoxic storage, in accordance with previous reports. The magnitudes of the increases (20-40 µg/L after two

weeks of anoxic storage) are slightly above those previously reported in a similar experiment (Franco-Luesma & Ferreira, 2016b), and double or even quadruple those found to accumulate after 1 year of reductive aging at room temperature (Franco-Luesma & Ferreira, 2016a). These higher increases were expected since the wines studied in the present work had been selected because of their known tendency to develop sulfur off-odors. Increases of these magnitudes have been demonstrated to cause strong sensory problems (Franco-Luesma et al., 2016), causing decreases in the intensities of fruity and floral attributes, and delivering notes of rotten eggs (H_2S) or camembert (MeSH).

In the case of free MeSH, increases are also strongly related to the wine and to the time of storage but the influence of the levels of copper is not very large and tends to fade away at longer storage times, as seen in Figure 4b. The magnitude of the increase is particularly relevant in wine R1 (up to 12 µg/L), which exceeds by a factor of 3 the highest increases measured in red wines in previous works (Franco-Luesma & Ferreira, 2016a, 2016b). It is remarkable that attending to the levels of free H_2S and MeSH accumulated during the anoxic storage, the sensory profile of the reductive notes of wines R1 and R2 would be completely different (Franco-Luesma et al., 2016). Increases of DMS are also related to the wine, time of storage and to the wine copper content. The magnitude of the increases (between 10 and 20 µg/L in two weeks of anoxic storage) are in the low range of those reported after one year of anoxic storage (Franco-Luesma & Ferreira, 2016a).

4.2. About the increases of brine-releasable forms

The increases of BR-H_2S measured in the three wines of the experiment are much higher than those previously reported. In fact, in accelerated reductive storage experiments carried

out with bottled commercial wines, average levels of BR-H_2S in red wines did not significantly increase (Franco-Luesma & Ferreira, 2016b) and in the few samples in which that happened, increases were quite modest (the highest 6.7 µg/L). In most cases, levels of BR-H_2S remained approximately constant since the beginning of the experiment, while free H_2S steadily increased tending, but never reaching, the ~~to~~ levels of BR-H_2S. Taking into account that dilution in brine can cleave some H_2S-metal complexes (maybe some other weak associations), but cannot reduce disulfides or polysulfides, and cannot of course ~~can~~ release H_2S from cysteine, it was concluded that in bottled commercial wines the quantitatively most important process explaining the accumulation of free H_2S was the release of metal-complexed forms. Because of such prominent role, BR-forms were named as "total" forms.

In clear contrast, in the present work levels of BR-H_2S increased during anoxic storage in all wines and treatments. Increases were much higher than those previously reported: above 50 µg/L in R2 (Figure 2a), or above 80 µg/L in R3 after 7 weeks (Figure 5a). Remarkably, in some R2 treatments and in R3, levels of free H_2S after reductive storage were well above initial BR-H_2S levels. These results clearly indicate that initial wines contain a rather large fraction of H_2S precursors not detectable by the brine dilution method and that BR-H_2S levels are much less stable than previously reported. Because of all these observations, the BR-fraction is no longer referred to as "total" forms.

In the light of recent evidences presented by Kreitman et al. (Kreitman et al., 2016a, 2016b), those precursors not detectable by the brine dilution method may be disulfides, persulfides or polysulfides formed by the copper-catalyzed oxidation of H_2S and mercaptans in aeration treatments as recently described (Kreitman et al., 2016a). In fact, the decrease of BR- forms induced by the copper treatment observed in one of the copper

421 treatments of R2 and in all treatments of R3 in Figure 2a could be due to oxidation of part
422 of H_2S to complex forms containing Cu(I) and S(-I). Similarly, the low levels of initial BR-
423 H_2S measured in all R3 treatments may be related to the fact that this wine had been
424 previously micro-oxygenated and had spent a short period in the bottle before the
425 experiment. The strong increases in BR-H_2S observed during anoxic storage should be
426 attributed to the reduction of those "oxidized precursors" to S(-II) valence. The exact nature
427 of those oxidized precursors and the apparent reversibility of the redox reaction requires
428 further investigation.
429 Another aspect which deserves being mentioned is the elevated variability associated with
430 the determination of BR-forms noted in the present work. This is evident in the large error
431 bars noted in R1 in Figure 2a and in R3 in figure 5a. In addition, the design of the second
432 experiment made it possible to estimate the uncertainty associated with the analytical
433 determination. The study revealed that about one third of the duplicated measurements had
434 relative deviations above 15%. Although it was already known that the method for BR-
435 forms was more imprecise than that for free forms, those high variabilities had not been
436 previously observed. One possible reason could be related to the formation during the
437 treatments of copper-sulfide and copper-cysteine colloids of large size, as proposed by
438 Kreitman et al. (Kreitman et al., 2016a) or copper-tartrate-sulfur particles as suggested by
439 Bekker et al. (Bekker, Mierczynska-Vasilev, et al., 2016) and in agreement with
440 observations made by Luther et al. in aqueous systems (Luther & Rickard, 2005; Luther et
441 al., 2002). Large colloids may follow Poisson-like distributions in the small volumes
442 handled for the analysis of BR-forms which would help explaining poor analytical
443 reproducibility.
444

