Elusive chemistry of hydrogen sulfide and mercaptans in wine

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Journal of Agricultural and Food Chemistry, **2018**, 66: *2237-2246*

ABSTRACT

This paper summarizes, discusses and complements recent findings about the fate of H_2S and methanethiol (MeSH) during wine storage. Analytical assays to determine free volatile sulfur compounds (VSCs) and brine-releasable (BR-) VSCs in combination with accelerated reductive (AR) aging and micro-oxygenation (MOX) assays allow characterizing the different categories of species able to produce H_2S and MeSH and the processes of interconversion. Each wine seems to contain a specific total amount of H_2S and MeSH distributed into free, metal-complexed and oxidized forms (di and polysulfides) interconnected through reversible redox equilibria whose external expression is wine redox potential. Oxidation transforms all mercaptans likely into non-volatile disulfides and hydrodisulfides. In anoxia, these molecules are spontaneously and quantitatively reduced back. The concomitant accumulation of major wine thiols would provoke complex dissociation and the release of free H_2S and MeSH. Additionally, total amounts can increase due to the metal-catalyzed desulfhydration of cysteine and methionine.

Key words: reductive off odors, sulfides, disulfides, copper, iron,

INTRODUCTION

In wine science, reduction refers to a series of off-odors related to an array of sulfur compounds displaying characteristic notes such as rotten eggs, decaying seaweed, putrefaction or cooked cabbage.[1] These off-odors are of great concern in the wine industry, since they can develop after the wine has been bottled when no remedial action is possible.[2] It has been estimated that they constituted nearly a third of the faults encountered in the wines sent to one of the largest international contests.3(Goode/Harrop) These off-odors are primarily caused by the sulfhydryl compounds (-SH) H_2S and MeSH, and eventually other mercaptans and volatile sulfur compounds (VSCs).[1] They are normal by-products of alcoholic fermentation[4,5] but they are easily purged out by the stream of CO_2 produced during fermentation and, in most cases, only little subthreshold levels remain not posing any sensory problem. However, in a number of situations related to different causes, such as N-deficient musts,[5] specific strains of yeasts,[6] or less frequently, B group vitamin deficiencies, or a too high protein or glutathione level in must7,8 suprathreshold levels of these compounds can accumulate. Additionally, the widespread use of reductive winemaking techniques in which the contact of O_2 is minimized throughout the winemaking process has increased the frequency of appearance of this problem.[9] If these compounds are formed, winemakers decrease their levels by copper fining, aeration or addition of lees.[10-13] In general, these remedial actions have an immediate impact decreasing the intensity of the sensory problem, but their long term effectiveness is under question. By experience, winemakers know that once a wine shows a tendency to develop reductive off-odors, it seems to become susceptible to suffer this problem again during its shelf-life,[14] particularly when it is stored in a bottle closed with a closure with low permeability to oxygen.[15] This suggests that these treatments affect the

odor-active molecules but not to their different precursors. Up to this date, these precursors have not been identified.

One reason why the progress in research is being so difficult lies in the complicated chemistry of sulfur. This element can be present in different redox states, has the ability to concatenate making bonds with itself and can form a large number of combinations not only with carbon atoms, but with some metal cations present at low concentrations in wine, such as copper, iron or zinc. Furthermore, small molecules or simple complexes tend to aggregate forming clusters containing S atoms in different redox states.[16-18] Additionally, the long time span required to observe the capricious appearance of reductive off-odors during wine aging has not helped. This complicated scenario has just recently begun to be understood when new chemical assays have made it possible to recognize the existence of different species of these molecules interconnected via different chemical equilibria[19-23]. Additional recent works revealing key details about the interactions between sulfides, copper, iron and oxygen[18,22-24] have provided an essential theoretical framework to understand the relationship between the different species.

The present paper summarizes the most relevant recent findings and adds new complementary experimental material or data analyses essential for understanding unclear aspects. With all the pieces at hand, a basic theoretical model explaining the underlying chemistry of these deleterious molecules has been formulated.

Basic tools for the study of the processes related to reductive off-odors

Three different categories of analytical strategies have to be used in combination in order to get enough information about the species related to reductive problems:

1. Direct headspace methods analyzing vapors on the undisturbed, undiluted wine sample for measuring exclusively free forms of H_2S and mercaptans.[20,25] It should

be noted that strategies commonly used to gain sensitivity, such as the addition of salt[1,26] or SPME preconcentration[26-27] will affect equilibria between free and complexed forms which could bias the results.

2. Brine-dilution headspace methods. Two different strategies have been so far proposed. In the first one the vapors emanated from the wine strongly diluted in brine are pre-concentrated in a SPME fiber.[20,28] The second strategy concentrates the headspace on the brine-diluted wine directly on a gas adsorbing tube.[19] Since no redox reagents are involved, it can be assumed that only species in which sulfur is already as sulfide –complexes with metal cations and the free sulfhydryl fraction- will be detected in this brine releasable (BR) fraction. The complexed fraction can be obtained simply by subtracting the free fraction from the BR-fraction. Alternatively, it is possible to calculate the quotient between free and BR-forms, α, which provides the percentage of sulfide under free form. In previous works, the BR-fraction was improperly referred as the "total" fraction[20,21,29] because in many commercial red wines it remained relatively stable during reductive aging. Recent works with un-bottled or recently micro-oxygenated wines[12,23] have revealed, however, that these BR fractions are not stable and that therefore cannot be referred to as "total".
3. Accelerated reductive (AR) assays. Two different types of these assays have been proposed. The first one consists of heating a volume of wine in a completely anoxic environment for some weeks;[21] the second one involves the addition of a strong reducing agent such as TCEP, eventually together with a Cu(I) chelator, such as BCDA.[19,22] Then, these reduced wines can be further analyzed by the methods in categories 1 and 2. In the case of the accelerated anoxic strategy, it has been demonstrated that the levels of both free and BR-forms obtained after 12.5

days at 50ºC correlate well with the levels of these forms measured in the wines after 1 year of anoxic storage.[29]

Changes in free and BR-forms during anoxic storage

Increases in free forms and in α: release. The most obvious effect of storing a wine under completely anoxic storage is the increase in the levels of free forms of H_2S and MeSH,[12,21,23,29] which is the cause of the appearance of reductive off-odors. Such increase is due in part to the increase in α, the percentage of H_2S or MeSH under free forms. This can be clearly seen in Figure 1, which represents the evolution of this parameter during the accelerated anoxic storage of bottled red wines.[21] Similar results have been observed with other wines and storage conditions[12,21,23,29] meaning that one of the most relevant processes taking place during anoxic storage of wines is the dissociation of metal-complexed forms to release free sulfhydryl forms. In bottled red wines release accounted for 90% of the observed increase in free H_2S, while in whites and rosé wines it could explain just 24% of the increases of free MeSH.[21]

