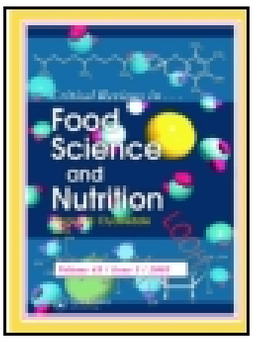

Critical Reviews in Food Science and Nutrition

ISSN: 1040-8398 (Print) 1549-7852 (Online) Journal homepage: http://www.tandfonline.com/loi/bfsn20

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To cite this article: Gal Y. Kreitman, Ryan J. Elias, David W. Jeffery & Gavin L. Sacks (2018): Loss and formation of malodorous volatile sulfhydryl compounds during wine storage, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2018.1427043

To link to this article: https://doi.org/10.1080/10408398.2018.1427043

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https://doi.org/10.1080/10408398.2018.1427043

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Loss and formation of malodorous volatile sulfhydryl compounds during wine storage

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ABSTRACT

Volatile sulfur compounds (VSCs), particularly low molecular weight sulfhydryls like hydrogen sulfide (H_2S) and methanethiol (MeSH), are often observed in wines with sulfurous off-aromas. Recent work has shown both H_2S and MeSH can increase up to a few μM (> 40 μg/L) during anoxic storage, but the identity of the latent sources of these sulfhydryls is still disputed. This review critically evaluates the latent precursors and pathways likely to be responsible for the loss and formation of these sulfhydryls during wine storage based on the existing enology literature as well as studies from food chemistry, geochemistry, biochemistry, and synthetic chemistry. We propose that three precursor classes have sufficient concentration and metastability to serve as latent sulfhydryl precursors in wine: 1) transition metal-sulfhydryl complexes, particularly those formed following Cu(II) addition, which are released under anoxic conditions through an unknown mechanism; 2) asymmetric disulfides, polysulfanes, and (di)organopolysulfanes formed through transition-metal mediated oxidation (e.g., Cu(II)) of sulfhydryls or pesticide degradation, and released through sulfitolysis, metal-catalyzed thiol-disulfide exchange or related reactions; 3) *S*-alkylthioacetates, primarily formed during fermentation, and releasable hydrolytically. Some evidence also exists for S-amino acids serving as precursors. Based on these findings, we propose a "decision tree" approach to choosing appropriate strategies for managing wines with sulfurous off-aromas.

KEYWORDS

Sulfur-like off-aromas; reduced wines; wine storage; latent sulfhydryl precursors

1. Introduction

Volatile sulfur compounds (VSCs) are well established as key contributors to the aromas of foods and beverages, often acting as character impact compounds (Mestres et al., 2000; Rauhut, 2009; Davis and Qian, 2011; McGorrin, 2011; Robinson et al., 2014). The resulting effects on product quality can be either positive or negative, depending on concentration and VSC in question, with no better example than wine. Several classes of VSCs have been detected in wine including sulfhydryls (-SH containing compounds), thioacetates and sulfides (Table 1). Certain VSCs are considered desirable in wine when present at concentrations above their sensory thresholds, particularly higher molecular weight polyfunctional thiols (MW >100 g/mol) such as 3-sulfanylhexan-1-ol (3SH), which contribute pleasant grapefruit, passionfruit, and blackcurrant aromas (Tominaga et al., 1996, 1998; Roland et al., 2011).

In contrast, lower molecular weight VSCs are implicated as a major cause of malodors reminiscent of rotten egg, sewage, cabbage or burnt rubber in wine and other systems (Mestres et al., 2000; McGorrin, 2011; Waterhouse et al., 2016b; Block, 2017). As early as 1873, the German biochemist J.L.W. Thudicum described a "reduced sulphur scud" in which "Sulphuretted hydrogen [H_2S] is first formed, and causes the wine to stink horribly" (Thudicum, 1873). In modern texts, the terms "reduced" or "reductive" can refer to either major malodorous species (especially sulfhydryls) which possess sulfur in low oxidation states (i.e., chemically reduced forms; see Table 1), or to the fact that these faults are most commonly observed under anoxic conditions and in wines with a low redox potential (P. Ribéreau-Gayon, et al., 2006). Reduced malodors are one of the most common faults observed in modern commercial wines – they were reportedly responsible for 26–29% of all wine faults at the International Wine Challenge over the years 2006–2008 (Goode and Harrop, 2008) – and thus are an important issue for the €28 billion (2015) global wine industry (OIV, 2016). Sulfhydryls are readily oxidized, and although historical numbers are lacking, it is likely that malodor faults have become more common in recent years with the increased popularity of reductive winemaking (i.e., minimizing oxygen exposure) and use of low oxygen transmission rate (OTR) closures, especially screwcaps (Limmer, 2005; Ugliano, 2013).