4.3. About the effects of copper treatments

Results demonstrate that copper treatments are effective at the immediate removal of free forms of H_2S and MeSH from the wine headspace, ~~and quite~~but are more limited ~~at~~ in removing DMS, in agreement with the strength of the Cu-S bonds of the three molecules. Levels of H_2S became in all cases close to the analytical detection limits, while MeSH was removed by more than 75%. Such decreases will have a deep effect on the sensory properties of the wines (Franco-Luesma et al., 2016; Siebert et al., 2010), creating the impression that the problem has been solved. However, after anoxic storage, levels of free H_2S and MeSH strongly increase in all samples. The effects of the copper treatments are more easily interpreted with the help of figure 4. In the case of H_2S, the figure suggests that all samples from the same wine, regardless of the copper treatment, tend to accumulate in the headspace the same amount of H_2S during the anoxic storage, but they do it at different rates. The rate of accumulation of free H_2S is inversely proportional to the copper level of the wine. Then, for a short time of storage, levels of free H_2S are inversely proportional to the copper level of the sample, as observed in Figure 1a. In this regard, it can be concluded that the copper treatment, carried out as detailed in the experimental section, delays but does not prevent the accumulation of H_2S during the anoxic storage. If the copper treatment was combined with some additional fining or filtration process results may be different.

In the case of free MeSH, the effectivity of the copper treatments is much more limited, as shown in figures 1b and 4b. The reasons for that limited effectivity have to do with both the weaker bonds Cu-S of this molecule and with the relevance of de novo formation in this case, as previously observed (Franco-Luesma & Ferreira, 2016a, 2016b). The case of DMS is surprising, because as shown in Figures 1c and particularly in 4c, the effects of the copper treatments were weak but, they do not disappear at longer storage times, as observed

for free H_2S and MeSH. This suggests that the effects of copper treatments in this case must be of a completely different nature. It seems that there was an effective removal of this molecule during the copper treatment (Figure 1c and 4c), that the removal was related to the dose of copper, but that the dosage with the commercial product was less effective in wines R1 and R2. As the increases during anoxic storage are independent on the copper dosage, it can be concluded that the treatment does not affect the precursors of this molecule.

Copper treatments, on the other hand, have a practically null ability to remove BR-forms, as was seen in Figures 2 and 5. This inevitably means, as recently demonstrated by different authors, that copper does not remove H_2S and MeSH nor their precursors from the wine (Bekker, Mierczynska-Vasilev, et al., 2016; Clark, Grant-Preece, et al., 2015; Clark, Wilkes, & Scollary, 2015). In addition, as can be seen in some of the treatments with high levels of copper (R1 in Fig 2a and R3 in Fig 5a) copper seems to be also able to induce a larger formation of BR-forms from unknown precursors. We cannot conclude from those observations that the addition of copper implies the development of higher levels of reductive off-odors, since we have not been able to register higher levels of free H_2S or MeSH in copper treated samples. This could be a matter of time, however, since as aforementioned, the major role of copper seems to be to delay the release of free forms from bound forms.

5. Conclusion

In conclusion, the present research has revealed that wines affected by reductive problems contain relevant amounts of H_2S precursors different to those measured by the dilution in

brine method. Those precursors are most likely reduced to BR-H_2S during anoxic aging, which may suggest that they were formed by oxidation during aeration or micro-oxygenation treatments applied to remove reductive off-odors. Copper treatments have two major effects on VSCs. On the one hand they are efficient at removing from the headspace H_2S, MeSH and a small fraction of DMS. On the other hand, they determine the rate at which free forms of H_2S (but not of MeSH) are released from bound forms. Higher levels of copper result in much slower release rates. However, after 7 weeks of reductive aging, levels of free H_2S and MeSH were high and similar regardless of the copper treatment. Copper treatments had no clear effects on levels of BR-H_2S and BR-MeSH, and high copper doses, can even induce the accumulation of higher levels of BR-H_2S.

Acknowledgements

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624

625 **Figure Captions**

626

627 Scheme 1. Experimental procedure followed in the 2nd ~~essay~~assay carried out on wine R3.

628

629 Figure 1. Effects of the treatments with copper on the levels of a) free H_2S, b) free MeSH
630 and c) DMS of the three tested wines immediately after the treatment (OR) or after 2 weeks
631 of accelerated reductive aging.

632

633 Figure 2. Effects of the treatments with copper on the levels of a) BR-H_2S and b) BR-

MeSH of the three tested wines immediately after the treatment (OR) or after 2 weeks of accelerated reductive aging at 50ºC (AR).

Figure 3. Fraction of H_2S present as free forms (as % of the BR-fraction). Effects of the anoxic storage and of the copper treatments.

Figure 4. Evolution of the contents of wine R3 in a) free H_2S, b) free MeSH and c) DMS during its accelerated anoxic storage at 50°C for seven weeks.

Figure 5. Evolution of the contents of wine R3 in a) BR-H_2S and b) BR-MeSH during its accelerated anoxic storage at 50°C for seven weeks.

Figure 6. Proportions of H_2S (a) and MeSH (b) under free forms (as % of the corresponding BR-fraction) in the second experiment. Effects of the copper treatment and evolution with anoxic storage.

