Reductive off-odors in wines: Formation and release of H_2S and Methanethiol during the accelerated anoxic storage of wines

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Abstract

In order to better understand the processes involved in the development of H_2S and methanethiol (MeSH) along anoxic storage of wines, 24 wines were stored in strict anoxia at 50°C for 3 weeks. Free and total forms of H_2S and MeSH were measured at different times. Results showed that: 1) All wines contain relevant proportions of bonded forms of H_2S and MeSH (93% and 47% on average); 2) such % decreases with age; 3) levels of total forms are related to wine metal composition; 4) anoxic storage brings about an increase of free forms, a strong decrease in the percentage of bonded forms, and except for H_2S in red wines, an increase in total forms. Both de novo formation and release contribute to reductive off-odors. Release is predominant for reds and H_2S, while at 50°C, de novo formation dominates for whites and rosés and MeSH

Keywords: Reduction, Copper, Sulfur, Bonded H_2S, Wine aging, de novo formation

1. Introduction

Reductive off-odors are a not infrequent outcome of wine production, particularly of bottle aging (Mestres, Busto, & Guasch, 2000; Park, Boulton, Bartra, & Noble, 1994; Siebert, Solomon, Pollnitz, & Jeffery, 2010; Ugliano, Dieval, Siebert, Kwiatkowski, Aagaard, Vidal, et al., 2012; Ugliano, Kwiatkowski, Vidal, Capone, Siebert, Dieval, et al., 2011) and are responsible for an important proportion of faulty wines. Such a problem is mostly caused by the development of H_2S and MeSH (Ugliano, et al., 2011), although a number of different other volatile sulfur compounds (VSCs) have been also identified (Mestres, Busto, & Guasch, 2000; Park, Boulton, Bartra, & Noble, 1994). A third relevant molecule, dimethyl sulfide (DMS), is also frequently included within the group of reductive problems. However, DMS strongly differs from H_2S and MeSH both in sensory effects (Escudero, Campo, Farina, Cacho, & Ferreira, 2007; Lytra, Tempere, Zhang, Marchand, de Revel, & Barbe, 2014; Segurel, Razungles, Riou, Salles, & Baumes, 2004) and in chemical origin and properties (Segurel, Razungles, Riou, Trigueiro, & Baumes, 2005), and should be considered apart.

It is usually thought that the most relevant source of reductive off-odors is the alcoholic fermentation and in fact, the development of the characteristic H_2S and MeSH odors during this key wine making step is sometimes clearly observed. H_2S can be directly formed by Saccharomyces from elemental sulfur (Schutz & Kunkee, 1977), sulfates or more easily from the sulfite (Jiranek, Langridge, & Henschke, 1995) usually added as antioxidant and antimicrobial agent. The formation is typically stronger in musts with low

54 levels of assimilable nitrogen (Jiranek, Langridge, & Henschke, 1995), although the factors
55 determining its synthesis are far for being clearly understood (Ugliano, Fedrizzi, Siebert,
56 Travis, Magno, Versini, et al., 2009). The demonstrated influence of methionine (Barbosa,
57 Mendes-Faia, & Mendes-Ferreira, 2012; Spiropoulos, Tanaka, Flerianos, & Bisson, 2000) or
58 cysteine levels (Jiranek, Langridge, & Henschke, 1995; Moreira, Mendes, Pereira, de Pinho,
59 Hogg, & Vasconcelos, 2002) on the formation of H_2S and MeSH undeniably suggests that
60 the formation of these components in fermentation depends on many factors. In the
61 event of an excessive formation of these compounds, winemakers try to control their
62 levels by copper finning, aeration or addition of lees (Clark, Grant-Preece, Cleghorn, &
63 Scollary, 2015; Ugliano, Kwiatkowski, Travis, Francis, Waters, Herderich, et al., 2009;
64 Viviers, Smith, Wilkes, & Smith, 2013).
65 The reasons why these molecules accumulate during bottle aging, more often in those
66 wines in which these compounds were previously formed in fermentation, are not clearly
67 known (Ugliano, Kwiatkowski, et al., 2009). Several hypothesis have been formulated
68 along the years, such as the hydrolysis of thioacetates (Leppanen, Denslow, & Ronkainen,
69 1980) or thioethers (Waterhouse & Laurie, 2006), the reduction of disulfides (Bobet,
70 Noble, & Boulton, 1990), the reaction between cysteine and wine α-dicarbonyls
71 (PripisNicolau, deRevel, Bertrand, & Maujean, 2000), the transition-metal catalyzed
72 reduction of sulfate or sulfite (Swiegers, Bartowsky, Henschke, & Pretorius, 2005) or the
73 metal catalyzed desulfhydration of sulfur containing amino acids (Gruenwedel & Patnaik,
74 1971; Viviers, Smith, Wilkes, & Smith, 2013). All these possibilities involve the “de novo”

formation of H_2S or MeSH from a specific precursor through a specific chemical route. Because the problem is most often observed in the absence of oxygen it is generally assumed that those processes involving a chemical reduction are the most likely candidates, and notably the reduction of disulfides is most often mentioned in the technical literature.

However, an important factor that should be additionally taken into account in order to understand the observed increases of H_2S and MeSH along wine bottle aging is the presence of relevant amounts of these two compounds under the form of non-volatile complexes with Cu(II) and other metal cations, as recently demonstrated (Franco-Luesma & Ferreira, 2014). The finding is further supported by the recent observation that Cu(II) is not easily removed from the wine by precipitation as CuS at the normal levels found in wineries (Clark, Grant-Preece, Cleghorn, & Scollary, 2015). These complexes are reversible, so that the simple dilution of the wine with brine releases back into the headspace H_2S and MeSH. This dilution coupled to a SPME preconcentration constitutes the base for the analytical determination of total (=free+bonded) forms (Lopez, Lapena, Cacho, & Ferreira, 2007). Such determination has to be complemented with a gentle direct headspace injection for the exclusive analysis of free forms to have a clear picture of the total balance of free and bonded forms (Franco-Luesma & Ferreira, 2014). The existence of those interconvertible species means that studies using a method sensitive exclusively to free forms, or exclusively to total forms, will see just half of the picture and most surely will not be able to make a clear diagnosis. For instance, in a previous aging

96 experiment involving 16 different red wines, and in which VSCs were monitored using the
97 strategy measuring total forms, no H_2S increase was observed even in the samples stored
98 under complete anoxia (Ferreira, Bueno, Franco-Luesma, Cullere, & Fernandez-Zurbano,
99 2014). Such an unexpected result raised the question of whether the increases of H_2S
100 observed in other reports using methods sensitive to free forms were in fact the
101 unnoticed result of the release of already present bonded forms or whether they were the
102 result of a genuine "de novo" formation which simply did not happen in that sample set.
103 The present paper aims at determining whether the often observed increases of H_2S and
104 MeSH along the anoxic storage of wines are the result of the de novo formation from
105 precursors or are rather the result of the release of complexed forms.
106
107 **2. Material and methods**
108 *2.1. Solvents and Chemical Standards*
109 Ethanol and methanol were purchased from Merk (Darmstadt, Germany). Water with
110 resistance of 18.2 MΩ·cm at 25 °C was purified in a Milli-Q system from Millipore
111 (Bedford, Germany).
112 Chemicals used for the analytical characterization were of analytical reagent grade and
113 were supplied by Merck (Darmstadt, Germany), Panreac (Barcelona, Spain), Sigma-Aldrich
114 (Madrid, Spain), Lancaster (Eastgate, UK), Scharlau (Barcelona, Spain), Oxford Chemicals
115 (Hartlepool, UK), Fluka (Madrid, Spain), ChemService (West Chester, PA, USA),
116 Extrasynthèse (Genay, France) and SAFC (Steinheim, Germany). Purity of chemical

117 standards is over 95% in all cases and most of them are over 99%. TSK Toyopearl gel HW-
118 50F was purchased from Tosohaas (Montgomery-ville, PA, USA).

119 *2.2. Wines*

120 Twenty-four Spanish wines were purchased from a local retailer in Zaragoza.
121 Denomination of origin, vintage, alcohol % (v/v), free and total SO_2, pH and metal content
122 are detailed in Table 1 of the Supplementary material.