Increases in BR-forms. The BR fraction can also suffer relevant changes during aging. This is particularly evident in the case of MeSH, whose BR-levels continuously increase in a fairly linear way during anoxic storage, regardless of the temperature.[21,29,30] Those increases accounted for 50-75% of the total increments of free MeSH observed in AR-aging and were particularly important in white and rosé wines. At room-temperature, normal bottled wines were found to produce between 3 and 7.5 ng/L of BR-MeSH per day of anoxic storage.[29] Wines particularly affected by reductive off-odor problems will likely form higher amounts. These increases in BR-forms were attributed to the "de novo" formation of MeSH, most likely from the metal catalyzed desulfhydration of methionine,[30] as will be later confirmed.

100 The evolution of BR-forms of H_2S during anoxic aging is more complex and at least three
101 clearly different patterns can be identified in the literature,[12,21,23,29] as summarized in
102 Figure 2. The figure shows data from a normal bottled red wine taken from reference[21]
103 and from two other cases taken from reference.[12]
104 The stability pattern followed by W1 seems to be the main evolution pattern followed by
105 normal bottled red wines.[21] Only in 4 out of 16 cases there was a slight (3-6.7 µg/L)
106 increase in BR-levels after 3 weeks of AR-aging.[21] In white and rosés, however, levels of
107 BR-H_2S tended to increase continuous and linearly with aging time (4.6-12.6 µg/L after
108 3 weeks of AR-aging).[21] The evolution pattern followed by W2 in the figure is expected
109 to be followed by wines having suffered some previous oxidation. W2 (R3 in the
110 reference[12]) is a red wine which had been subjected in the cellar to a MOX treatment 2
111 months before the analysis. In this case, BR-H_2S strongly increase during the first 2 weeks
112 of reductive aging, to plateau in the final period. The initial increases are likely due to the
113 reduction of BR-H_2S forms previously oxidized during the MOX treatment.[23] Finally,
114 W2_Cu is this same wine after a copper fining treatment which left a final copper content
115 close to 0.5 mg/L. In this case, BR-H_2S forms continuously increase during the complete
116 aging process reaching values well above those observed for W2, suggesting that
117 increased levels of copper induce a "de novo" formation of BR-forms.
118 Therefore, increases in BR-forms seem to have two different sources:
119 1. The reduction of previously oxidized BR-forms[23]
120 2. The "de novo" production likely from amino acids
121 This second possibility will be specifically addressed in the following section.
122
123 **De novo formation of H_2S and MeSH from metal catalyzed desulfhydration of S-**
124 **amino acids.** The metal catalyzed desulfhydration of S-amino acids has been

125 demonstrated to occur in aqueous solutions at high temperatures.[31] In wine, the likely
126 involvement of cation metals in the formation of H_2S and MeSH has been previously
127 observed.[12-14] In another report, increases of BR-MeSH in some samples were correlated
128 to the levels of methionine and aluminum of the wine.[30]
129 Additional clues about the involvement of S-amino acids and metals on the accumulation
130 are found in Table 1. The table summarizes results from a PLS-modeling carried out on
131 data reported in reference[21] relating increases of BR-forms to the wine chemical
132 composition. Although the models are complex, they reveal the existence of an evident
133 link between increases of BR-H_2S and BR-MeSH and the corresponding S- containing
134 amino acids, and in some cases, also with metals. The model for red wines (1, Table 1)
135 suggests that the de novo production of H_2S is positively related to the cysteine content
136 of the wine. In the case of whites and rosés the model is more explicit (2, Table 1) and
137 suggests that increases in BR-H_2S are basically the result of the metal catalyzed
138 desulfhydration of cysteine. Similarly, the accumulation of BR-MeSH in red (3, Table 1)
139 and white and rosé wines (4, Table 1) are related to the wine levels of methionine, and in
140 the case of white and rosé wines, also to metals.
141 Finally, results of an experiment specifically carried out to check the ability of metal
142 cations to catalyze the desulfhydration of cysteine and methionine are summarized in
143 Figure 3. Results confirm that copper can significantly induce the formation of BR-forms
144 of these VSCs and that polyphenols are essential for the catalytic reaction (data not
145 shown). The negative effects played by Mn and Al, as suggested in model 2 (Table 1),
146 were also confirmed, while the effects of Fe and Zn on H_2S and MeSH formation were
147 not. The understanding of the specific effects played by metal cations and their different
148 chelators including polyphenols, will have to be addressed in future research.
149

Pools of species precursors of free H_2S and MeSH

Taking into account all the previous elements, it can be concluded that the tendency of a specific wine to release free H_2S and MeSH during reductive aging depends on its content in three different pools of compounds:

1. BR-forms (free plus metal complexed forms)
2. Oxidized precursors, such as disulfides or polysulfides
3. Cysteine, methionine and other S-containing species together with a catalytic system able to induce their desulfhydration. This pool is particularly important in MeSH production

The AR-aging assays hardly differentiate between 2 and 3, because reduction and desulfhydration can take place simultaneously during AR-aging. A fine assignment will require the use of chemical reducing agents, such as TCEP,[19,22] after demonstrating that they not promote desulfhydration, and/or specific HPLC-MS strategies devoted to measure oxidized precursors.[22]

Leaving aside the catalytic systems able to induce "de novo" formation, it can be considered that each wine contains a fixed pool of H_2S and MeSH, which will be referred as the "**present total amount**" of these molecules, distributed into three differentiable fractions: free; metal bound; and oxidized precursors. The following part of the paper will be devoted to understand how the three fractions are related.