Figure(s)

Click here to download Figure(s): figures_Revised_19_3_17.docx

Scheme 1

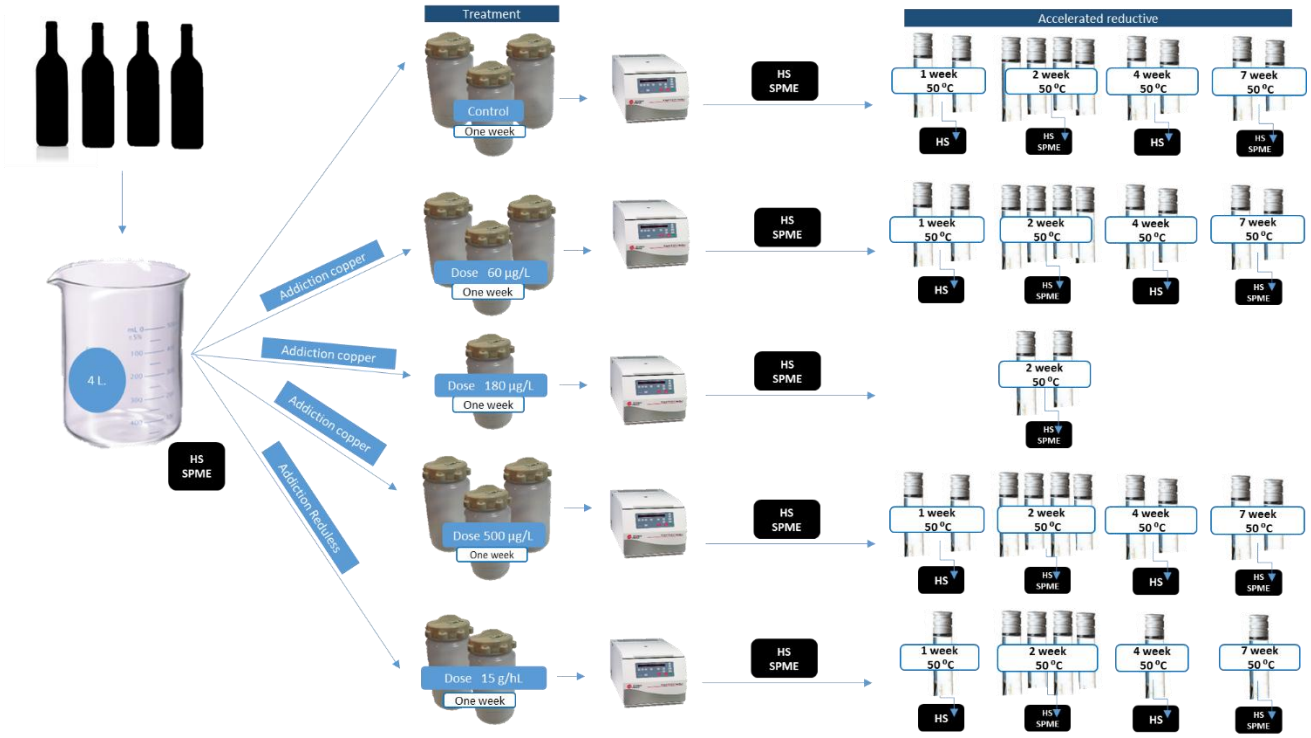
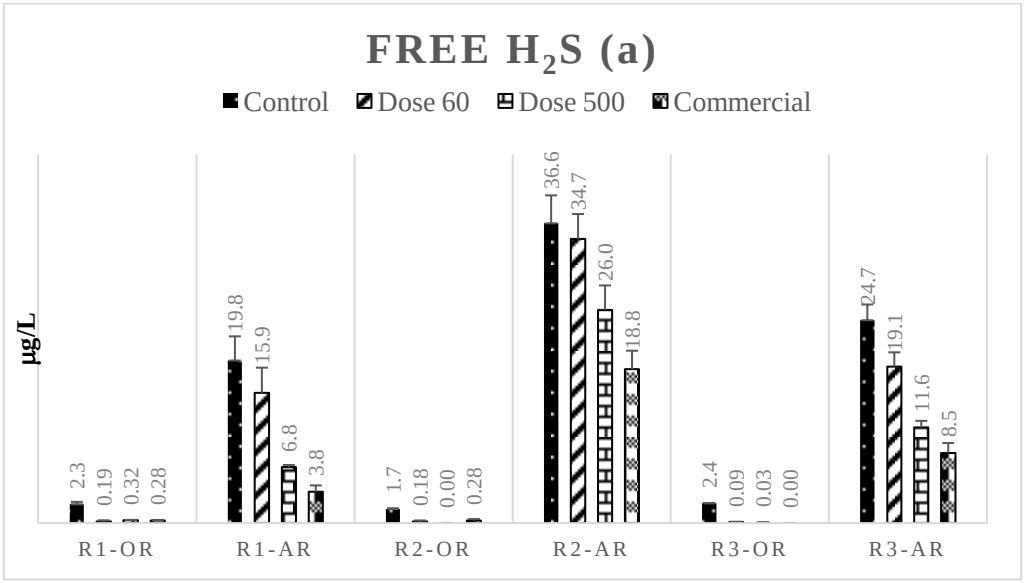
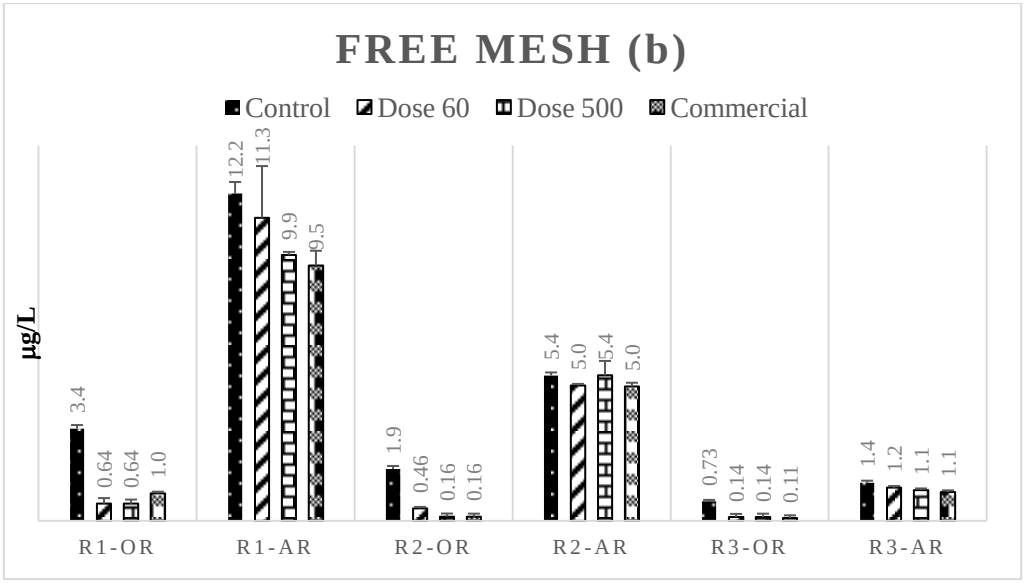