123 *2.3. Accelerated reduction*

124 Wines were opened inside an anoxic glove box (Jacomex, France) containing Argon
125 atmosphere. Average levels of O_2 inside the anoxic chamber were below 2 ppm (v/v). The
126 levels of dissolved O_2 in the recently opened wines were measured with a calibrated
127 OptiOx SG 9 oxygen sensor (Mettler Toledo-España, Barcelona). The sensor was calibrated
128 daily following the manufacturer directions with water-saturated air by introducing it in a
129 test tube containing a wetted sponge for 5 minutes and activating the calibration function.
130 Wines were stirred for some minutes within the Ar chamber just letting the very small
131 amounts of dissolved oxygen (<0.2 mg/L) dissipate to ensure that at the beginning of the
132 experiment the levels of this molecule were below the limit of detection of the sensor (1
133 μg/L). The wine was then distributed in 60 mL tubes with highly hermetic closures (WIT,
134 Blanquefort, France). The tubes were completely filled avoiding any headspace. The tubes
135 were further enclosed in a vacuum thermos-sealed plastic bag with known O_2
136 permeability (< 9 cm^3 / m^2 24 h) supplied by Amcor (Barcelona, Spain). After this, the
137 bagged tubes were taken out of the Ar chamber and were then incubated in a water bath

138 at 50°C (Grant Instruments, Cambridge, UK) for 1.5, 5.5, 12.5 or 21 days, prior to further
139 analysis. Owing to the complicated logistics of the experiment, only one tube per wine
140 was used for analysis at each sampling point and was further discarded. Analyses of VSCs
141 were carried out in each tube in triplicate as described below.
142 The reproducibility of the accelerated reduction procedure was checked in an
143 independent experiment in which 20 tubes, 10 filled with the same red wine and 10
144 others with the same white wine were incubated at 50°C for 12.5 (5 tubes/wine) and 21
145 days (5 tubes/wine) and analyzed as described below.Some tubes filled with a wine model
146 (12% ethanol (v/v); pH 3.5; 5 g/L tartaric acid) and containing also a PreSens Pst6 oxygen
147 sensor (Presens GmbH, Regensburg, Germany) were similarly prepared within the anoxic
148 chamber and were further incubated at 50°C for 21 days. The O_2 permeated was
149 measured with the Nomasense O2 analyzer (Nomacorc, Aubel, Belgium). The readings
150 were in all cases below the detection limits of the system, implying that the O_2 permeated
151 into the tubes was below 60 ng, which for our purposes can be considered negligible.
152 *2.4. Chemical analysis*
153 Total and free VSCs, color, Total Polyphenol Index, pH, total and free SO_2, Trolox
154 equivalent antioxidant capacity (TEAC) and Folin-Ciocalteu were measured in all sampling
155 points. Metals, polyphenols and amino acids were measured only in the initial wines.
156 The methods and instruments for the analysis of free and total VSCs are described in
157 references (Franco-Luesma & Ferreira, 2014) and (Lopez, Lapena, Cacho, & Ferreira,
158 2007). Briefly, for the analysis of total forms, the wine is introduced into the anoxic

159 chamber and 0.1 mL are pipetted into a 20 mL standard headspace vial, already containing
160 4.9 mL of a NaCl brine. The internal standard solution (EMS, PrSH and thiophene at 20
161 µg/L) is also added, the vial is sealed, taken out of the chamber and immediately (without
162 idle time in the sampler tray) preconcentrated by headspace SPME and further analyzed
163 by GC with pulsed flame photometric detection (pFPD).
164 For the analysis of free forms, the wine is also introduced into the anoxic chamber, and 12
165 mL with 40 µL of the internal standard solution (EMS, PrSH and thiophene at 2 mgL^{-1}) are
166 directly transferred into a 20 mL standard headspace vial. The vial is then sealed, taken
167 out of the chamber and immediately analyzed. The analysis consists of a direct headspace
168 injection in the GC-pFPD system. In both determinations, the areas are normalized to
169 those of the internal standard (PrSH for H_2S, MeSH and EtSH and EMS for DMS, DES,
170 DMDS and DEDS) and interpolated in calibration graphs built by the analysis of a synthetic
171 calibrated wine.
172 Color was quantified as the absorbances at 420, 520 and 620 nm. Red wines were diluted
173 10 times with purified water, and rosés and whites were measured directly. Total
174 Polyphenol Index was estimated as absorbance at 280 nm, red wines were diluted 100
175 times, rosés 50 and whites 20 with purified water. All absorbance measurements were
176 made in triplicate using 1 cm cuvettes and a UV-VIS spectrophotometer UV-17000 Pharma
177 Spec from Shimadzu (Kyoto, Japan).
178 TEAC and Folin-Ciocalteau assays were adapted from procedures described by Rivero-
179 Perez et al. (Rivero-Perez, Muniz, & Gonzalez-Sanjose, 2007) and Singleton et al.

(Singleton, Orthofer, & Lamuela-Raventos, 1999) respectively. Absorbance measurements were taken using 1 cm cuvettes at a UV-VIS spectrophotometer UV-17000 Pharma Spec from Shimadzu (Kyoto, Japan).

Cu, Fe, Mn, Ni, Sn and Zn in wines were determined by ICP-OES with a Thermo Elemental IRIS Intrepid, as indicated in the method proposed by Gonzalvez et al. (Gonzalvez, Armenta, Pastor, & de la Guardia, 2008), microwave-assisted digestion was used as sample treatment.

Analyses of the polyphenolic matter was performed following the method described by Gonzalez-Hernandez et al. (Gonzalez-Hernandez, Avizcuri-Inac, Dizy, & Fernandez-Zurbano, 2014). Two mL of wine were filtered by 0.45 μm and fractionated by Gel Permeation Chromatography (GPC) in an automated fraction collector from Gilson (Middleton, WI, USA) with a Vantage L column (120 mm x 12 mm) from Millipore (Bedforf, Ma, USA) packed with TSK Toyopearl gel HW-50F (Tosohaas, Montgomery Ville, PA, USA). Two fractions were collected and brought to dryness under vacuum. Fraction 1 was dissolved in 2 mL of formic acid/water (5:95, v/v) and it was further analyzed by UPLC–DAD-MS for quantifying anthocyanins and by UPLC–MS for quantifying flavonols, flavanols, hydroxycinnamic acids, phenolic acids, aconitic acid and resveratrol. Fraction 2 was dissolved in 2 mL of methanol.

The analyses of amino acids were made following a precolumn derivatization procedure with aminoquinolyl-N-hydrosysuccinimidyl carbamate (AQC) using a quaternary high-performance liquid chromatography (HPLC) eluent system as it is described in (Hernandez-

Orte, Ibarz, Cacho, & Ferreira, 2003). A summary of all this data can be found in Table 2 of the supplementary material.

2.5. Statistical analysis and data treatment

Simple statistical calculations were carried out with Excel 2013 (Microsoft, WA, USA). For the comparison of means of different groups of samples, normal t-test assuming equal variances were carried out. For the comparison of means of the same samples at different times, paired t-test statistics were used. Regression analysis was used in order to assess the significance of temporal evolutions. Models were built using Partial Least-Squares (PLS) regression with The Unscrambler 9.7 (CAMO Software AS, Oslo, Norway). Models have been produced combining correlation analysis and further PLS modeling. The simplest models explaining as much as possible the variability of the parameter and producing minimum predictive errors were searched by progressively reducing the number of variables in the model. In all the cases models were developed and validated by cross-validation, a strategy in which the model is built iteratively leaving out one sample per iteration. The predictive power of these models is assessed via the root mean square error (RMSE), which is estimated as well by cross validation, computing the predictive error for each sample in the iteration in which it was left out.

218 **3. Results and discussion**

219 *Free and complexed forms of H_2S and mercaptans in wines*

220 Twenty four different wines were analyzed for their levels in free and total VSCs following
221 a previously developed procedure (Franco-Luesma & Ferreira, 2014). With the analytical
222 methods used, only H_2S, MeSH and DMS were found above the method detection limits.
223 Results of the analysis are summarized in Table 1. As seen in the table, most wines contain
224 small amounts of free H_2S and MeSH and only in four cases the levels of these molecules
225 were not detectable. At these levels, these molecules are most likely not causing a
226 reductive off-odor problem. In contrast, levels of DMS are high enough so that this
227 molecule will exert a notable sensory effect, and depending on the aromatic environment
228 will enhance fruity notes or will even produce an unpleasant effect (Lytra, Tempere,
229 Zhang, Marchand, de Revel, & Barbe, 2014; Segurel, Razungles, Riou, Salles, & Baumes,
230 2004).