Historically, several VSCs were implicated in causing reductive malodors (Goniak and Noble, 1987; Park et al., 1994; Mestres et al., 2000; Pripis-Nicolau et al., 2004; Francis and Newton, 2005; Park, 2008) but only three are routinely observed at suprathreshold concentrations in faulted wines: hydrogen sulfide (H_2S), methanethiol (MeSH), and dimethyl sulfide (DMS) (Siebert et al., 2010). Other malodorous VSCs, e.g., ethanethiol (EtSH) and methyl thioacetate (MeSAc), appear to rarely exceed their sensory thresholds in commercial wines described as reduced (Siebert et al., 2010). Of the three major malodorous VSCs, the origins of DMS are most

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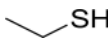
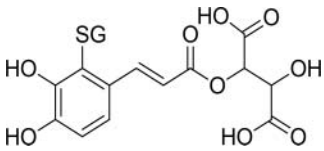
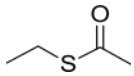
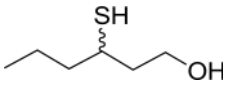
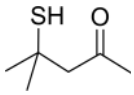
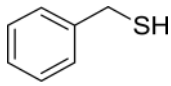
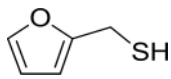
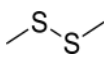
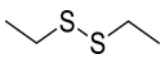
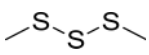
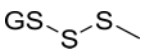
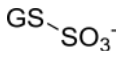
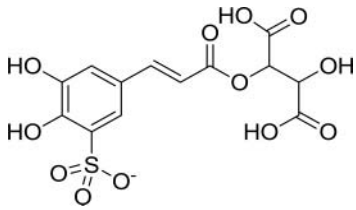
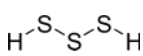
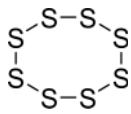
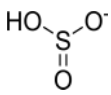
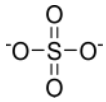
Table 1. Classes of S-compounds discussed in detail in this manuscript. Examples, odor descriptors and thresholds from Waterhouse, et al and references therein (Waterhouse *et al.*, 2016b) unless otherwise noted. Origins are discussed in detail in text; normal font indicates that a formation route is well-established to occur in wine, *italics* indicates putative based on model systems. Abbreviations: G = grape, F = fermentation, S = storage, W = winemaker addition.

Compound class	S redox state	Examples	Structure[a]	Odor	Odor Threshold (ng/L) [b]	Origin
			Organic forms			
Alkyl thiol	−2	Methanethiol (MeSH)		putrefaction	2000	F, S
		Ethanethiol (EtSH)		onion, rubber	1000	F
Alkyl sulfide	−2	Dimethyl sulfide (DMS)		cabbage, truffle	25000	S
Alkyl aryl sulfide (Quinone-thiol adduct)	−2	2-*S*-Glutathionyl caftaric acid			n.a.	G, S
Thioacetate	−2	Methyl thioacetate (MeSAc)		cheese, egg	50000	F
		Ethyl thioacetate (EtSAc)		garlic, onion	10000	F
Polyfunctional thiol	−2	3-sulfanylhexan-1-ol (3SH)		grapefruit, passionfruit	60	F
		4-methyl-4-sulfanylpentan-2-one (4MSP)		box tree, guava	3	F
Aryl thiol	−2	Benzenemethanethiol (BMT)		smoke, struck flint	0.3	F
		2-Furanmethanethiol (2-Furfurylthiol (FFT))		roasted coffee	0.4	F
Alkyl disulfide	−1	Dimethyl disulfide (DMDS)		onion, cabbage	29000	F, *S*
		Diethyl disulfide (DEDS)		onion	4000	F, *S*
Organopolysulfane (monoorganopolysulfane)	0, −1	Methyl disulfane (Starkenmann *et al.*, 2016)		flinty	Unknown	*F, S*
Diorganopolysulfane	−1, 0, −1	Dimethyl trisulfide (Leppänen *et al.*, 1979)		truffle	100	*F, S*
		Glutathionyl methyl trisulfide (Kreitman *et al.*, 2017)			n.a.	S
S-Alkylthiosulfate (Bunte salt, *S*-sulfonate)	−1, +5	Glutathione-*S*-sulfonate (Arapitsas *et al.*, 2016)			n.a.	S

(*Continued on next page*)

Table 1. (*Continued*).

Compound class	S redox state	Examples	Structure	Odor	Odor Threshold (ng/L)	Origin
Sulfonate	+4	5-Sulfo-(*E*)-caftaric acid (Hayasaka *et al.*, 2017)			n.a.	S
			Inorganic forms			
Sulfide	−2	Hydrogen sulfide (H_2S)		rotten egg	1000	F, S
Polysulfane	−1, 0, −1	Trisulfane		flint, matches	Unknown	*F, S*
Elemental sulfur	0	S_8 (major allotrope)			Unknown	G
Bisulfite	+4				n.a.	F, W
Sulfate	+6				n.a.	G, S, W

[a]GS = glutathionyl group.
[b]n.a. = not applicable because compound is non-volatile.

straightforward – the primary pathway appears to be through non-enzymatic hydrolysis of grape-derived *S*-methylmethionine (Segurel et al., 2005; Loscos et al., 2008). Once formed, DMS appears to be stable, and its accumulation is well correlated with wine age and storage temperature (Segurel et al., 2005).

The origins of the two main malodorous sulfhydryl compounds (H_2S and MeSH) that arise during alcoholic fermentation are well studied (Rankine, 1963; Eschenbruch, 1974; Stratford and Rose, 1985; Kilmartin et al., 2001; Ugliano and Henschke, 2009). One major source of H_2S is sulfide (S^{2-}) formed by yeast as an intermediate in the sulfate reduction sequence pathway during the biosynthesis of sulfur-containing amino acids (Swiegers et al., 2005). Conditions which impede incorporation of S^{2-} into these amino acids, e.g., low levels of nitrogen, the absence of certain co-factors, or the metabolic efficiency of different yeast strains, can result in extracellular diffusion of S^{2-} and formation of H_2S (Bell and Henschke, 2005; Smith et al., 2015). Yeast catabolism of cysteine (Cys) and methionine (Met) may also serve as a source of H_2S and MeSH, respectively (Walker and Simpson, 1993). Finally, elemental sulfur, S(0), present from fungicide residues, can be readily converted to H_2S during fermentation (Acree et al., 1972) through non-enzymatic reaction with yeast-derived glutathione (GSH) (Sluiter, 1930) although enzymatic pathways may also be involved (Araujo et al., 2017). In addition to having a direct sensory effect, H_2S can react with other electrophilic components of wine to lead to the formation of additional VSCs, e.g., reaction with (*E*)-2-hexenal can yield 3SH during fermentation (Araujo et al., 2017).