Figure 1


FREE H_2S (a)
Control
Dose 60
Dose 500
Commercial
µg/L
2.3
0.19
0.32
0.28
19.8
15.9
6.8
3.8
1.7
0.18
0.00
0.28
36.6
34.7
26.0
18.8
2.4
0.09
0.03
0.00
24.7
19.1
11.6
8.5
R1-OR
R1-AR
R2-OR
R2-AR
R3-OR
R3-AR


FREE MESH (b)
Control
Dose 60
Dose 500
Commercial
µg/L
3.4
0.64
0.64
1.0
12.2
11.3
9.9
9.5
1.9
0.46
0.16
0.16
5.4
5.0
5.4
5.0
0.73
0.14
0.14
0.11
1.4
1.2
1.1
1.1
R1-OR
R1-AR
R2-OR
R2-AR
R3-OR
R3-AR

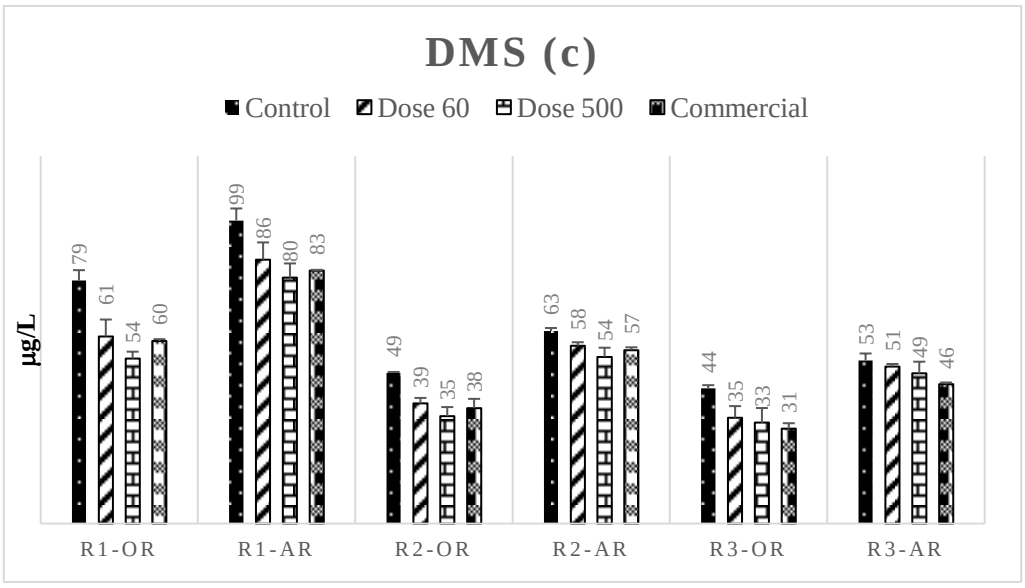
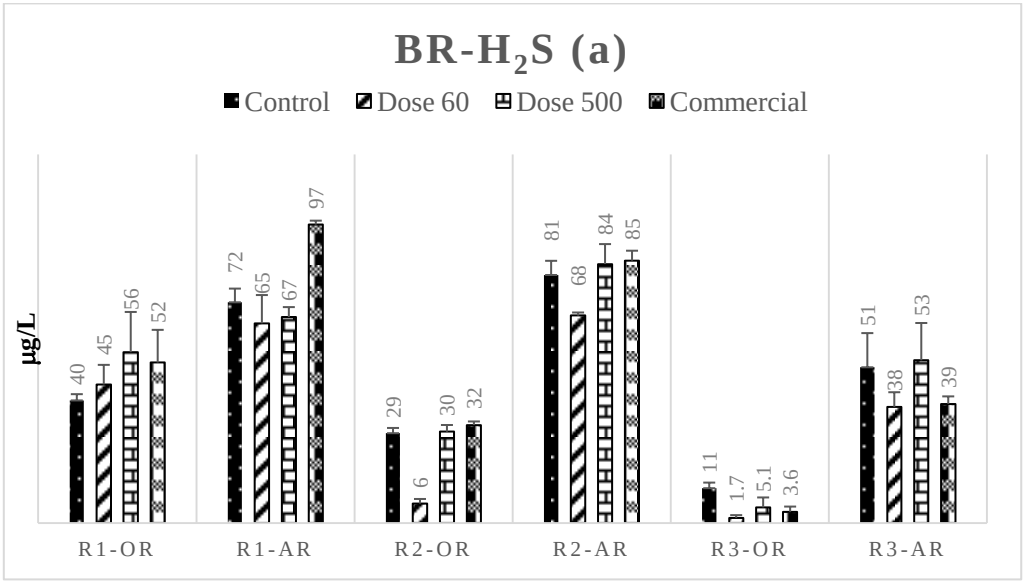

DMS (c)
Control
Dose 60
Dose 500
Commercial
µg/L
79
61
54
60
99
86
80
83
49
39
35
38
63
58
54
57
44
35
33
31
53
51
49
46
R1-OR
R1-AR
R2-OR
R2-AR
R3-OR
R3-AR