231 Confirming previous results (Franco-Luesma & Ferreira, 2014), both H_2S and MeSH are
232 present under non-volatile forms, most likely as complexes with Cu^{2+}, Fe^{2+} and even Zn^{2+}.
233 Results in the table indicate that, on average, free H_2S constitutes just a 6% of the total
234 amount of H_2S in a red wine and 8% in a white or rosé. Similarly, free MeSH only accounts
235 on average for 38% of the total levels of a red wine while a 69% of the MeSH contained in
236 whites and rosés is under free forms. In clear contrast, DMS was not found present in
237 complexed forms in agreement with previous work (Franco-Luesma & Ferreira, 2014).
238 Levels of total H_2S are relatively high, likely more than enough to cause an aromatic

problem if those complexed forms would be released. Levels of free and total H_2S are not correlated, while in the case of MeSH, there is a positive correlation (significant at $P<0.01$) between free and complexed forms, suggesting that in this case the differential metal content of the wine is not that critical in determining the proportion in bonded forms.

Another interesting question is the relationship between VSCs content and wine age, which applies only to the 16 red wines in the study, since whites and rosés were all from the same vintage. Regression analysis revealed that the average level of free H_2S increased by 0.38 ± 0.11 μg/L per year of aging (significant at $P<0.01$), while the average level of total H_2S remained approximately constant (see supplementary material Figure 1a). Consequently, the bonded fraction decreases from 96.9 ± 0.89% in wines from 2012 to 87.8 ± 4.0% in wines from 2008 or older, which suggests that there is an average release of 1.9 ± 0.7% of bonded forms per year of aging (significant at $P<0.05$).

Similar results were observed in the case of MeSH and regression analysis revealed the existence of an average increase of 0.23 ± 0.06 μg/L per year of aging (significant at $P<0.01$), while levels of total forms were not found to significantly increase with age (see supplementary material Figure 1b). The proportion of bonded forms changed from 74.1 ± 7.2% in 2012 wines to 40 ± 6.2% in wines from 2008 or older (significant at $P<0.01$), suggesting an average release of 8.1 ± 2.7% of bonded forms per year of aging. Finally, in the case of DMS, regression analysis revealed that its levels increase on average 6.9 ± 1.7 μg/L per year of aging (significant at $P<0.01$). No further comment on this aroma molecule will be presented in this paper.

Relationship between actual contents and wine composition

Total H_2S levels of whites and rosés are strongly related to the wine content in metals. Although univariately there is only a significant correlation with wine Zn levels ($r = -0.67$, significant at $P<0.05$), PLS modeling shows that in fact the wine content in total H_2S is nearly completely determined by its contents in four metals. As the model reveals (model 2 in Table 2), the total H_2S levels of wine is directly related to its levels of copper and manganese and negatively related to its levels of iron and zinc. The variance explained by the model (by cross validation) is 89.6% and the predictive error is just 1.37 µg/L. In case of red wines, wines from 2008 or older had to be excluded from the model, but for the other 11 younger wines PLS modeling confirmed that the levels of total H_2S were positively related to wine copper levels and negatively related to the levels of Zn and Fe, as shown in Table 2 (model 1).

While there are two possible known causes explaining the positive correlation between total H_2S and copper, the negative role played by Zn and Fe is less obvious. In terms of catalytic action, addition of Zn to commercial wines did not produce but an increase in H_2S levels, while the effects of iron were not clear (Viviers, Smith, Wilkes, & Smith, 2013); hence, catalytic action is not consistent with the negative coefficients in the models. The same happens to the demonstrated potential ability of Zn^{2+} and Fe^{2+} to reversibly trap H_2S in synthetic hydroalcoholic solutions (Franco-Luesma & Ferreira, 2014). A potential explanation would be that must deficiencies in Zn and Fe would be responsible for yeast overproduction of H_2S. In fact, it is known that Zn is an essential element for

281 Saccharomyces cerevisiae and that its deprivation can cause sluggish fermentations
282 (Bromberg, Bower, Duncombe, Fehring, Gerber, Lau, et al., 1997; Gauci, Beckhouse,
283 Lyons, Beh, Rogers, Dawes, et al., 2009), and there is also a report demonstrating that the
284 activity of Zn-Cu superoxide dismutases is enhanced by H_2S (Searcy, Whitehead, &
285 Maroney, 1995). However, and to the best of our knowledge there are no literature
286 reports demonstrating a direct relationship between low levels of this element or of iron
287 and high production of H_2S in fermentation, as the models may suggest.
288 In the case of copper, it is known that winemakers use it to remove excesses of H_2S, so
289 that wines coming from fermentations with high production of this molecule may have
290 more residual copper. Besides, wines naturally containing more copper may have
291 accumulated more H_2S produced in fermentation.
292 In the case of reds, the model suggests that total H_2S levels are related to the wine
293 chromatic parameters and to the wine levels in methionine and cysteine (Table 2, model
294 1). Chromatic parameters may be just indicating the negative influence of wine oxidation
295 (A420 and A520) on total H_2S levels. The negative relationship between wine total levels
296 of H_2S and methionine may be attributed to the demonstrated suppressing effect of this
297 amino acid on H_2S production by yeast (Barbosa, Mendes-Faia, & Mendes-Ferreira, 2012;
298 Spiropoulos, Tanaka, Flerianos, & Bisson, 2000). The similar relationship of cysteine is
299 more difficult to explain, because in fact there is a known relationship between the must
300 levels of this amino acid and the formation of H_2S (Jiranek, Langridge, & Henschke, 1995;
301 Moreira, Mendes, Pereira, de Pinho, Hogg, & Vasconcelos, 2002). On the other hand, it

302 has been reported that high cellular levels of cysteine were related to H_2S suppression
303 (Spiropoulos & Bisson, 2000). Our poor understanding about the factors that determine
304 the residual amounts of these amino acids in wine after fermentation does not make it
305 possible to propose a clear hypothesis.
306 In the case of methanethiol, two independent models with a quite similar structure have
307 been also derived for reds and for whites and rosés, as seen in Table 2 (models 3 and 4).
308 One of the most remarkable observations is that, in clear contrast with the models for
309 H_2S, the levels of total MeSH contained in the wine are inversely related to copper levels,
310 confirming that the role of copper as trapping agent of MeSH is not really important in this
311 case. Secondly, as was previously observed, in both cases total MeSH levels are related to
312 the actual content in free MeSH. Thirdly, methionine plays a negative role in both models,
313 as was already observed for H_2S in red wines, but in apparent contrast with the known
314 relationship between must methionine levels and the production of methanethiol by
315 Saccharomyces (Perpete, Duthoit, De Maeyer, Imray, Lawton, Stavropoulos, et al., 2006)
316 or by lactic bacteria (Pripis-Nicolau, de Revel, Bertrand, & Lonvaud-Funel, 2004) and with
317 the relationship between wine methionine and de novo formation of MeSH during anoxic
318 storage (Ferreira, Bueno, Franco-Luesma, Cullere, & Fernandez-Zurbano, 2014). This
319 suggests that the residual amount of methionine in wine is not a major source of the total
320 MeSH found in wine before anoxic aging, which would support a major fermentation
321 origin for this compound.

322 Finally, the models also assign a consistent negative influence of the levels of some
323 important antioxidants on the wine total MeSH content. The negative correlation between
324 MeSH formation and resveratrol has been previously observed in an independent
325 experiment (Ferreira, Bueno, Franco-Luesma, Cullere, & Fernandez-Zurbano, 2014).

326 *Evolution of H_2S and MeSH during anoxic aging*

327 The wines were stored in strict anoxia at 50°C for 3 weeks, and samples were analyzed at
328 days 1.5, 5.5, 12.5 and 21. This type of accelerated aging was selected because in
329 preliminary trials we could observe that the rates of increase of H_2S and MeSH were fast
330 enough for the purposes of the experiment and that there was no evident formation of
331 artefacts. At higher temperatures such as 70°C, strong increases of free and total forms of
332 H_2S and MeSH together with decreases in DMS were observed, suggesting strong
333 decomposition of S-amino acids (data not shown). In addition, an aging experiment at
334 25°C with a set of similar wines has been also conducted. Results will be presented in a
335 forthcoming paper, but confirm that the accumulation of free H_2S and free MeSH at 25°C
336 and 50°C are strongly correlated. The reproducibility of the accelerated anoxic aging
337 turned out to be very high, as revealed by the low RSDs for the overall process obtained in
338 an independent experiment (see methods). The overall variability (given as RSD(%)) was
339 3.9%, 2.2% and 1.6% for the levels of free H_2S, MeSH and DMS, respectively. For total
340 forms RSDs were 5.3% and 3.3 % for total H_2S, and MeSH, respectively. In all cases the
341 major contributor to uncertainty was the analytical determination.