Although enzymatic formation of H_2S and MeSH is well documented, the importance of non-enzymatic pathways has only been recently recognized. Several reports have shown that these two compounds (as well as reduced aromas more generally) can increase in wines bottled under low oxygen conditions, e.g., in a sealed ampule or under a tin-lined screwcap, and particularly in the presence of transition metals (Table 2). As a caveat, some of the methods employed in these studies utilized brine or NaCl addition, which can disrupt metal-sulfhydryl complexes and overestimate free sulfhydryls, as described in Section 3. From a winemaking perspective, this appearance of H_2S, MeSH, or related sulfhydryls during bottle storage is particularly problematic, since the winemaker has little recourse at this stage other than recalling and/or rebottling the wine.

Table 2. Literature reports of the appearance of reduced aromas or increases in free H_2S/MeSH during wine storage.

Citation	Treatments	Observations
(Skouroumounis *et al.*, 2005)	Riesling and Chardonnay wines bottled for 5 years under one of 4 closures or in sealed ampules, with and without ascorbic acid	Wines stored under lower oxygen conditions (screwcap or sealed ampule) had greater "*reduced struck flint/rubber*" aroma
(Kwiatkowski *et al.*, 2007)	Cabernet Sauvignon bottled for 2 years under one of 3 closures and with varying headspace volumes (*ullage*)	Wines stored under lowest oxygen conditions (screwcap, low ullage) had greatest "*reduced struck flint/rubber*" aroma
(Lopes *et al.*, 2009)	Sauvignon blanc bottled for 2 years under one of 3 closures or in a sealed ampule, with varied concentrations of ascorbic acid and/or SO_2	Wines stored under lowest oxygen conditions (sealed ampule, Saran-tin screwcap) had signficantly higher *"reduced"* aroma after bottling, and H_2S increased from 1.4 μg/L at bottling to >20 μg/L. Intermediate H_2S concentrations observed for other closures.
(Ugliano *et al.*, 2011)	Air- or N_2-saturated Sauvignon blanc bottled under synthetic closures for 6 months, and then stored in either anerobic (N_2) or aerobic environments. Wines were bottled with varied concentrations of glutathione and/or copper sulfate	Significant increase of H_2S in several treatments, with highest concentrations (up to 4.5 μg/L) observed in wines with lowest oxygen exposure and added glutathione and copper sulfate.[a]
(Viviers *et al.*, 2013)	Chardonnay and Shiraz wines, stored in N_2-flushed Cornelius kegs for up to 12 months. Wines were treated with individual or combinations of transition metals (Cu, Fe, Mn, Zn, and Al)	Increases in H_2S and MeSH observed for several treatments – in general, largest increases (Day 365 vs. Day 1) observed for Cu-containing treatments.[a]
(Kwasniewski, 2013)	Chardonnay produced from grapes with S(0) pesticide residues. H_2S from fermentation was removed by sparging with inert gas prior to bottling for 3 weeks under screwcap	Detectable H_2S (~ 1 μg/L) after 3 weeks bottle storage in several treatments
(Ferreira *et al.*, 2014)	16 Spanish red wines, bottled with varying oxygen levels, stored for up to 6 months	Increase in MeSH (up to 6.9 μg/L) in 12 of 16 wines stored under low oxygen conditions.[b]
(Bekker *et al.*, 2016c)	Chardonnay and Shiraz wines, stored in N_2-flushed Cornelius kegs for up to 12 months. Wines were treated with or without copper sulfate addition, and with varying pH	Increases in H_2S and MeSH observed for control and all treatments. Highest H_2S (42.7 μg/L) observed at 3 months for Chardonnay with higher pH (3.48) and 0.5 mg/L Cu addition.[a]
(Franco-Luesma and Ferreira, 2016a)	21 wines (13 reds, 5 whites, and 3 rosés) stored anaerobically for up to 379 days	Increases in free H_2S (up to 12.9 μg/L) and free MeSH (up to 3.5 μg/L). Increase in free H_2S well correlated with initial total H_2S concentration (free + complexed)
(Bekker *et al.*, 2016a)	Verdelho and Shiraz wines with or without Cu^{2+} and SO_2. Stored in Cornelius kegs for up to 12 months	Increases in H_2S (>40 μg/L) for treated wines containing both Cu^{2+} and SO_2.[a]

[a]Measurements performed by headspace sampling following addition of 2 g NaCl to 10 mL of wine. Although the approach should primarily measure free forms, some complexed ("brine-releasable") forms may also have been measured.
[b]Measurements performed by SPME extraction following 50:1 dilution with concentrated brine, and therefore reflect the sum of both free and complexed ("brine-releasable") MeSH.

Oxygen availability has a clear role in limiting accumulation of H_2S and MeSH during storage – either through their oxidation or by inhibiting their formation (Goode, 2005; Ugliano, 2013) – but the identity of the latent precursors of these sulfhydryl compounds in wine remains uncertain. Two early hypotheses regarding the formation of MeSH (and other thiols) during wine storage were:

i) Symmetric disulfides, formed by oxidation of their corresponding (and more potent) thiols, were proposed to re-form thiols via reduction, e.g., diethyl disulfide as a precursor of ethanethiol (Bobet et al., 1990).
ii) *S*-Alkylthioacetates, formed through enzymatic acetylation of thiols during fermentation, were proposed to re-form thiols via acetate hydrolysis, e.g., methyl thioacetate as a precursor of MeSH (Rauhut et al., 1998).