Figure 2


BR-H2S (a)
Control
Dose 60
Dose 500
Commercial
µg/L
40
45
56
52
72
65
67
97
29
6
30
32
81
68
84
85
11
1.7
5.1
3.6
51
38
53
39
R1-OR
R1-AR
R2-OR
R2-AR
R3-OR
R3-AR

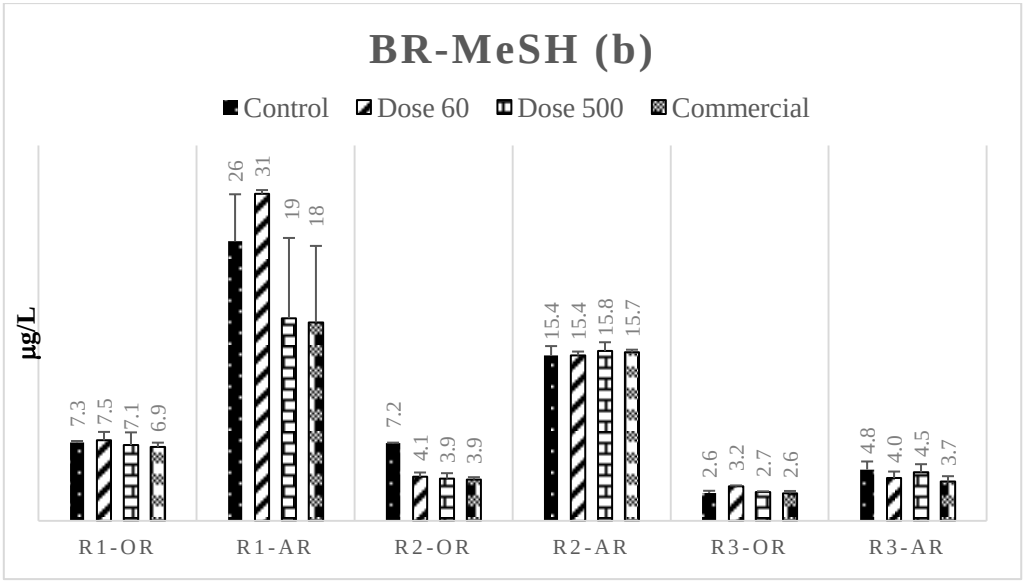
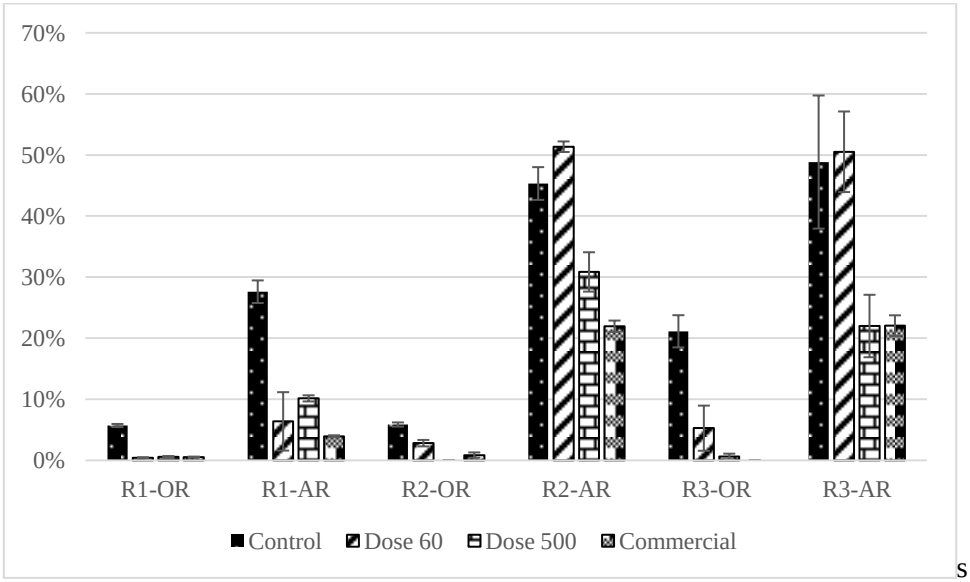
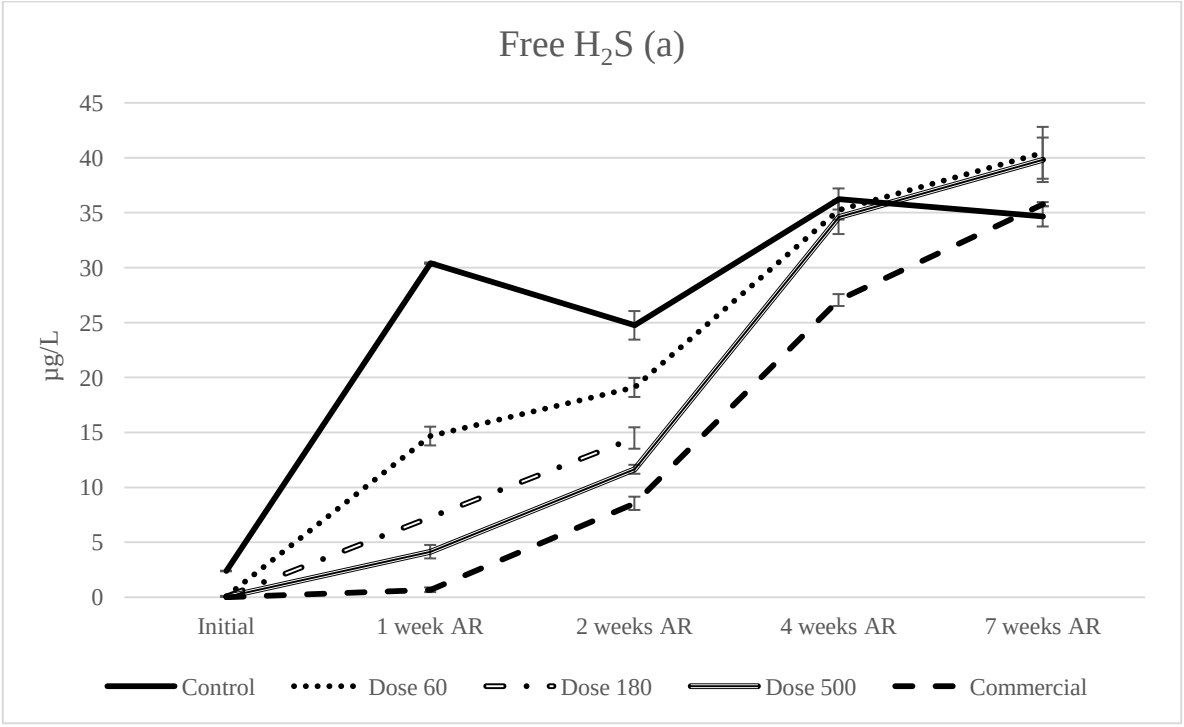