342 The anoxic aging brought about important increases in the levels of free H_2S, as
343 summarized in Figure 1 and Table 3. The figure gives the evolution of the average contents
344 of free and total H_2S for the 16 red wines (Figure 1a) and the 8 whites and roses (Figure
345 1b) along the anoxic storage. Figure 1c shows the evolution of the proportion of
346 complexed forms of H_2S during the storage. In the case of red wines the total content of
347 H_2S remained relatively stable along the whole process. The average increment, as
348 detailed in Table 3, was 1.58 µg/L which did not reach the level of statistical significance
349 (paired t test, P=0.07). It can be concluded that for red wines and on average, there is no
350 significant de novo formation of this molecule during the storage. On the contrary, the
351 average levels of free H_2S increased continuously becoming at the end of the storage just
352 slightly smaller than total levels. The average total increase of free H_2S was above 16 µg/L,
353 as detailed in Table 3 (paired t test, significant at $P<10^{-8}$). As seen in Figure 1c, the average
354 proportion of complexed forms decreases from the initial 94% (Table 1) to a meager 23%
355 after the 3 weeks (paired t test, significant at $P<10^{-8}$). The consequence of the process is
356 therefore a neat transformation of complexed non-volatile forms into free H_2S.
357 In the case of white and rose wines, shown in Figure 1b, levels of total H_2S increased
358 slightly but significantly, becoming on average 9.4 µg/L higher than the initial contents
359 after the storage (paired t test, significant at P<0.0001). For these types of wines the levels
360 of free H_2S increased very fast in the first week of storage and then the rate of increase
361 slowed down. This is also seen in Figure 1c, which confirms that for whites and rosés, the
362 release of complexed forms is very fast and seems to be nearly completed in less than one

363 week. Remarkably, in the last sampling point there is a slight increase in the proportion of
364 bonded forms. Such increase is statistically significant (paired t test, P=0.026).
365 Going into more detail, in a reduced number of 4 red wines there was a slight but
366 significant (regression analysis, in all cases P<0.05) increment of total H_2S, as can be seen
367 in Table 3. Increments were in three of the cases smaller than 4 μg/L and in the other case
368 it was of 6.7 ± 0.8 μg/L (Significant at P<0.01). The increments of free H_2S of red wines
369 were in all cases but one, between 10 and 26 μg/L. The odd sample released only 2.9 μg/L
370 in the three weeks and after the process it still contained 89% of H_2S in bonded forms. The
371 proportion of complexed forms at the end of the process was smaller than 12% for half of
372 the samples, and smaller than 35% for all but two. For whites and roses, increments in
373 total H_2S level were significant (P<0.05) in all the wines and ranged from 4.6 to 12.6 μg/L,
374 as detailed in Table 3, while increments in free forms were relatively homogeneous
375 ranging from 14 to 33 μg/L. For these wines, at the end of the process the % in bonded
376 forms ranged from 0 to 54%.
377 The mass balance of the process is summarized in Table 4. As detailed in the table, it can
378 be estimated that out of the 16.2 μg/L of free H_2S that on average will accumulate in red
379 wines during the anoxic storage, 14.7 μg/L can be attributed to the release. This value has
380 been estimated by applying the decrease of the fraction bonded (-71%) to the total initial
381 content (20.8 μg/L in Table 1). Therefore, it can be said that attending to our data, the
382 release of H_2S from complexes is the dominant process in reds, explaining on average
383 90.3% of the observed increase in free H_2S potentially responsible for an off-odor. In the

384 case of whites and rosés, however, the de novo formation of H_2S becomes more relevant,
385 so that the release from complexed forms explains just 58% of the free H_2S accumulated
386 in the wine during the aging.
387 Results for the evolution of MeSH along the anoxic aging are shown in Figure 2 and in
388 Table 3. In this case there are clear average increments of total forms both in red and in
389 white and rosé wines. In red wines, the average increment is 1.4 µg/L (paired t test,
390 significant at $P<10^{-10}$), while in whites and rosés it reaches nearly 3 µg/L (paired t test,
391 significant at P<0.0001). In both types of wines the proportion of complexed forms
392 significantly decreases (paired t test, significant at $P<10^{-10}$ for reds and <0.01 for whites
393 and rosés), faster in the case of whites and rosés as shown in Figure 2c, so that in the last
394 sampling points average levels of free and total forms are coincident. The release and
395 formation of this molecule in red wines is relatively homogeneous, and increments of free
396 values range from 1.8 to 4.3 µg/L, while increments of total forms range between 0.88
397 and 1.91. In white and roses there is more variability, and increments in free forms range
398 a factor 4 (from 1.9 to 7.7), while total forms range from 1.58 to 4.38, as shown in Table 3.
399 The overall balance of the process for MeSH is summarized in Table 4. In this case, it is de
400 novo formation the dominant process causing the accumulation of the free form of this
401 molecule in the headspaces of wines. It can be seen that in the case of reds, the release
402 from complexed forms just accounts for a 47.5% of the free MeSH accumulated, while in
403 whites and rosés, release from complexes become nearly irrelevant, since only 24 % of the
404 amounts of free MeSH accumulated in these types of wines are attributed to the release.

405 It should be taken into account that the mass balance summarized in table 4 refers to
406 accelerated anoxic storage at 50°C. It can be expected that the pattern will not change
407 dramatically at lower temperatures, at least in relative terms, but different proportions of
408 de novo formation and release will surely be observed. It is remarkable that two of the
409 main observations derived from a previous work carried out at 25°C with red wines
410 (Ferreira, Bueno, Franco-Luesma, Cullere, & Fernandez-Zurbano, 2014), namely the
411 inexistence of relevant de novo formation of H_2S and the significant de novo formation of
412 MeSH, are coincident with the results presented here. All those data make it possible to
413 state that the release of free forms from already present complex forms and the de novo
414 production of small quantities of H_2S (except in reds) and MeSH during storage is a fairly
415 common outcome of wines. A corollary to this is that if the wine is bottled using a
416 perfectly hermetic closure, both processes may lead to the accumulation in the media of
417 significant amounts of free H_2S and MeSH, which undoubtedly will have consequences on
418 the wine sensory profiles. Questions which will have to be further addressed are the time
419 frame at which these phenomena take place at normal wine storage temperatures, the
420 chemical origin of bonded forms and the chemical nature of the processes causing de
421 novo formation.
422 In summary, this paper has revealed that the observed accumulation of free H_2S and
423 MeSH along the accelerated anoxic storage of wines is caused both by de novo formation
424 from precursors and by release from already existent Cu(II)-H_2S and Cu(II)-MeSH
425 complexes. While release from complexes seems to be the major cause explaining the

accumulation of H_2S, particularly in red wines, de novo formation becomes the dominant source of MeSH, particularly in white and rosé wines.

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555

556

FIGURE CAPTIONS

Figure 1. Evolution of the mean total (1a) and free (1b) contents and of the mean proportions in complexed forms (1c) of H_2S along the accelerated reductive aging period. Experimental points represent the average of the 16 (reds) and 8 (whites and rosés) samples. Error bars are the standard error of the corresponding means (S/√16 for reds and S/√8 for whites and rosés).

Figure 2. Evolution of the mean total (2a) and free (2b) contents and of the mean proportions in complexed forms (2c) of MeSH along the accelerated reductive aging period. Experimental points represent the average of the 16 (reds) and 8 (whites and rosés) samples. Error bars are the standard error of the mean (S/√16 for reds and S/√8 for whites and rosés)

Table(s)

Table 1. Free and total contents of H_2S, MeSH and DMS (for this compound free=total) of the wines in the study. Data expressed in µg/L