Surprisingly, the importance of these precursors does not appear to have been validated with a broad spectrum of wines, and recent work has questioned whether these compound classes are the only (or even the major) latent sources of MeSH (Ugliano, 2013; Ferreira et al., 2014, 2017). Additionally, the early hypotheses did not consider potential precursors of H_2S during bottle storage. As discussed later in this review, several other plausible candidate latent precursors include metal sulfide complexes (Section 3), and asymmetric disulfides, polysulfanes, and (di)organopolysulfanes (Section 4).

Considering the frequency of reductive faults in wine, establishing the major latent precursors and pathways responsible for H_2S and MeSH formation during bottle storage is of importance to wine producers, as well as to makers of other alcoholic beverages (e.g. beer, cider). This report will critically review proposed or established non-enzymatic reactions responsible for formation of malodorous sulfhydryl-containing compounds during wine storage. Likely pathways should satisfy two criteria:

1) The putative precursor should be metastable during typical bottle storage conditions, i.e., the conversion of precursor to free sulfhydryl should occur on the order of several weeks to a couple of years at room temperature.
2) The precursor concentration should be large enough to generate concentrations of H_2S and MeSH reported to develop during storage (up to 1–2 μM).

Many proposed pathways regarding formation of H_2S and MeSH are reversible or competitive, so reactions responsible for the loss of these compounds are also discussed. Finally, because mechanistic studies on sulfur redox chemistry in wine are limited, insights from relevant studies conducted in physiological and biogeochemical systems have been included.

2. Sulfhydryls and wine oxidation products

Sulfhydryl compounds, especially H_2S, are nucleophilic and can participate in reversible or non-reversible reactions with electrophiles. Fermentation produces a highly reductive

environment and yeast will largely consume electrophilic compounds, produced in crushed grapes (e.g., (*E*)-2-hexenal) or via glycolysis (e.g., acetaldehyde), as electron acceptors. As a result, electrophiles in wine (e.g., quinones, aldehydes) most likely to react with sulfhydryls are those resulting from non-enzymatic wine oxidation.

2.1. Reactions of sulfhydryl compounds with major wine oxidation products

Exposure of wine to oxygen (O_2) is well known to decrease concentrations of sulfhydryl compounds, and intentional exposure of stored wine to O_2 (e.g., through micro-oxygenation) is used as means to decrease undesirable reductive aromas (Nguyen et al., 2010; Ugliano, 2013; Day et al., 2015). Triplet state oxygen ($^3O_2$) is unreactive towards singlet state species and therefore requires the presence of transition metal catalysts or photosensitizers to react with organic compounds in wine (Danilewicz, 2003; Elias and Waterhouse, 2010). Thorough discussion of metal-mediated reactions of wine components with O_2 can be found elsewhere (Danilewicz, 2013; Waterhouse et al., 2016c), and the most important steps are shown in Fig. 1A. The initial reaction of Fe(II) with O_2 yields a reactive superoxide species ($[Fe(III)\text{-}O_2^{\bullet}]^{2+}$) and H_2O_2. Once generated, H_2O_2 can either be consumed by bisulfite (HSO_3^-) (used in wine as an antioxidant) or undergo metal-catalyzed reduction *via* the Fenton reaction to generate highly-reactive hydroxyl radicals ($HO^{\bullet}$) capable of oxidizing organic compounds in wine at rates proportional to their concentrations (Danilewicz, 2003; Elias and Waterhouse, 2010). Due to its high abundance in wine, ethanol (ca. 2 M) is the primary target of $HO^{\bullet}$, resulting in formation of an intermediate 1-hydroxyethyl radical (1-HER) and eventually acetaldehyde (Fig. 1A) (Elias and Waterhouse, 2010). Although $HO^{\bullet}$ and 1-HER can directly oxidize sulfhydryls in model systems, there is no evidence of either pathway occuring to any extent in real wines due to the presence of other wine components (e.g. glycerol and polyphenols) (Kreitman et al., 2013).

The [Fe(III)] species formed during initial oxidation will oxidize catechols to *o*-quinones (Fig. 1B). These quinones may be reduced back to catechols by ascorbic acid or HSO_3^-, or can undergo Michael-type addition reactions with HSO_3^- or other wine nucleophiles, including sulfhydryls (Fig. 1B) (Nikolantonaki and Waterhouse, 2012; Nikolantonaki et al., 2014). These quinone-sulfhydryl adducts have been characterized in both real and model wine systems, and appear to be a major pathway for loss of sulfhydryl compounds following oxidation (Blanchard and Darriet, 2004; Laurie et al., 2012; Nikolantonaki et al., 2012).

A recent publication has questioned the importance of quinone adduct formation in explaining the loss of sulfhydryls (Vela et al., 2018). The authors reported that VSCs are lost following micro-oxygenation but can be regenerated following high temperature anoxic accelerated aging (50 °C for up to 7 weeks; see Section 7.1 for further discussion). The authors state that this is evidence against quinone-sulfhydryl reacions, since these adducts are expected to be stable. However, as discussed in the next section, evidence for quinone adduct stability is still lacking.