BR-MeSH (b)
Control
Dose 60
Dose 500
Commercial
µg/L
7.3
7.5
7.1
6.9
26
31
19
18
7.2
4.1
3.9
3.9
15.4
15.4
15.8
15.7
2.6
3.2
2.7
2.6
4.8
4.0
4.5
3.7
R1-OR
R1-AR
R2-OR
R2-AR
R3-OR
R3-AR

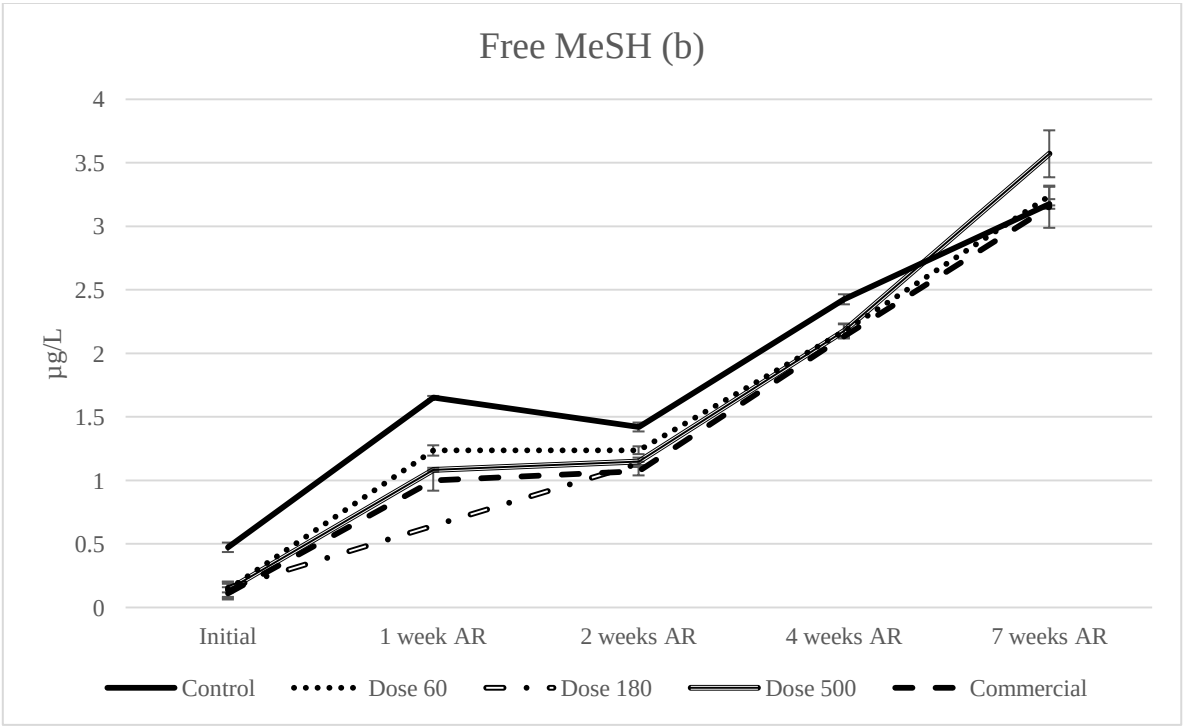
Figure 3


70%
60%
50%
40%
30%
20%
10%
0%
R1-OR
R1-AR
R2-OR
R2-AR
R3-OR
R3-AR
Control
Dose 60
Dose 500
Commercial

s

Figure 4


Free H_2S (a)
45
40
35
30
25
20
15
10
5
0
µg/L
Initial
1 week AR
2 weeks AR
4 weeks AR
7 weeks AR
Control
Dose 60
Dose 180
Dose 500
Commercial


Free MeSH (b)
4
3.5
3
2.5
2
1.5
1
0.5
0
µg/L
Initial
1 week AR
2 weeks AR
4 weeks AR
7 weeks AR
Control
Dose 60
Dose 180
Dose 500
Commercial