Wine redox potential

It is important to differentiate between the thermodynamic redox potential and the measured redox potential since this last one will reflect exclusively the redox pairs which are electrochemically active at the surface of the electrode. In the many cases in which the solution contains redox pairs interconnected by non-reversible or very slow reactions

175 at the electrode surface, the experimentally determined potential will not reflect the true
176 thermodynamic redox potential but a figure related to the potential at which the oxidation
177 and reduction currents in the electrode equilibrate.[32] Those currents are generated by the
178 redox pairs active in that electrode and will typically evolve as the slow redox reaction
179 progress.[33] In practice, this means that redox measurements are effective only in
180 anaerobic solutions[34] or in solutions dominated by iron ions.[33,35]
181 In the case of wine, the redox potential is a standard measurement[36] whose experimental
182 value strongly depends on wine pH, O_2 concentration,[32,37] electrode material and ethanol
183 levels,[32,38] but not on the wine content in polyphenolic antioxidants.[39,40] In the absence of
184 O_2, the changes observed in wine redox potential during its oxidation or reduction seem
185 to be related to the changes in the ratios Fe(II)/Fe(III) and cysteine/cystine (or
186 GSH/GSSG), as will be shown.
187 The effect of cysteine on the redox potential of wine models containing or not copper and
188 iron can be appreciated in Figure 4. In the absence of cysteine and in the presence of
189 Fe(II), the redox potential of the system is around 103±2 mV. The addition of Cu(II) has
190 no effect on the potential, while the presence of Cu(I) makes the potential decrease
191 slightly but significantly to 82±3 mV. The addition of cysteine causes a strong decrease
192 in the redox potential, indicating that this amino acid is active in the electrode. The final
193 redox potential depends primarily on the concentration of cysteine and on that of Fe(II),
194 but the presence of either Cu(II) or Cu(I) does not seem to have much effect. The most
195 negative redox potential is attained in the wine model containing only cysteine.
196 The dependence between the redox potential and the molar fraction of reduced forms of
197 a selected number of redox pairs present in wine models is summarized in Figure 5. As
198 can be seen for the three Fe(II)/Fe(III) systems represented in the figure, the redox
199 potential associated to a given redox pair is extremely dependent on the specific

compositional parameters of the solution. This means that redox potential cannot be used to compare between wines, but still it will be useful to monitor how the main redox pairs present in the wine evolve during oxidation or reduction. Another remarkable observation is that the pair Fe(II)/Fe(III) determines the potential in the right part of the plot (E>50 mV), while the system cysteine/cystine determines redox potential in the left part (E<50 mV).

The pivotal role of redox potential in the distribution of species of H_2S and MeSH

The most obvious change observed during reductive storage of wine is a decrease in the wine redox potential. This can be seen in Figure 6 which represents the evolution of the average redox potentials of two sets of wines during their anoxic storage at 50°C.[21]

In the case of red wines, there is a continuous decrease during the storage (significant at $P<10^{-7}$), while in whites and rosés the decrease takes place only in the first 12 days of storage (significant at $P<0.01$), while it increases again in the last sampling point (significant at $P<0.01$). Similar results have been obtained in the anoxic storage of wines at room temperature[29] and in the AR-aging of wines subjected to MOX.[23] Decreases in the redox potential indicate that wines become enriched in the reduced forms of the redox pairs active at the electrode surface. The source of those electrons is not well understood, but it could be related to the presence in wine of many polyphenols with antioxidant properties which may undergo spontaneous condensation reactions even in strict anoxia.[41]

Redox potential plays an essential role determining the relationship between the three differentiable fractions (free; metal bound; oxidized precursors) forming the "present total amount" of H_2S and MeSH contained in a wine.

The relationship between BR-forms (free + metal bound) and redox potential can be seen in the plot in Figure 7. The plot compiles data from wine W2 in reference[23] and represents

225 the BR-H_2S levels of the 12 samples -initial wine + 3 MOX samples + (2 x 4) AR-aging
226 derivatives each- derived from the MOX experiment carried out with this particular wine
227 versus their corresponding measured redox potentials. The three MOX samples are those
228 with redox potentials above -50 mV, while the initial wine and the 8 samples subjected
229 to AR-aging all had negative redox potentials. As discussed in such reference, the figure
230 is completely equivalent to the characteristic sigmoid observed in a redox titration in
231 which a reducing agent (H_2S) is quantitatively oxidized by an oxidant (O_2 mediated by
232 copper and iron, as suggested[18,24]). It is also most evident the similarity between the
233 sigmoid shown in the plot and the function relating the molar fraction of cysteine to the
234 redox potential shown in Figure 5. The linearization of the sigmoid by means of the logit
235 transformation[23] suggested that it corresponds to a two-electron reversible redox process
236 with a formal potential around -82 ± 2.2 mV. Since reduced samples were obtained both
237 from the initial wine and from the MOX samples the process has to be highly reversible,
238 and is consistent with a "total amount" of H_2S close to 160 µg/L in this particular wine.
239 Such total amount can be present as BR-forms (sulfide) or as oxidized forms, non-
240 detectable by the BR-method, and the proportion of both forms is determined by the redox
241 potential.
242 The distribution of BR-forms into free and metal-complexed forms is also determined by
243 the redox potential. This was seen in in references[23,29] and is further supported in Figure
244 8, which shows unpublished data from the experiment described in reference.[21] The plots
245 represent the average α fractions of free H_2S and free MeSH of 16 red wines and 8 whites
246 and rosé wines subjected to AR-aging different times (1.5, 5.5, 12 and 21 days). As was
247 already seen in Figure 6, redox potentials become more negative during AR-aging and,
248 concomitantly, the metal-complexed fraction decreases so that α increases. The close
249 correspondence between α and redox potential suggests that complex formation and

250 dissociation are also highly reversible processes. This is particularly evidenced by the fact
251 that the reversion in the redox potential suffered by whites and rosé wines after 21 days
252 of AR-aging (see Figure 6) is followed by a decrease in α for H_2S, as seen in Figure 8.
253 The α/redox potential plots are less sigmoid in shape than those observed in figures 5 and
254 7, which may be compatible with the partial displacement of the complex equilibria by
255 the addition of a competing complexing agent, such as cysteine. Additionally, as reported
256 in reference,[23] the process of release of free forms by dissociation of metal complexed
257 forms, takes place at potentials significantly slightly more reductive than those at which
258 oxidized precursors are reduced into BR-forms. This observation indicates that in a
259 spontaneous reduction process, the reduction of oxidized precursors takes place before
260 complex cleavage. This in fact could imply that cleavage is the result of the competing
261 action of the wine major thiols (cysteine and glutathione) formed by reduction of their
262 oxidized forms.
263 There are more evidences supporting this hypothesis. First, the amounts of free H_2S
264 released after AR-aging in the wines in the reference[21] are significantly correlated to $1/C_{Cu}$
265 ($r=0.750$ and $r=0.950$, both significant at $P<0.001$, for reds and for whites and rosés,
266 respectively) and similar observations have been made in the copper-fining study.[12] Since
267 among wine cations, copper has highest bonding constants with sulfhydrils, wines
268 containing more copper will release a smaller fraction for an equivalent production of
269 competing thiol. Second, some satisfactory models for predicting the amounts of free H_2S
270 and MeSH released from their complexed forms have been derived and are given in Table
271 1 (models 5 to 8). The two models for H_2S suggest that a high release of this molecule
272 will require a high BR-H_2S/Cu ratio and a low redox potential. In the case of MeSH, a
273 high release requires also high levels of complexed MeSH and a high complexed
274 MeSH/Cu ratio. The models support, essentially, that the increases in free forms