2.2. Potential for regenerating free sulfhydryls from quinone-sulfhydryl adducts

There is abundant research showing the formation of catechol-sulfhydryl adducts during wine oxidation (Nikolantonaki and Waterhouse, 2012; Nikolantonaki et al., 2012, 2014). However, there have been no reports regarding the reversibility of these Michael-type reactions under wine conditions, and thus whether these adducts could serve as latent precursors of H_2S and MeSH. The major quinone adduct, 2-*S*-glutathionylcaftaric acid ("Grape Reaction Product", Table 1), can be detected in aged wines, suggesting the complexes are stable (Cheynier et al., 1986). However, reaction of H_2S with quinones is kinetically-favored over the reaction of quinones with other nucleophiles and reductants in wine-like model systems (Nikolantonaki and Waterhouse, 2012), and release of H_2S or MeSH could hypothetically occur during wine storage, particularly at higher temperatures like those employed in accelerated aging assays (Vela et al., 2018). H_2S can be rapidly released from its 4-methylcatechol adduct (4-methyl-5-sulfanylcatechol) at pH 6 in the presence of dithiothreitol (DTT) or tris(2-carboxyethyl) phosphine (TCEP) (Kwasniewski, 2013). Similarly, addition of DTT, TCEP, or sodium sulfite at pH 8 to a protein-phenol adduct (generated by reaction of the cysteine residues with quinones) resulted in regeneration of free phenol and sulfhydryl groups (Jongberg et al., 2015). Whether reverse reactions are relevant under wine conditions is an interesting direction worthy of future study.

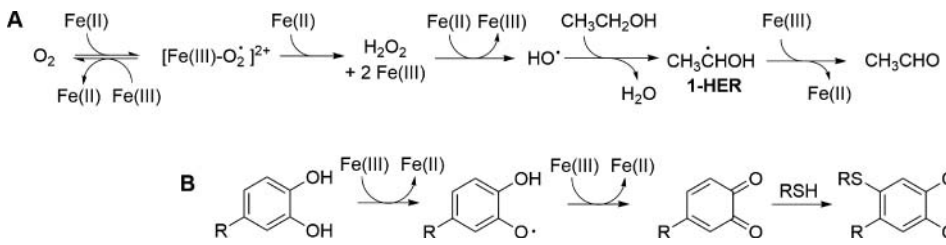


Figure 1. (A) Proposed reaction scheme of Fe(II) with oxygen to produce Fe(III) and hydrogen peroxide in wine, followed by the Fenton reaction and oxidation of ethanol to 1-hydroxyethyl radical (1-HER), which forms acetaldehyde under low oxygen conditions. (B) Proposed reaction scheme for loss of sulfhydryls via coupled oxidation in wine, showing *o*-catechol being oxidized to *o*-quinone in the presence of Fe(III), and subsequent Michael-type nucleophilic addition of sulfhydryl to give a catechol-thiol adduct.

2.3. Reactivity of sulfhydryls with other carbonyl compounds

Aldehydes formed during fermentation or wine oxidation, such as acetaldehyde (Fig. 1A), glyceraldehyde, etc. (Lea et al., 2000; Elias et al., 2008), can potentially form adducts with nucleophilic sulfhydryl compounds, resulting in hemithioacetals and thioacetals under acidic conditions. These reactions would be analogous to (and in competition with) reactions of carbonyl compounds with HSO_3^- (Boulton et al., 1999; De Azevedo et al., 2007). Hypothetically, the adducts could serve as latent sulfhydryl precursors in wine, as has been proposed for cysteine adducts of aldehydes in beer (Baert et al., 2015). Thiol-aldehyde adducts have not been detected in wine, however, and based on existing literature should be at negligible concentrations – studies report low equilibrium binding constants, with $K \approx 20\ M^{-1}$ for a range of thiols and aldehydes, including GSH (Lienhard and Jencks, 1966; Sonni et al., 2011). An interesting exception appears to be reaction of oak-derived furfural and H_2S to generate stable 2-furfurylthiol (Blanchard et al., 2001). However, considering its trace concentrations ($< 1\ \mu g/L$), this compound would be an unlikely latent source of H_2S. The bi-substitutional ability of H_2S may result in its reaction with multiple aldehydes (Starkenmann et al., 2008), although reports under wine-like conditions are limited to the case of substituted trithiolanes, e.g. *trans*-3,5-dimethyl-1,2,4-trithiolane, and related cyclic compounds, which can be formed in model wine systems containing high levels of acetaldehyde (>100 mg/L) and H_2S (>1 mg/L) (Rauhut et al., 1993). More recent work has shown that trithiolane and other cyclic species do not appear in real wines, putatively because bisulfite in wines would bind acetaldehyde and render it unavailable for reaction (Bertrand and Beloqui, 2009). Finally, wines also contain low mM concentrations of hydroxycinnamic acids bearing the electrophilic α,β-unsaturated carboxylic side chain. Bouzanquet et al. have reported the formation of an irreversible GSH-hydroxycinnamic acid product in a model wine system through a free radical mechanism (Bouzanquet et al., 2012), but analogous reactions involving H_2S and MeSH in real or model systems have not been studied.

3. Metal-sulfide complexes

In addition to catalyzing wine oxidation through redox cycling, transition metals are capable of complexing sulfhydryl compounds. Historically, reactions of copper with Cys residues of proteins were studied as a cause of wine haze (*copper casse*) (Joslyn and Lukton, 1956; Lukton and Joslyn, 1956). As described later in this section, the sulfhydryl-binding properties of copper are also exploited by winemakers to treat reductive wines, and these complexes could potentially serve as latent precursors of H_2S, MeSH, or other sulfhydryls (Franco-Luesma and Ferreira, 2014, 2016b).