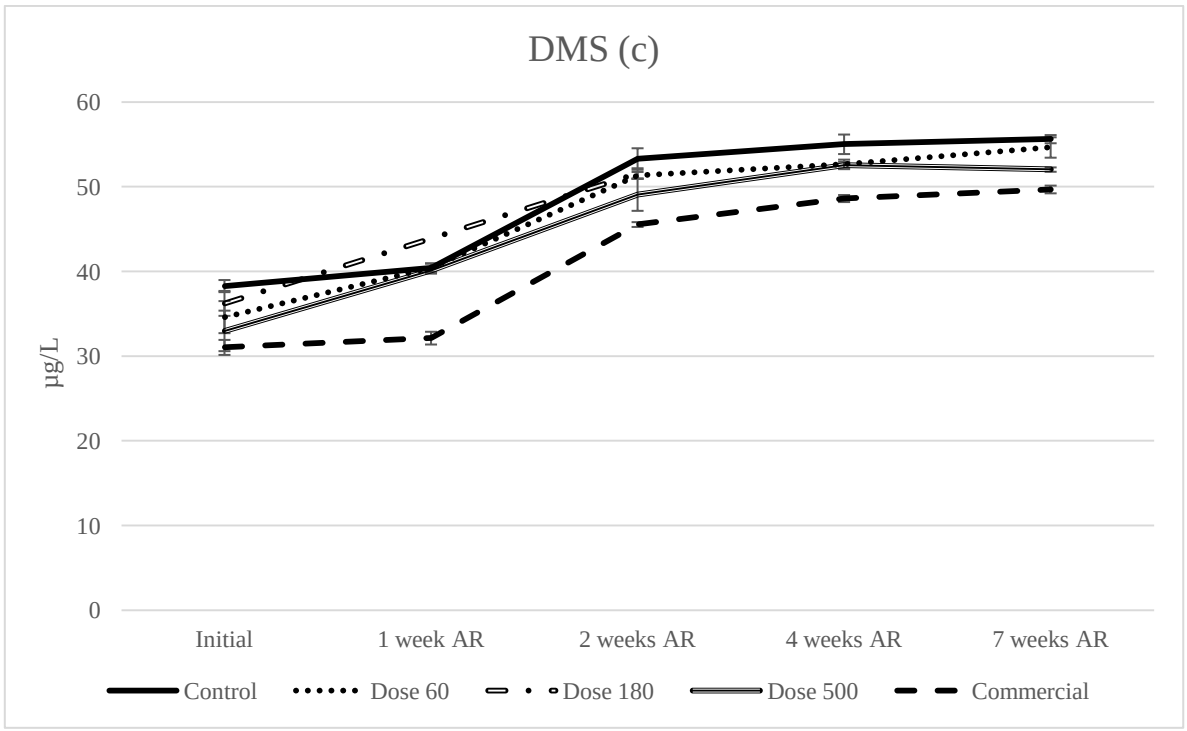
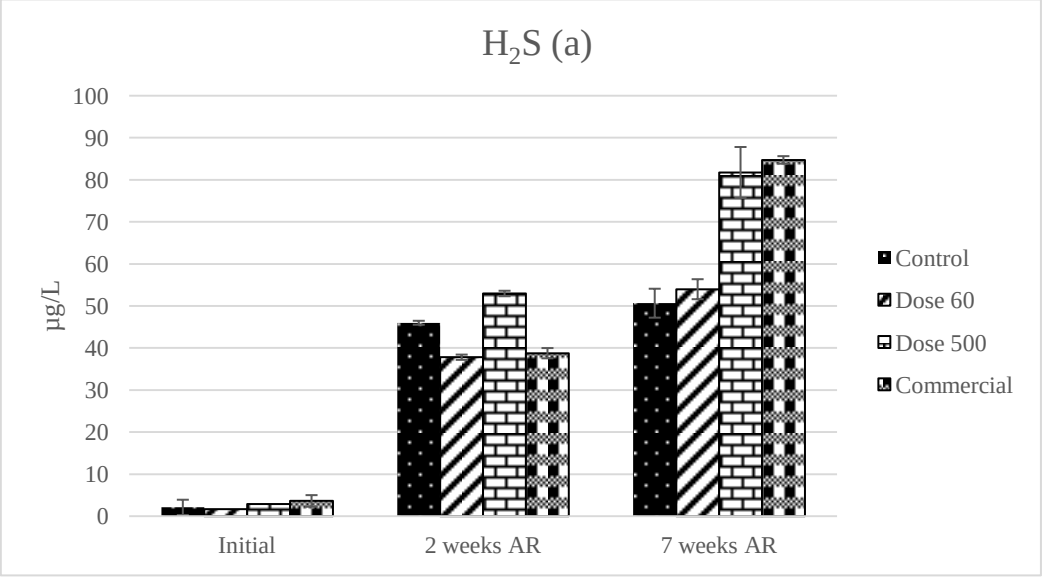

DMS (c)
60
50
40
30
20
10
0
µg/L
Initial
1 week AR
2 weeks AR
4 weeks AR
7 weeks AR
Control
Dose 60
Dose 180
Dose 500
Commercial

Figure 5


H2S (a)
100
90
80
70
60
50
40
30
20
10
0
µg/l
Initial
2 weeks AR
7 weeks AR
Control
Dose 60
Dose 500
Commercial