275 responsible for the appearance of reductive off-odors are due to the dissociation of metal
276 complexes induced by a drop in the redox potential. In practice this confirms that the
277 presence of copper makes more difficult and retards (but does not impede) the release of
278 free forms.
279
280 **Conclusions, hypotheses and perspectives**
281 All the previous facts lead us to formulate the following set of conclusions and
282 hypotheses:
283 1. Each wine contains a specific present total amount of H_2S and MeSH distributed into
284 different species. Depending on the wine, such total amount can increase with time
285 due to the metal-catalyzed desulfhydration of cysteine and methionine (de novo
286 formation). De novo formation is a poorly known process which constitutes a relevant
287 and common source of MeSH and is less frequent and important in the case of H_2S
288 2. Such total amounts of H_2S and MeSH are distributed into BR-forms and into oxidized
289 forms interconnected through reversible redox equilibria. These equilibria are of the
290 type:
291
292 $$H_2S + RSH \leftrightarrow RSSH + 2H^+ + 2e^-$$
293 $$MeSH + RSH \leftrightarrow RSSMe + 2H^+ + 2e^-$$
294 $$2\ RSH \leftrightarrow RSSR + 2H^+ + 2e^-$$
295 $$H_2S + RSSR \leftrightarrow RSSSR + 2H^+ + 2e^-$$
296
297 Where RSH can be cysteine or glutathione, the major thiols of wine, and RSSH is a
298 hydrodisulfide, RSSR is cystine or oxidized glutathione (or the mixed disulfide) and
299 RSSSR is a trisulfide. RSSH and RSSSR are examples of oxidized forms of H_2S and
300 RSSMe of MeSH. Formation of some of these putative molecules has been recently
301 described in model solutions and even in wine by Kreitman et al.[22] The oxidation will
302 follow, most likely, the mechanisms recently described by these same authors.[24] The

role played by copper should be complex since the reaction takes place via a copper complex, but some copper complexed forms seem to be quite resistant to oxidation.[17,23,42]

3. The degree of the displacement of the chemical equilibria schematized in the previous paragraph depends on the wine redox potential; or more precisely, impacts the wine redox potential since this parameter seems to depend on the cysteine/cystine (or GSH/GSSG) ratios. At positive redox potentials (>50 mV), most wine mercaptans will be as oxidized forms. It should be considered that Fe(III) and the thiol group cannot be simultaneously present, unless they are strongly protected by complex formation, as can be deduced from the mechanisms proposed[24] and from previous evidence presented by Rozan et al.[38] Results recently presented in reference[23] seem to confirm this, since the residual low levels of BR-H_2S found in the MOX samples with higher redox potentials, were directly correlated to the copper levels of the wine
4. BR-forms of H_2S and MeSH are further distributed into free (odor active) and into metal complexed (odorless) forms. Complexes are mainly with Cu(II), Cu(I), Fe(II) and even Zn(II).

$$M^{2+} + H_2S \longleftrightarrow MS + 2H^+$$
$$2Cu^+ + H_2S \longleftrightarrow Cu_2S + 2\,H^+$$
$$M^{2+} + 2\,CH_3SH \longleftrightarrow CH_3SMSCH_3 + 2H^+$$
$$Cu^+ + CH_3SH \longleftrightarrow CH_3SCu + H^+$$

The strength of the complexes decreases in the order Cu(II)>Cu(I)>Fe(II)>Zn(II) and H_2S>MeSH. Copper is a particular case as it has been demonstrated that in Cu(II)-S(-II) complexes there is a transference of charge between the Cu and S atoms, becoming temporally Cu(I)-S(-I), as described by Luther et al[16] and Kreitman et al.[18] The displacement of the different complex formation/dissociation equilibria to release free forms will be most likely caused by the accumulation of large amounts

of cysteine and glutathione formed by reduction of the corresponding disulfides accumulated when the wine has been previously in contact with oxygen:

$RSSR + 2H^+ + 2e^- \longrightarrow 2RSH$ (where R is Cys or Glu and the reduction is possibly induced by the spontaneous condensation of polyphenols)

$$MS + 2RSH \longleftrightarrow RSMSR + H_2S$$
$$Cu_2S + 2RSH \longleftrightarrow 2CuSR + H_2S, \text{ or}$$
$$Cu\text{-}SH + RSH \longleftrightarrow CuSR + H_2S$$

$$CH_3SMSCH_3 + 2RSH \longleftrightarrow RSMSR + 2CH_3SH$$
$$CH_3SCu + RSH \longleftrightarrow RSCu + CH_3SH$$

5. Wine redox potential tends spontaneously to become reductive by reasons that are not understood today. Preliminary data suggest that spontaneous polyphenol condensation reactions taking place in anoxia, could be a source of electrons which would first completely reduce Fe(III) to Fe(II) and later all disulfides and hydrodisulfides and maybe also polysulfides, hydropolysulfides, polysulfanes, to mercaptans and H_2S. In this sense, the sulfur atom seems to be the ultimate sink for the electrons that wine polyphenols tend to spontaneously release. It can be hypothesized that the redox potential will drop until all S(-I) has been reduced to S(-II) as long as the wine contains polyphenols able to undergo those spontaneous condensation reactions. Therefore, in the absence of oxygen, wine tends to a state of equilibrium which depends on its polyphenolic and total thiol content and which is characterized by a more or less negative redox potential at which the predominant forms will be free sulfhydryls in equilibrium with their metal complexed forms. Note that only at this negative redox potential, BR-forms represent the present total content in H_2S and MeSH of that wine and that α will be maximum, close to 1. It can be also hypothesized that the maximum value of α will depend on the RSH/Cu ratio of the wine.