3.1. Stability constants of metal-sulfide complexes

The complexation of many transition metals with H_2S and other sulfhydryls has been well-studied in geochemical processes due to its role in dissociation of bulk minerals and generation of soluble or insoluble metal-sulfide structures (Baumgartner et al., 1982; Blesa et al., 1986; Amirbahman et al., 1997; Herszage et al., 2003; Luther and Rickard, 2005; Rickard and Luther, 2006). The stability constants (K_c) for metal sulfide complexes of transition metals are reported in Table 3. Larger stability constants indicate more favorable H_2S binding, and follow the order of Cu > Zn > Fe > Mn. Corresponding stability constants for metal-thiol complexes are lacking but are expected to follow similar trends. Solubilities of bulk metal sulfides generally follow the inverse trend of stability constants – higher K_c values correlate with lower solubility products (K_{sp}) – but as seen in Table 3 the relationship is not linear. As a caveat, determining the solubility of Cu(II)S is complicated by the fact that Cu(II) may be partially reduced to Cu(I) during analysis (as described later).

Ultimately, K_{sp} values are of little use in predicting actual metal-sulfhydryl solubility in most real systems, including wine, because the concentration of solubilized metal-sulfhydryl clusters can be much higher than the concentration of free copper or sulfhydryl species characterized by K_{sp} values (Rickard and Luther, 2006). This discrepancy is particularly important for copper-sulfhydryls, as discussed in Section 3.2.1. Additionally, although complexation may decrease activity of the sulfhydryl compound (e.g., decreased volatility or participation in nucleophilic reactions), redox reactions between complex-bound metals and sulfhydryls may still occur, as discussed subsequently.

3.2 Complexes of sulfhydryl groups with specific transition metals

3.2.1 Copper-sulfhydryl complexes

Copper is naturally present in grapes, and copper-based pesticide treatments in the vineyard may cause carryover into the

Table 3. Experimental stability constants (log K_c) at ionic strength of 0.7 M and solubility products (-log K_{sp}) for metal sulfides in pure water. Values determined at 25 °C and pH 7. Values adapted from (Rickard and Luther, 2006) and sources within (Renders and Seward, 1989; Zhang and Millero, 1994; Luther *et al.*, 1996; Al-Farawati and Van Den Berg, 1999).

Metal	Median wine concentration (mg/L)	-log K_{sp} of metal sulfide	Solubility of metal sulfide (mg/L)	Complex	Redox state	log K_c of metal sulfide
Mn	0.97	13.5	6	$[MnHS]^+$	Mn(II)	
Fe	0.88	18.1	6×10^{-2}	$[FeHS]^+$	Fe(II)	
Cu	0.15	36.1	3×10^{-14}	$[CuHS]^{+*}$	Cu(II)	6.5
				$[CuS]^{0*}$	Cu(II)	11.2
				$[CuHS]^0$	Cu(I)	12.1
Zn	0.54	24.7	8×10^{-9}	$[ZnHS]^+$	Zn(II)	6.1
				$[ZnS]^0$	Zn(II)	11.7

*Cu(II) is likely partially or fully reduced to Cu(I) during analysis.

juice (Provenzano et al., 2010). However, the concentration of copper is known to decrease during fermentation due to its adsorption and removal by yeast cells (*lees*) (Junghans and Straube, 1991; Blackwell et al., 1995). Because of this, copper concentrations of freshly fermented wines are generally less than legal limits (Ribereau-Gayon et al., 2006), which vary among countries but typically range from 0.5 – 1 mg/L (Clark, Wilkes, et al., 2015). Leaching from brass fittings or other winery materials is a potential source of copper, but in modern wineries the major source of copper in finished or packaged wine is likely due to the intentional addition of Cu(II) salts to treat sulfurous off-aromas. This addition, known as copper fining, has been used in winemaking for decades (Rauhut, 1993; Clark, Grant-Preece, et al., 2015) and protocols can be found in most wine production texts (Zoecklein et al., 1995).

Because copper sulfide (and copper sulfhydryls) have low K_{sp} values in water (Table 3), it was long assumed (incorrectly) that these forms were completely precipitated and removed following racking or filtration (Zoecklein et al., 1995). However, the K_{sp} value is based on the concentrations of copper and sulfide ions – it does not consider the solubility of copper sulfide complexes or nanoparticles which can remain dispersed well in excess of their solubilities predicted from pure solutions (Ma et al., 2006). For example, in ocean water, low nM concentrations of complexed copper and biologically relevant thiols can be detected, whereas the concentration of free copper is reported to be only 10^{-14} M (Moffett and Dupont, 2007). Similarly, in beer, copper and H_2S can exceed 0.1 mg/L and 0.05 mg/L, respectively (Walker, 1995). The work noted that addition of a large molar excess of Cu(II) salt could decrease H_2S headspace concentration, but without precipitation of a copper-sulfhydryl salt (Walker, 1995).