	H_2S			MeSH			
	free	**total**	**% bonded**	**free**	**total**	**% bonded**	**DMS**
Red Wines							
AA	3.4 ± 0.1	44.9 ± 2.9	92%	1.6 ± 0.0	2.7 ± 0.1	42%	40.6 ± 0.0
BL	2.2 ± 0.3	20.4 ± 0.0	89%	1.8 ± 0.0	4.2 ± 0.0	57%	35.8 ± 0.2
CH	0.9 ± 0.0	13.3 ± 0.0	94%	1.5 ± 0.0	2.2 ± 0.1	33%	40.9 ± 0.6
FG	0.7 ± 0.1	15.1 ± 0.3	95%	0.9 ± 0.0	1.7 ± 0.0	45%	23.1 ± 0.1
LH	1.1 ± 0.1	19.4 ± 2.5	94%	1.1 ± 0.0	2.7 ± 0.2	60%	19.6 ± 0.3
MF	nd	30.2 ± 0.3	>99%	nd	1.9 ± 0.0	>79%	11.7 ± 0.1
MZ	nd	17.1 ± 1.7	>99%	nd	1.1 ± 0.1	>63%	13.8 ± 0.1
PC	0.6 ± 0.0	22.0 ± 1.8	97%	0.7 ± 0.1	2.4 ± 0.0	72%	12.4 ± 3.3
RD	0.7 ± 0.1	14.9 ± 2.0	95%	0.8 ± 0.0	2.7 ± 0.3	72%	9.2 ± 0.1
SL	0.3 ± 0.3	28.4 ± 1.4	99%	nd	2.3 ± 0.1	>83%	23.8 ± 1.1
TP	0.7 ± 0.0	21.9 ± 3.4	97%	0.8 ± 0.0	2.0 ± 0.1	60%	47.3 ± 0.5
TS	0.6 ± 0.1	21.3 ± 4.3	97%	0.7 ± 0.0	2.3 ± 0.2	68%	26.2 ± 0.2
TZ	2.9 ± 0.0	12.1 ± 0.1	76%	1.3 ± 0.0	1.9 ± 0.0	29%	57.3 ± 0.4
UB	0.7 ± 0.0	19.9 ± 1.9	97%	1.3 ± 0.0	2.2 ± 0.2	42%	62.0 ± 0.2
VN	1.8 ± 0.2	16.9 ± 0.7	90%	0.9 ± 0.0	1.9 ± 0.1	51%	36.4 ± 0.2
VV	0.8 ± 0.0	15.0 ± 0.3	95%	0.9 ± 0.0	2.0 ± 0.1	54%	18.8 ± 0.1
Average	**1.1 ±0.1**	**20.8 ± 0.7**	**94%**	**0.9 ± 0.0**	**2.3 ±0.1**	**62%**	**29.9 ± 0.3**
SD	**0.99**	**8.1**	**6%**	**0.55**	**0.67**	**23%**	**16.4**
Maximum	**3.44**	**44.9**	**>99%**	**1.82**	**4.20**	**>83%**	**62.0**
Minimum	**<0.2**	**12.1**	**76%**	**<0.4**	**1.08**	**29%**	**9.2**
Whites & Roses							
AB	0.4 ± 0.4	28.0 ± 0.2	99%	1.5 ± 0.03	2.1 ± 0.1	27%	10.9 ± 0.1
CC	1.1 ± 0.3	21.4 ± 0.5	95%	2.4 ± 0.01	3.0 ± 0.0	22%	14.1 ± 0.0
EN	3.8 ± 0.4	23.9 ± 0.1	84%	1.8 ± 0.07	2.7 ± 0.2	33%	44.8 ± 1.2
FR	3.9 ± 0.9	29.1 ± 0.6	86%	2.3 ± 0.06	2.5 ± 0.1	10%	40.1 ± 1.2
HJ	3.4 ± 0.0	19.9 ± 1.4	83%	1.1 ± 0.07	2.3 ± 0.1	54%	24.8 ± 0.3
IL	nd	24.0 ± 0.4	>99%	0.8 ± 0.04	2.0 ± 0.0	60%	22.6 ± 0.1
MR	3.6 ± 0.9	28.5 ± 2.7	87%	2.0 ± 0.05	3.3 ± 0.4	39%	28.5 ± 1.1
VT	nd	31.0 ± 3.5	>99%	2.7 ± 0.02	2.8 ± 0.2	4%	7.0 ± 0.0
Average	**2.0 ±0.3**	**25.7 ± 0.8**	**92%**	**1.8 ±0.02**	**2.6 ± 0.1**	**31%**	**24.1 ± 0.4**
SD	**1.81**	**4.0**	**7%**	**0.66**	**0.44**	**20%**	**13.5**
Maximum	**3.94**	**31.0**	**>99%**	**2.73**	**3.26**	**60%**	**44.8**
Minimum	**<0.2**	**19.91**	**83%**	**0.80**	**1.99**	**4%**	**7.04**

nd, not detected. The estimated limit of detection are 0.2 µg/L (H_2S) and 0.4 µg/L (MeSH)

Table 2. Summary of the PLS models relating the actual wine content in total H_2S and total MeSH with the wine chemical composition

Nº	Parameter	EVar	RMSE	Model (regression coefficients)
1	Reds H_2Stot	78%	3.90	5.00 **+ 0.227 Cu + 0.237 A620** -0.205 A420 – 0.195 A520 – 0.075 Fe – 0.145 Zn – 0.43 Cysteine – 0.146 Methionine
2	Wh&Ros H_2Stot	90%	1.37	6.41 **+0.303 Cu + 0.359 Mn** – 0.487 Fe – 0.583 Zn
3	Reds MeSHtot	82%[1]	0.19	7.11**+ 0.117 FreeMeSH + 0.169 H_2Stot/Cu** – 0.140 Cu – 0.14 t-caftaric acid -0.153 caffeic acid – 0177 c-resveratrol – 0.176 t-resveratrol -0.175 proanthocyanidin A2 -0.167 TEAC– 0.160 Methionine
4	Wh&Ros MeSHtot	81%	0.20	6.18 **+ 0.179 FreeMeSH + 0.217 H_2Stot/Cu** – 0.164 Cu -0.20 t-resveratrol -0.18 vitisin A – 0.188 pyranoanthocyanins -0.058 Methionine

[1]One sample with maximum contents excluded

Table 3. Increments of free and total contents of H_2S and MeSH measured in the wines in the study after 21 days of anoxic aging at 50°C. Data expressed in µg/L