6. When the wine comes into contact with oxygen, the following set of reactions will most likely take place:[22-24]

a) Formation of Fe(III)

$$Fe(II) + ½\ O_2 + H_2O \longrightarrow Fe(III) + H_2O_2$$

b) Oxidation of Cu(I) –complexed with S(-II)- to Cu(II)

$$Fe(III) + Cu(I)\text{-}SH \longrightarrow Fe(II) + Cu(II)\text{-}SH$$
$$Fe(III) + Cu(I)\text{-}SCH_3 \longrightarrow Fe(II) + Cu(II)\text{-}SCH_3$$
$$Fe(III) + Cu(I)\text{-}SR \longrightarrow Fe(II) + Cu(II)\text{-}SR$$

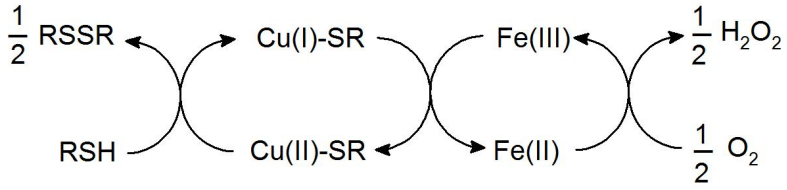
c) Formation of Cu(II)-S(-II) dimers

$$Cu(II)\text{-}SH + RSH \longrightarrow RS\text{-}Cu(II)\text{-}SH + H^+$$
$$Cu(II)\text{-}SCH_3 + RSH \longrightarrow RS\text{-}Cu(II)\text{-}SCH_3 + H^+$$
$$Cu(II)\text{-}SR + RSH \longrightarrow RS\text{-}Cu(II)\text{-}SR + H^+$$

d) Oxidation of S(-II) by Cu(II)

$$RS\text{-}Cu(II)\text{-}SH + RS\text{-}Cu(II)\text{-}SR \longrightarrow Cu(I)\text{-}SR + RSSH$$
$$RS\text{-}Cu(II)\text{-}SCH_3 + RS\text{-}Cu(II)\text{-}SR \longrightarrow Cu(I)\text{-}SR + RSSCH_3$$
$$2\ RS\text{-}Cu(II)\text{-}SR \longrightarrow Cu(I)\text{-}SR + RSSR$$

H_2O_2 will react with SO_2, the exogenous antioxidant of wine while is available, or will oxidize ethanol to produce acetaldehyde through the Fenton reaction. And if more oxygen is available, this will work in a continuous cycle as recently suggested.[24]



7. Even after having consumed relatively large amounts of O_2, (around 20 mg/L in ref. [23]), the process can be completely reversed recovering quantitatively the initial levels of "total" H_2S if the wine is again stored in anoxic conditions long enough. This suggests that disulfides and hydrodisulfides are quite resistant to oxidation. At present it is not clear when these compounds will be eliminated by reaction with quinones[43] or with other reactive oxygen species. It should be also considered that a wine containing in total 400 µM of thiols, is able to consume 100 µM of O_2 (3.2 mg) and will accumulate 200 µM of disulfides which will be a reservoir of oxidant able

392 to slowly consume up to 400 μM of electrons from polyphenols or other sources,
393 implying a deferred oxidation over time
394 8. During bottle aging wine receives a little ingress of oxygen through the closure whose
395 level depends on its specific oxygen transfer rate (OTR). The wine will reach an
396 equilibrium point at which the rate of formation of disulfides becomes similar to the
397 rate of their spontaneous reduction. Such equilibrium will be manifested by a specific
398 redox potential and by the corresponding levels of BR-forms and of α, meaning that
399 the OTR of the closure will influence the intensity of reductive off-odors.
400 The previous theory contains numerous statements which are just mere hypotheses, but it
401 seems to explain quite satisfactorily present evidence and provides a rational framework
402 which will help to design future research. It is also clear that further progress will require
403 a refinement of experimental techniques for characterizing and measuring the species
404 related to H_2S and mercaptans, as well as to characterize and measure the species related
405 to the changes in wine redox potential during anoxic storage.
406
407 **Supporting Information**. The detailed description of the experiments whose
408 unpublished results are presented in Figures 4 and 5 are given as Supporting Information
409

410 **ACKNOWLEDGEMENTS**

411 This work has been funded by the Spanish MINECO (projects ALI 2014-59840, RTC-
412 2015-3379 and RTC-2016-4935-2) and partly cofunded by the European Union
413 (FEDER). E.V. has received a grant from the Diputación General de Aragón. Funding
414 from Diputación General de Aragón (T53) and Fondo Social Europeo is acknowledged.
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Figure captions

Figure 1. Evolution of the fraction of H_2S and MeSH under free forms (α, in %) during the accelerated anoxic storage of red wines. Built with data from reference.[21] Data are the mean of 16 different wines

Figure 2. Evolution of BR-H_2S during the accelerated anoxic storage of three young red wines made with tempranillo (W1 from Ribera-Duero from ref,[21] W2 from Rioja was previously micro-oxygenated). W2_Cu is W2 treated with 0.5 mg/L of $CuSO_4$ [12]

Figure 3. Effect of metal cations on the formation of BR-H_2S and BR-MeSH during 2 weeks of AR-aging in wine models (11% v/v ethanol, 3 g/L glycerol, 5g/L tartaric acid, pH 3.5, 50 mg/L of gallic acid and of catechin, 20 mg/L of caffeic acid) containing 20 mg/L of L-Cys and L-Met. Metal cation concentration: Cu(II), 0.5 mg/L; Fe(II), 2 mg/L; Al(III), 1 mg/L; Mn(II), 0.8 mg/L; Zn(II), 0.6 mg/L. Experimental details given as SP.

Figure 4. Effect of the level of Cysteine on the redox potential of wine models (12% ethanol, 5 g/L tartaric acid, pH 3.5) containing or not 5 mg/L Fe(II), 0.6 mg/L Cu(II) or 0.6 mg/L Cu(I). All the models were carefully prepared in the anoxic chamber with Ar-bubbled solutions. Potentials were measured after 15 min, except the one containing just Fe(II), which required 2 days of stabilization. Experimental details given as SP.

Figure 5. Plots relating the ratio [reduced form]/[oxidized + reduced forms] to the redox potential in 4 different wine model systems, three containing different ratios of the Fe(II)/Fe(III) redox pair, and one of the Cysteine/Cystine. All models contained ethanol, 5 g/L tartaric acid with pH 3.5 and 5.0 mg/L of total iron. Iron pairs: 14%(v/v) ethanol; 12% ethanol, 1 g/L citric acid; 14% ethanol, 1 g/L citric acid + 1 g/L glutamic acid. Cysteine/Cystine pair: 12% ethanol, 45 mg/L Cys+Cystine, 5.0 mg/L Fe(II). Experimental details given as SP.