The mechanism for reaction of H_2S (or HS^-) with Cu(II) under aqueous conditions has been studied in model fresh and saltwater systems (Fig. 2) (Luther et al., 2002). Mixing of 100 μM solutions of Cu(II) and HS^- resulted in rapid formation of a neutral six-atom cluster complex (Cu_3S_3), which subsequently underwent redox self-reactions to generate $Cu(I)_3S_3$ and various soluble anionic cuprous species (e.g., $Cu(I)_3S_6^-$, $Cu(I)_3S_9^-$). When sulfide was titrated into low μM Cu(II) solutions, Cu_3S_3 was again formed, with eventual formation of other anionic species (e.g., $Cu(I)_4S_5^-$, $Cu(I)_4S_6^{2-}$). Based on these and other experiments, it appears that at copper and sulfide concentrations < 2 μM, $Cu_3(I)S_3$, $Cu(I)_4S_5^-$ and $Cu(I)_4S_6^{2-}$ clusters will predominate, while at concentrations > 10 μM, larger nanoparticles will begin to form the various anionic species (Luther and Rickard, 2005). Analogous experiments in model wine using 50–100 μM concentrations of Cu (II) and sulfide have shown rapid conversion of Cu(II) to Cu (I), and formation of complexes with an average H_2S:Cu ratio of 1.4 to 1 (Kreitman et al., 2016a). Importantly, both clusters and larger nanoparticles may stay dispersed in solution, and precipitation of copper sulfide minerals (e.g. Cu(I)S, covellite) is generally only observed at mM concentrations. (Luther et al., 2002)

The formation of Cu_xS_y complexes following Cu(II) addition explains why Cu can remain in solution following addition to wine. For example, a recent study demonstrated that > 80% Cu remained in solution when added to a wine containing an excess of H_2S, even after common separation steps like filtration (Clark, Grant-Preece, et al., 2015). Other work arrived at a similar conclusion and demonstrated that complexes of copper (added as $CuSO_4$) with H_2S, MeSH, or EtSH remained dispersed in a model wine, and that the sulfhydryls could be released through addition of a strong brine (Section 3.3) during high temperature anoxic storage, or during long-term storage at room temperature (Franco-Luesma and Ferreira, 2014, 2016b; Ferreira and Franco-Luesma, 2016).

Additional complications exist for predicting copper sulfide behavior in wine as compared to model systems. First, other wine constituents may decrease the activity of copper or sulfide. For example in beer, copper activity is thought to decrease due to the presence of proteins or other nitrogenous components (e.g., melanoidins) (Thorne et al., 1971), and other transition metals (e.g. Zn) could decrease sulfide activity. Another issue is that other organic constituents of wine may inhibit cluster aggregation, as suggested for tartaric acid (Clark, Grant-Preece, et al., 2015). Additionally, thiols like GSH and Cys can be present at much greater concentrations than H_2S in wine, and thiols are well-known to inhibit condensation of copper sulfides (Rickard and Luther, 2006) (Fig. 2). Although aggregates are not expected to precipitate spontaneously (Luther et al., 2002), larger aggregates presumably are more susceptible to physical removal than smaller species, which could explain why more Cu was removed by filtration (up to 60%) following addition to a sulfide-containing model wine as compared to a real wine (< 20%) (Clark, Grant-Preece, et al., 2015).

Similarly to its reaction with H_2S, Cu(II) can react with thiols to yield six-membered rings, with concurrent release of disulfides in addition to formation of Cu(I)-thiol complexes (Coucouvanis et al., 1980; Blower and Dilworth, 1987). For example, addition of GSH to Cu(II) at pH 7.4 resulted in

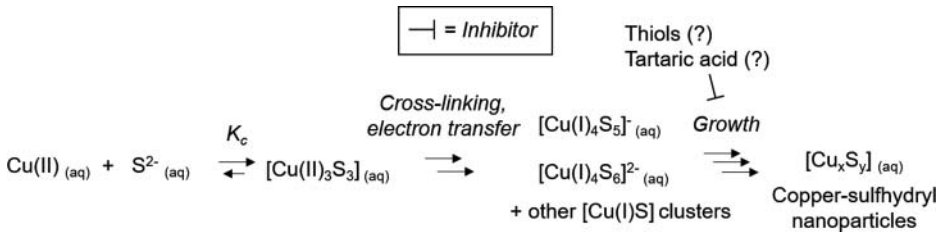


Figure 2. Mechanism for formation of soluble copper sulfide clusters and nanoparticles from Cu(II) and sulfide in a model seawater system (Luther et al., 2002). At low μM concentrations, as would be expected in wine, $[Cu(II)_3S_3]$ clusters are formed rapidly. Reduction of Cu(II) to Cu(I) then occurs via inner-electron transfer to form new [Cu(I)S] clusters, e.g. $[Cu(I)_4S_5]$. At sufficiently high copper and sulfide concentrations, formation of larger nanoparticles can occur, although growth may be inhibited by wine components such as thiols (e.g., GSH) and tartaric acid.

formation of both glutathione disulfide as well as a Cu(I)-GS complex. Similar results have been shown for other biologically-relevant thiols (Pecci et al., 1997; Gilbert et al., 1999). Recent studies in model wines containing free sulfhydryl compounds suggest that a similar pathway is followed (Fig. 3) (Kreitman et al., 2016a, 2016b), in which addition of Cu(II) results in the binding of one equivalent of Cu(II) with two sulfhydryl equivalents. Consistent with the previous biological studies (Pecci et al., 1997; Gilbert et al., 1999), the reaction between Cu(II) and sulfhydryls quickly leads to the formation of Cu(I)-complexes under wine conditions. In the presence of a single thiol, half of the sulfhydryl equivalents are ejected as symmetrical disulfides, while the remaining sulfhydryl equivalents form a copper-thiolate [Cu(I)-SR] complex, which further aggregates (Fig. 3) (Kreitman et al., 2016a). Interestingly, the [Cu(I)-SR] complex appears to remain redox active, and can react with Fe(III) to regenerate [Cu(II)-SR] species capable of further producing disulfides (Fig. 3) (Kreitman et al., 2016b). This cycle is discussed in more detail in Section 4.3.