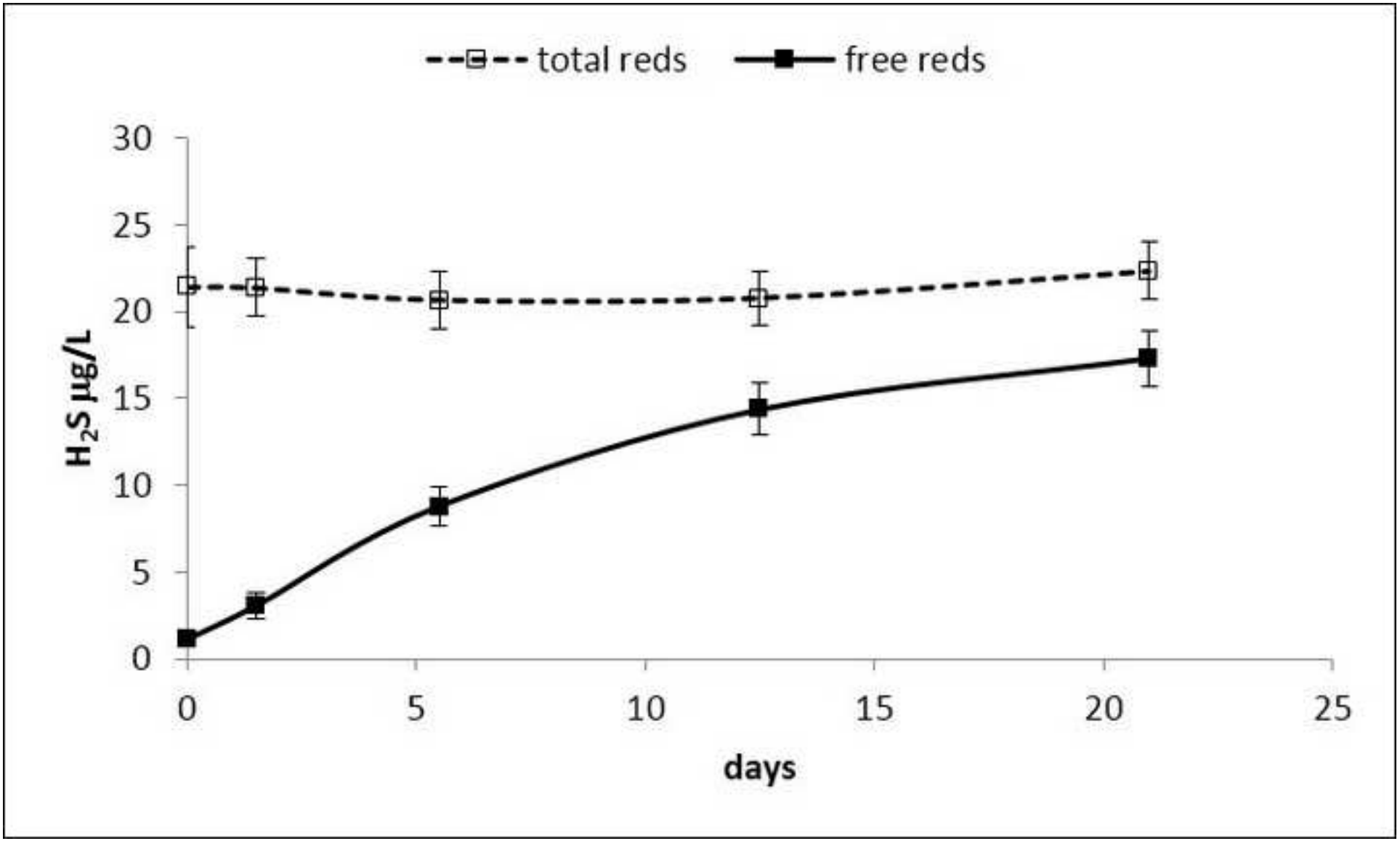
	H_2S			MeSH		
	Free	**Total**	**%bonded**	**Free**	**Total**	**%bonded**
Red wines						
AA	25.7 ± 0.2	ns	-59%	2.8 ± 0.09	1.3 ± 0.3	<-42%
BL	22.6 ± 0.8	ns	-89%	4.3 ± 0.12	1.4 ± 0.6	<-57%
CH	17.6 ± 0.1	3.9 ± 1.0	-94%	3.5 ± 0.06	1.9 ± 0.1	<-33%
FG	14.0 ± 0.2	ns	-67%	2.3 ± 0.04	1.7 ± 0.3	-41%
LH	23.0 ± 0.2	ns	-93%	2.6 ± 0.03	1.1 ± 0.4	-58%
MF	2.9 ± 0.2	ns	-11%	2.0 ± 0.003	1.2 ± 0.6	-66%
MZ	10.5 ±0.9	ns	-67%	2.2 ± 0.02	1.4 ± 0.2	-89%
PC	19.3 ± 0.3	ns	-86%	1.8 ± 0.08	1.0 ± 0.3	-45%
RD	13.7 ± 0.2	4.0 ± 2.2	-72%	2.3 ± 0.05	1.3 ± 0.4	-47%
SL	13.6 ± 0.7	ns	-52%	3.4 ± 0.06	1.8 ± 0.3	-82%
TP	15.9 ± 0.3	ns	-85%	2.1 ± 0.04	0.9 ± 0.2	-60%
TS	13.9 ± 0.2	ns	-67%	2.5 ± 0.06	1.1 ± 0.2	-65%
TZ	17.5 ± 0.01	3.4 ± 7.8	-76%	2.4 ± 0.01	1.3 ± 1.5	<-29%
UB	10.0 ± 0.3	ns	-41%	4.2 ± 0.09	1.6 ± 0.8	<-42%
VN	19.3 ± 0.6	6.7 ± 2.5	-79%	2.3 ± 0.10	1.8 ± 0.2	-38%
VV	20.6 ± 0.4	3.9 ± 0.3	-95%	2.6 ± 0.03	1.3 ± 0.2	<-54%
Average	***16.2 ± 0.4***	***1.6±3.8***	***-71%***	***2.7 ±0.06***	***1.4 ± 0.5***	***-60%***
SD	***5.73***	***3.06***	***22%***	***0.75***	***0.31***	***16%***
Maximum	***25.70***	***6.65***	***-95%***	***4.33***	***1.91***	***-89%***
Minimum	***2.87***	***-1.61***	***-11%***	***1.77***	***0.88***	***->29%***
Whites & Roses						
AB	20.3 ± 1.7	12.6 ± 8.7	-50%	4.4 ± 0.09	3.4 ± 0.5	<-27%
CC	32.9 ± 0.2	9.3 ± 0.8	-95%	7.7 ± 0.14	4.4 ± 0.4	<-22%
EN	16.8 ± 1.6	9.4 ± 4.1	-46%	3.1 ± 0.10	2.7 ± 0.2	-25%
FR	14.0 ± 0.7	8.6 ± 6.1	-34%	3.9 ± 0.07	3.7 ± 0.3	-9%
HJ	24.9 ± 0.03	12.2 ± 5.6	-71%	2.8 ± 0.09	2.7 ± 0.5	-32%
IL	27.5 ± 2.0	12.3 ± 7.8	-76%	3.8 ± 0.06	3.0 ± 0.3	-52%
MR	26.9 ± 0.5	6.5 ± 3.7	-75%	1.9 ± 0.09	1.6 ± 0.4	-19%
VT	16.2 ± 0.5	4.6 ± 4.9	-46%	2.7 ± 0.05	1.6 ± 0.2	<-4%
Average	***22.5 ± 1.4***	***9.4±***	***-61%***	***3.8 ±0.09***	***2.9 ±0.4***	***-31%***
SD	***6.64***	***2.89***	***21%***	***1.76***	***0.97***	***15%***
Maximum	***32.89***	***12.58***	***-95%***	***7.66***	***4.38***	***-52%***
Minimum	***13.98***	***4.64***	***-34%***	***1.89***	***1.58***	***<-4%***

ns: not significant

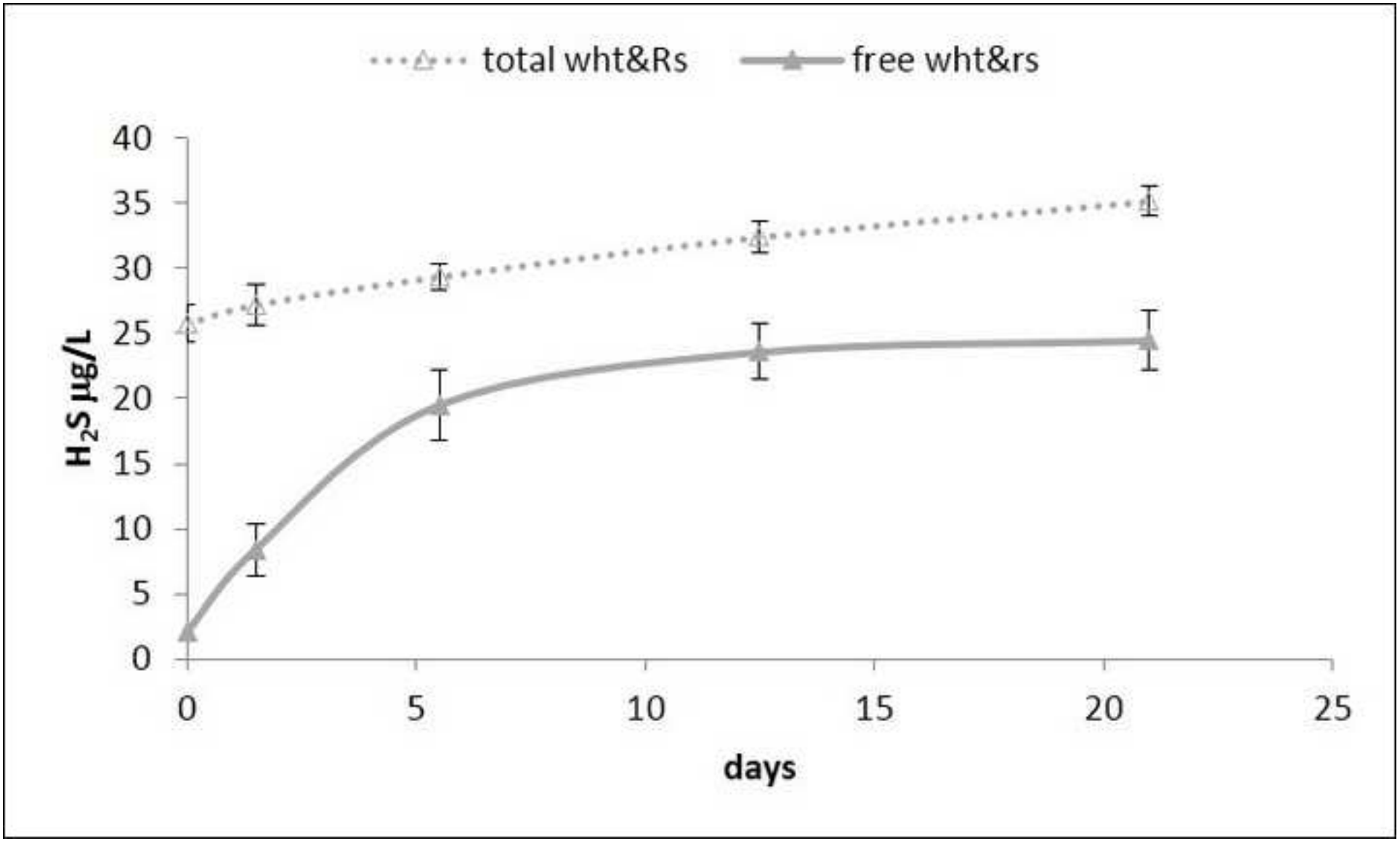
Table 4. Mean amounts of H_2S and MeSH formed along the anoxic storage and estimated contributions of the processes of de novo formation and release from complexes