Figure 6: Evolution of the average redox potential of 15 red wines and 8 whites and rosés stored in strict anoxia at 50°C. Error bars are standard errors of the corresponding means. Data taken from the work presented in ref.[21]

569 **Figure 7**. Relationship between the BR-H_2S level of a wine and its redox potential. Data
570 correspond to a red wine made from Syrah subjected to tree different MOX treatments. Initial
571 wine and the three MOX samples were further subjected to AR-aging (2 and 7 weeks)
572 **Figure 8**. Plot showing the relationship between the average redox potential of 16 red wines
573 stored at 50°C and the α fraction of H_2S present as free forms (expressed as % of BR-forms).
574 Error bars are the standard error of the means. Numbers indicate the AR-aging time in days

Table 1. PLS models explaining increases in BR-forms of H_2S and MeSH, in their corresponding proportions in free forms (α) and decreases in the redox potential during the anoxic storage of wines. These models have been built from data presented in reference.[21] Positively correlated compounds are boldfaced. Abbreviations are given in the legend

Nº	Wine type/Parameter	EVar	RMSE	Model (regression coefficients)
1	Red wines. De novo formation of H_2S	90%*	0.76	-0.31 + **0.224 LPP** + **0.227 Isorhamnetin** + **0.197 Vitisin A** + **0.177 Cysteine** + **0.163 Pyranoanthocyanins** – 0.182 epigallocatechin (thiolysis) – 0.169 proanthocyanidins
2	Whites and Rosés. Rate of increase of BR-H_2S (de novo formation rate)	97%	0.021	3.44 + **0.402 Fe** + **0.167 Cu** + **0.104 Cysteine** – 0.27 Mn – 0.27 Al – 0.225 freeSO_2
3	Red wines. Rate of increase of BR-MeSH (de novo formation rate)	88%*	0.0059	7.03 + **0.149 Methionine** + **0.164 Ethyl caffeate** + **0.170 Procyanidin A2** – 0.134 Initial redox potential -0.125 MP – 0.152 EC4b – 0.12 ECG -0.152 Procyanidins – 0.152 pH
4	White and rosé wines. Rate of increase of BR-MeSH (de novo formation rate)	91%	0.011	0.11 + **0.016 Methionine** + **0.0113 Zn** + **0.0142 pH** – 0.0199 Mn – 0.010 Initial redox potential
7	Red wines. Increase of free H_2S in 21 days	77%	2.94	3.27 + **0.216 BR-H_2S/Cu** + **0.155 Zn** – **0.233 Final redox potential**[a] – 0.247 *trans*-aconitic acid – 0.237 Cu– 0.151 Malvidin-3-O-glucoside – 0.141 A620
8	Whites & Rosés wines. Increase of free H_2S exclusively due to release from complexes	93%	1.02	5.64 + **0.332 BR-H_2S/Cu** – **0.212 ΔVmax**[a] – 0.427 Folin -0.272 t-coumaric acid – 0.227 vanillic acid
9	Red wines. Increase of free MeSH exclusively due to its release from complexes	87%	0.179	1.65+ **0.415 $MeSH_{complexed}$/Cu** + **0.457 $MeSH_{complexed}$** – 0.257 SPP
10	White & Rosés wines. Increase of free MeSH exclusively due to its release from complexes	88%	0.127	-0.324 + **0.263 $MeSH_{complexed}$** + **0.239 $MeSH_{complexed}$/Cu** + **0.221 TPI** +**0.241 MyrGal** – 0.222 Al

*two samples excluded; #one sample excluded

[a]*Since redox potential becomes more negative during the storage, the minus sign indicates that a higher drop implies higher release*

Abbreviations: LPP, large polymeric pigments; MP, mono pigments; EC4b, Epicatechin-4b-benyzlthioether; ECG, epicatechin-3-O-gallate; SPP, small polymeric pigments; mDP, mean degree of polymerization; TPI, total polyphenol index; MyrGal, Myricetin-3-galactoside