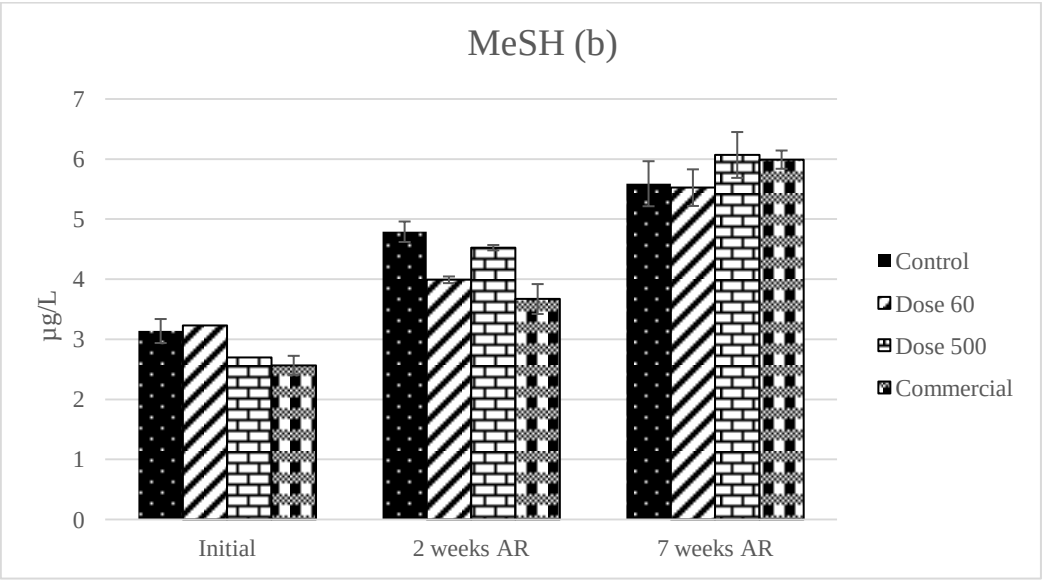
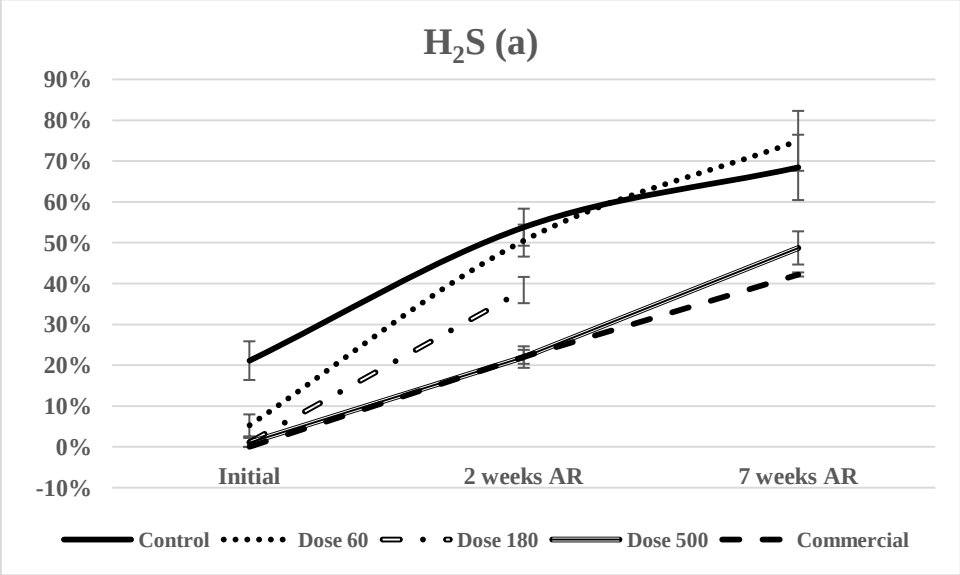
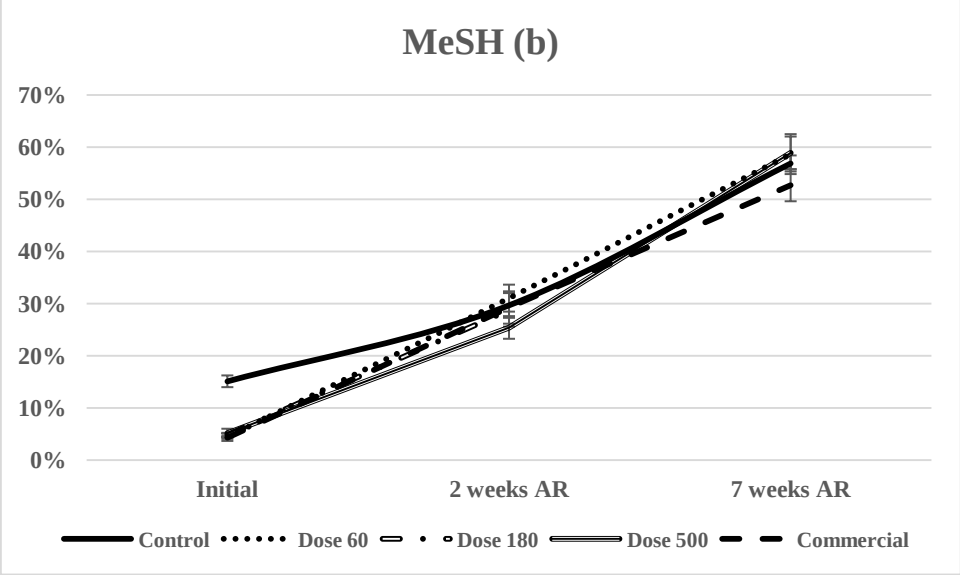

MeSH (b)
7
6
5
4
3
2
1
0
µg/l
Initial
2 weeks AR
7 weeks AR
Control
Dose 60
Dose 500
Commercial

Figure 6


H2S (a)
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
-10%
Initial
2 weeks AR
7 weeks AR
Control
Dose 60
Dose 180
Dose 500
Commercial


MeSH (b)
70%
60%
50%
40%
30%
20%
10%
0%
Initial
2 weeks AR
7 weeks AR
Control
Dose 60
Dose 180
Dose 500
Commercial