	H_2S		**MeSH**	
	Reds	Whites & rosés	Reds	Whites & rosés
Total increase of free forms (release + de novo) (µg/L)	**16.2**	**22.5**	**2.7**	**3.8**
Increase attributed to release (µg/L)	14.7	13.0	1.3	0.9
% increase attributed to release	90.3%	58.0%	47.5%	24.1%
% increase attributed to de novo formation	9.7%	42.0%	52.5%	75.9%
Bonded fraction at the beginning (%)	94%	92%	62%	31%
Bonded fraction after the storage (%)	23%	31%	2%	0%
Decrease of the fraction bonded (%)	**71%**	**61%**	**60%**	**31%**

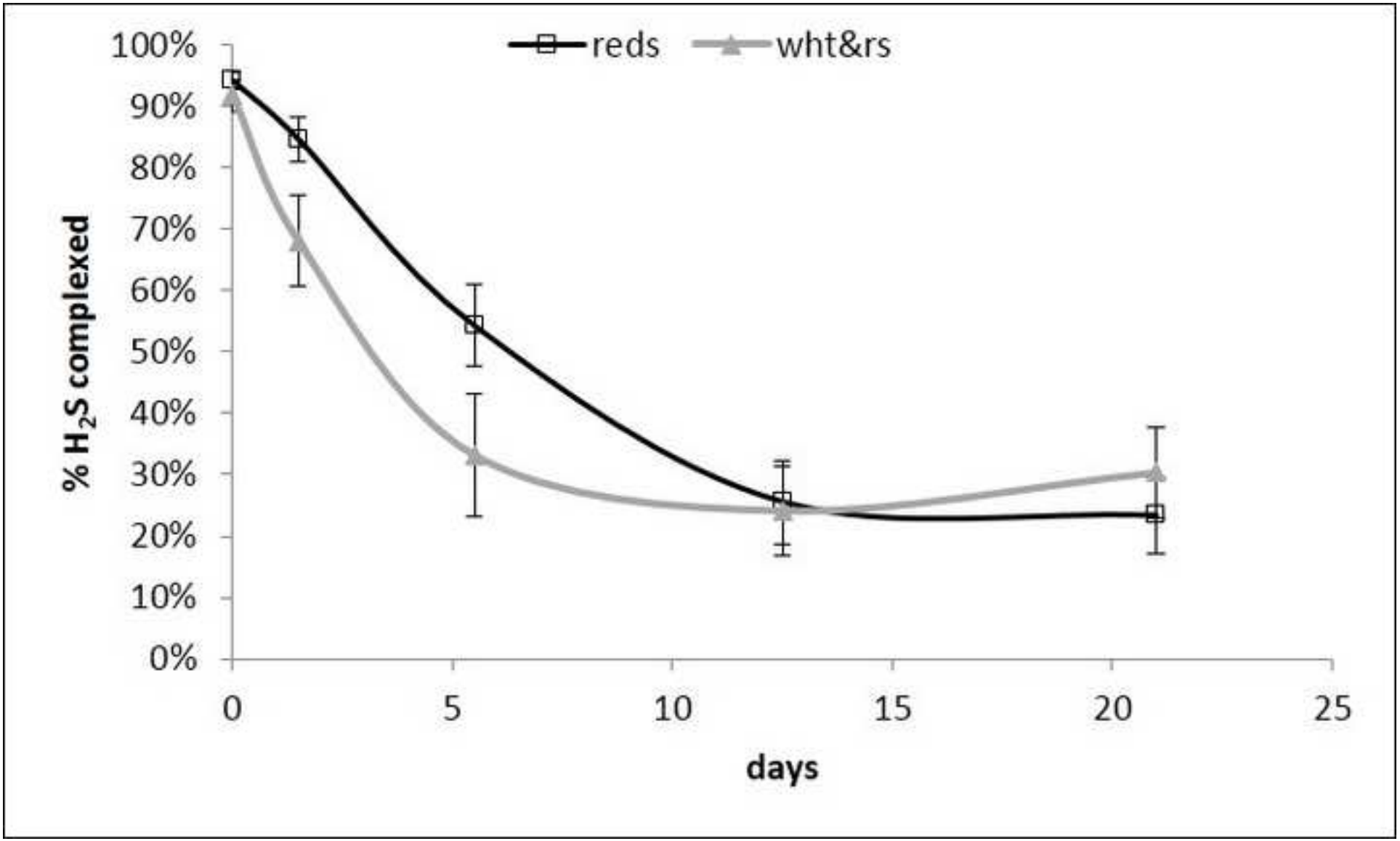
Figure(s) 1a



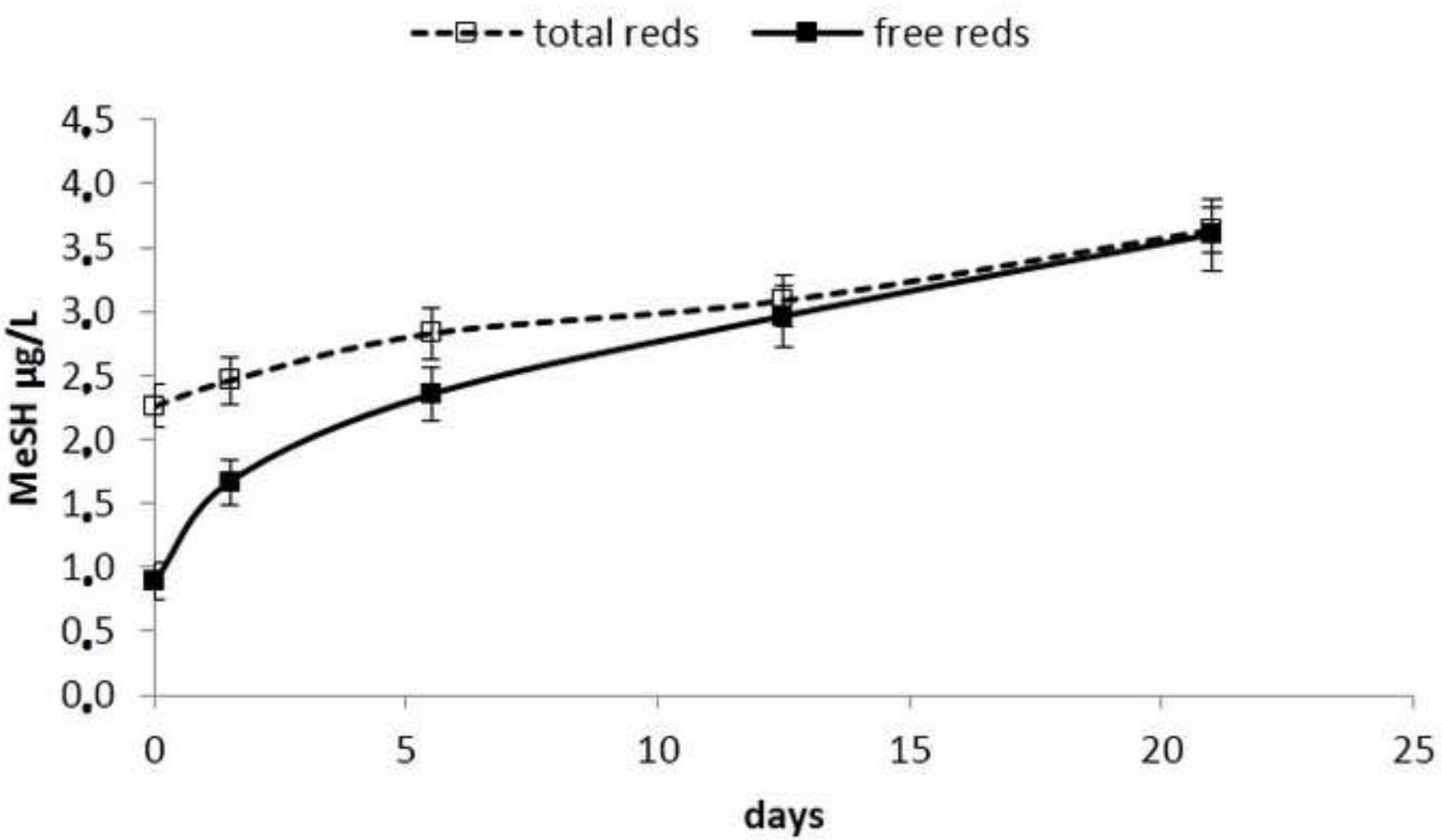
Figure(s) 1b


total wht&Rs