Figure 1

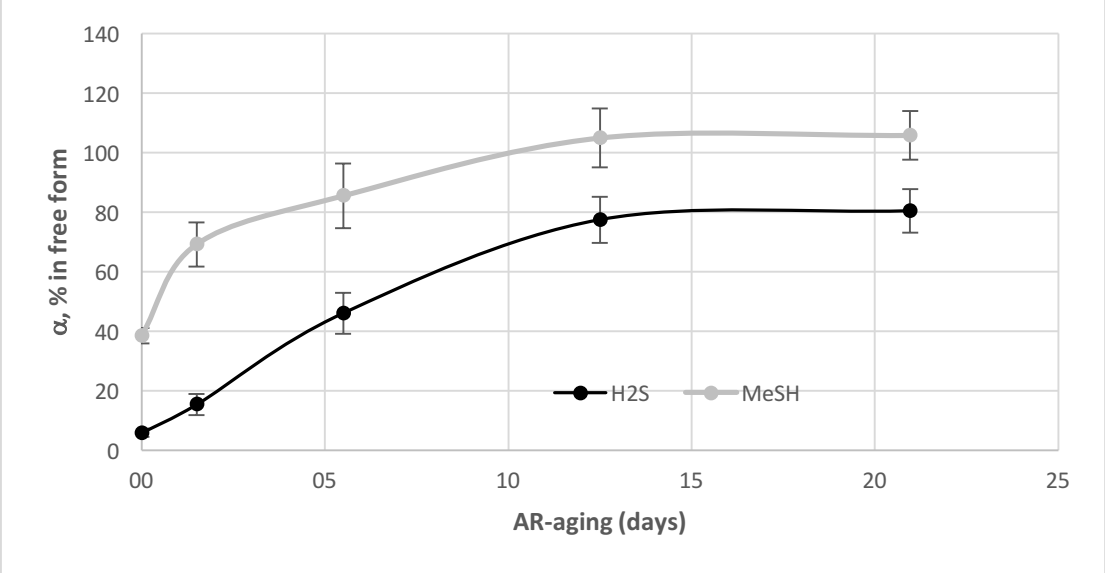

Figure 2

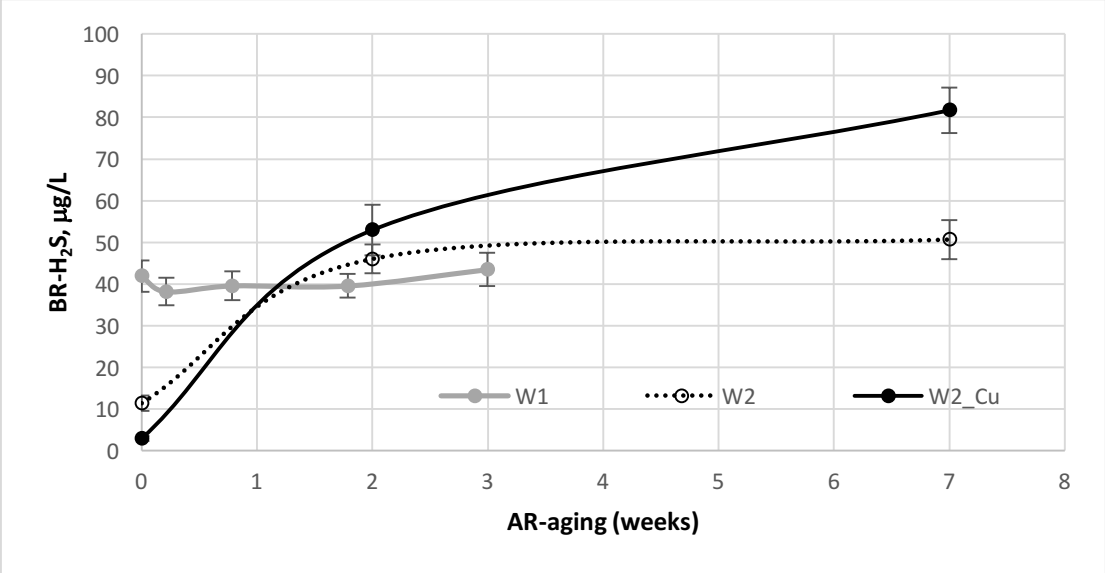

Figure 3

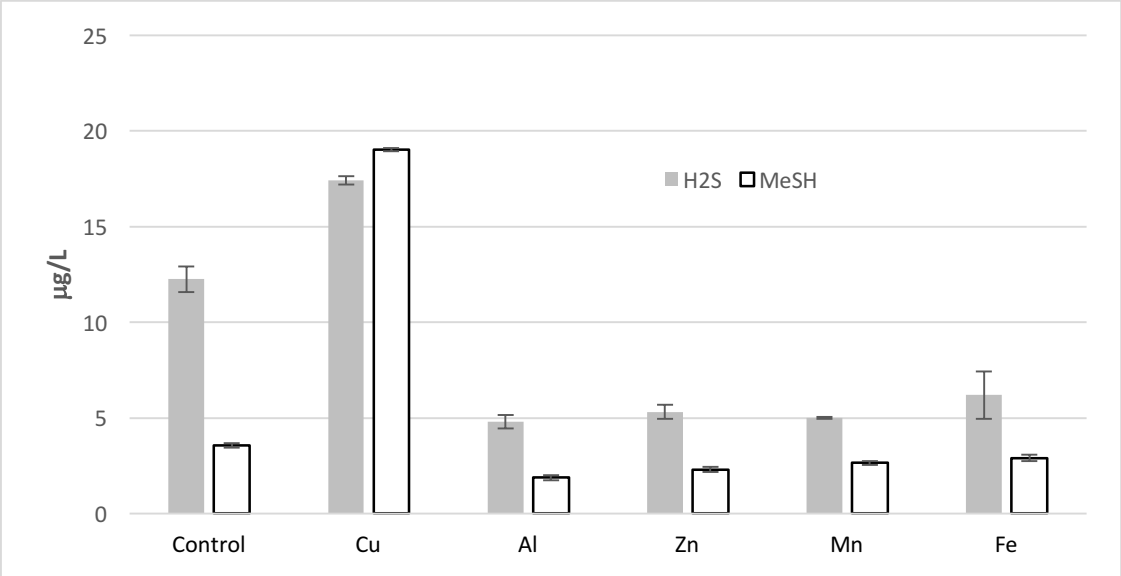

Figure 4