free wht&rs
40
35
30
25
20
15
10
5
0
H2S µg/L
0
5
10
15
20
25
days

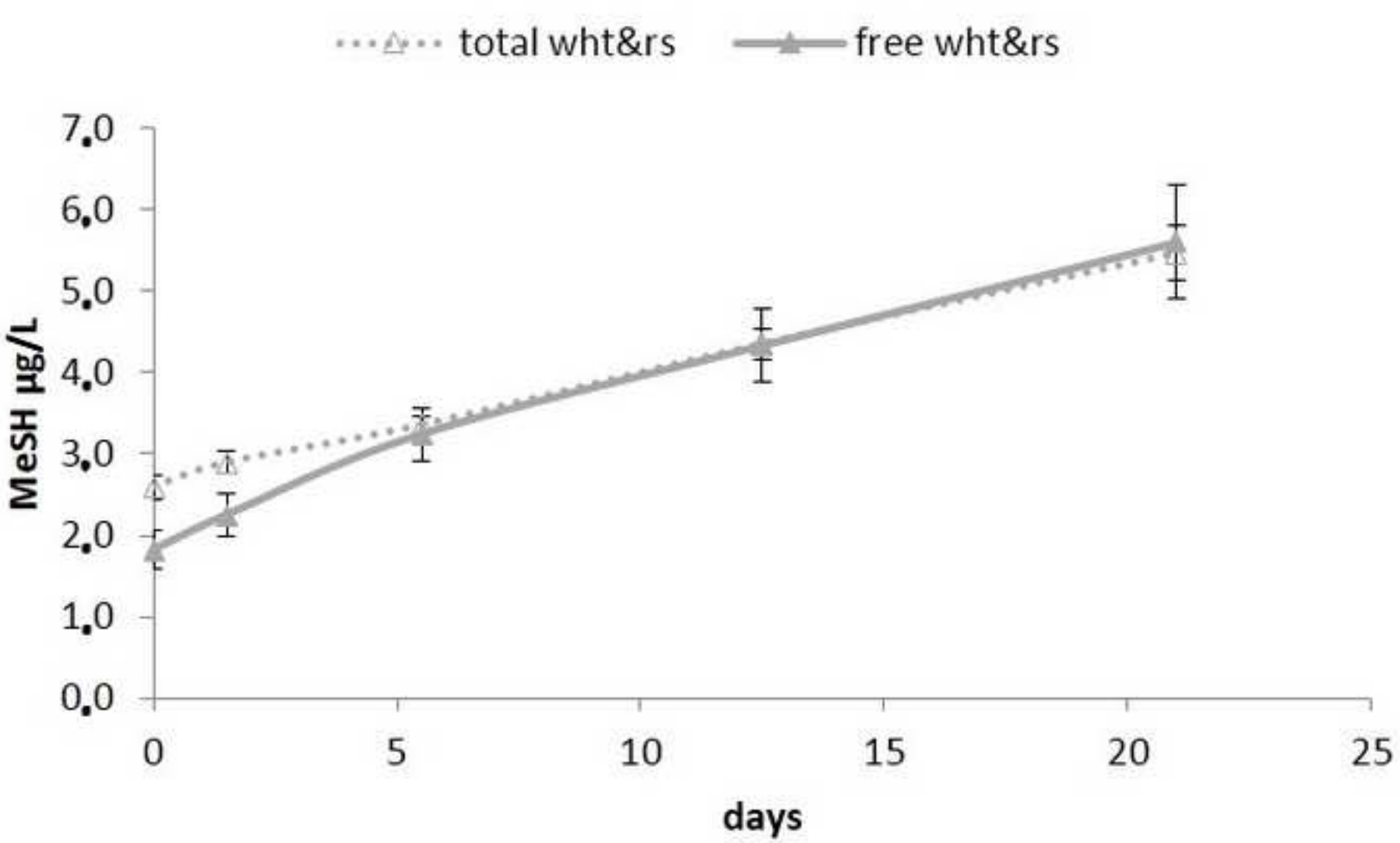
Figure(s) 1c


reds
wht&rs
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% H2S complexed
0
5
10
15
20
25
days

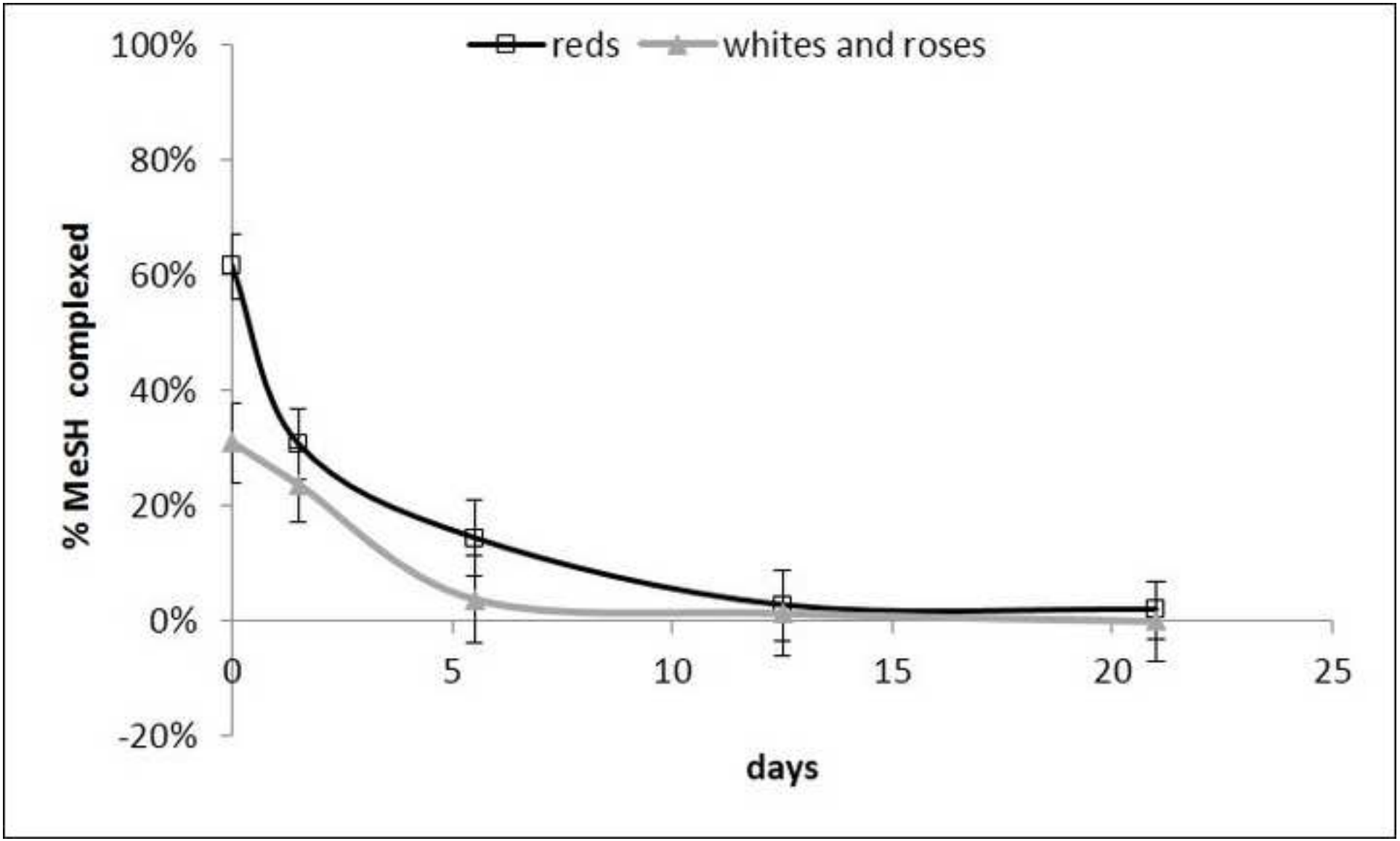
Figure(s) 2a


total reds
free reds
MeSH µg/L
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
5
10
15
20
25
days

Figure(s) 2b


total wht&rs
free wht&rs
MeSH µg/L
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
0
5
10
15
20
25
days

Figure(s) 2c


reds
whites and roses
100%
80%
60%
40%
20%
0%
-20%
% MeSH complexed
0
5
10
15
20
25
days