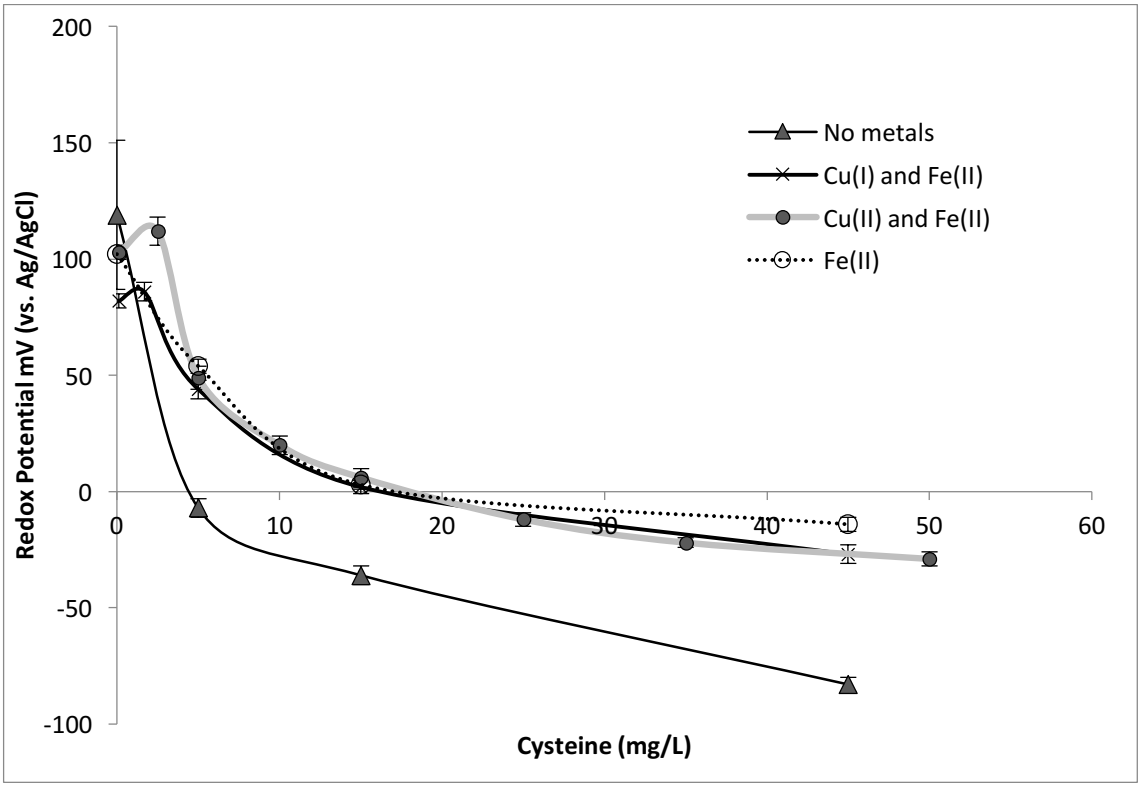

Figure 5

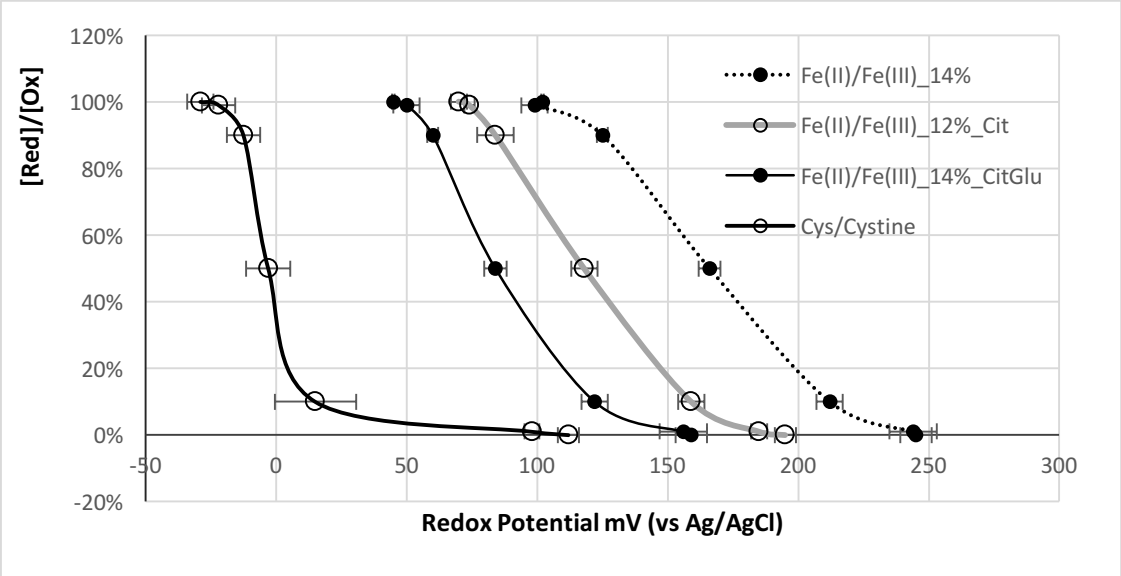

Figure 6

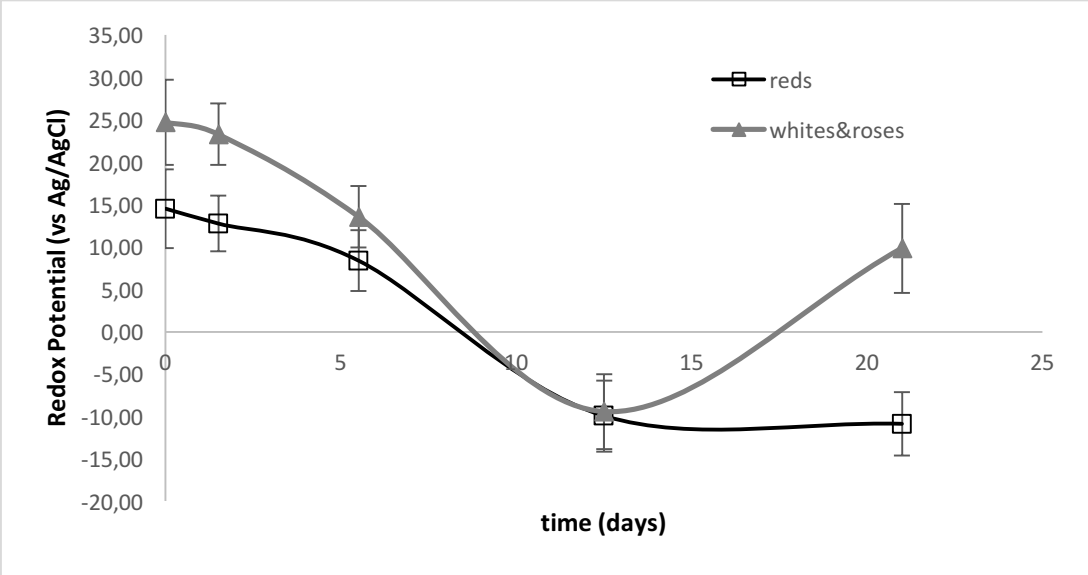

Figure 7

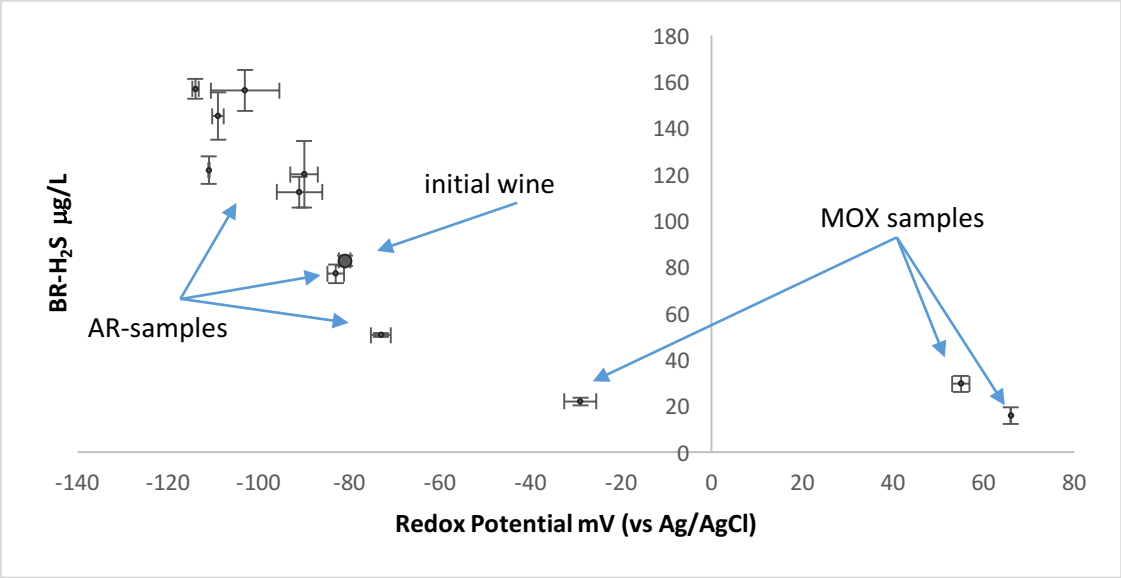

Figure 8