The reaction of Cu(II) and thiols to yield disulfides in wines has been suggested to proceed via a free radical intermediate (Smith et al., 2015); however, the addition of a free radical trapping agent (DMPO) to a model wine system containing sulfhydryls and Cu(II) had no effect on reaction kinetics or product (i.e., disulfide) yield, suggesting that disulfide and diorganopolysulfane formation shown in Fig. 3 proceeds through inner-sphere electron transfer without the release of free thiyl radical intermediates (Kreitman et al., 2016a). This observation is supported by previous research in model seawater systems, which also failed to show evidence of thiyl radical formation (Luther et al., 2002).

In model wine systems containing H_2S and multiple thiols (a situation that should be more analogous to real wines), putative mixed disulfides and diorganopolysulfanes can be formed in the presence of Cu(II) (Fig. 3) (Kreitman et al., 2017). These results are in agreement with work performed in model brandy, in which oxidation of H_2S, MeSH, and EtSH in the presence of Cu(II) resulted in formation of mixed disulfides and trisulfanes, which the authors speculate occurred via an organopolysulfane (i.e., RSSH) intermediate (Nedjma and Hoffmann, 1996). Related pathways could explain the presence of di- and trisulfanes (i.e., HSSH and HSSSH, associated with "flint" aroma) in white wines as reported recently (Starkenmann et al., 2016).

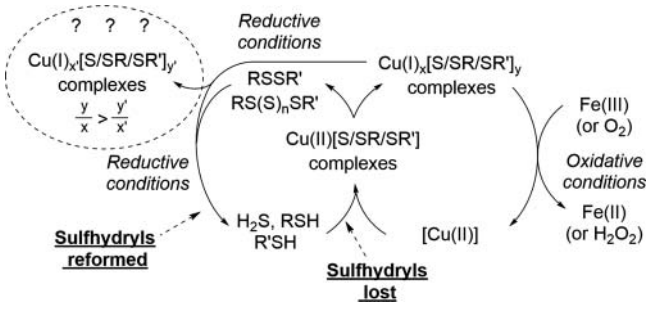


Figure 3. Pathway for formation of disulfides (RSSR'), diorganopolysulfanes ($RS(S)_nSR'$) and Cu(I)-sulfhydryl complexes ($Cu_x[S/SR/SR']_y$) from free sulfhydryl species in the presence of Cu(II), adapted from the literature (Kreitman *et al.*, 2016a). A pathway for the reformation of free sulfhydryls from disulfides and diorganopolysulfanes is also shown (Kreitman *et al.*, 2017). A speculative pathway for release of free sulfhydryls by conversion of $Cu_x[S/SR/SR']_y$ to $Cu_{x'}[S/SR/SR']_{y'}$ is depicted, where the latter complexes would have lower copper:sulfhydryl molar ratios ($\frac{y}{x} > \frac{y'}{x'}$).

3.2.2 Iron-sulfhydryl complexes

Iron has been well-studied for its role in wine oxidation reactions involving oxygen, polyphenols, and sulfite (Fig. 1). Similar to copper, iron can be introduced into grapes from the vineyard soil and local environment, and its presence is also affected by viticultural and enological practices (Almeida and Vasconcelos, 2003). However, the majority of iron in wines (average concentration of 1 – 2 mg/L) appears to be due to contamination, e.g., leaching from tanks or fittings, rather than intentional addition as is the case with copper (Almeida and Vasconcelos, 2003; Martin et al., 2012). The majority of free iron appears to be present as Fe(II) (Tašev et al., 2006; Elias and Waterhouse, 2010) due to wine's low pH and abundance of reducing compounds like phenolics, but the exposure of wine to oxygen increases the Fe(III):Fe(II) ratio (Danilewicz, 2016b; Kreitman et al., 2017).

Although iron plays a key catalytic role in non-enzymatic wine oxidation by generating intermediates (particularly quinones, Fig. 1) capable of reacting with sulfhydryls, studies on the direct reaction of iron with sulfhydryl compounds at wine pH are sparse. Model studies over the pH range 3 – 7 showed that GSH reacted spontaneously with Fe(III) to generate Fe(II) and GSSG (Hamed and Silver, 1983; Hamed et al., 1983). In wines, oxidation of thiols by Fe(III) to generate disulfides was speculated to occur through a radical-mediated mechanism (Danilewicz et al., 2008). However, recent work by Kreitman *et al.* in model wine systems indicates the reaction occurs in a concerted manner without the release of free thiyl radical intermediates. Although Fe(III) is capable of directly binding or oxidizing thiols, the reaction is relatively slow (24 h before a significant loss of thiols is observed) compared to reaction with Cu(II) (Kreitman et al., 2016b). As discussed above, the major redox species of iron in wine is Fe(II), and in contrast to Cu(I), there is little evidence that Fe(II) interacts strongly with thiols. In simple model systems performed under pH conditions representative of wine (pH < 4), GSH and Cys coordinated with Fe(II) through their carboxylate groups, and not their sulfhydryl groups (Hamed and Silver, 1983; Hamed et al., 1983). In real wines with high concentrations of tartaric acid and other carboxylic acids, the presence of Fe(II)-SR complexes should therefore be negligible.

Unlike thiols, H_2S could potentially remain bound to Fe(II) to some degree, appearing to form clusters with a mackinawite-like structure (Fe_2S_2 subunit), which may remain dispersed under acidic conditions (Rickard and Luther, 2007; Nielsen et al., 2008). Fe(III) can be generated during wine oxidation (Fig. 1A), and Fe(III)-catalyzed oxidation of sulfhydryls is also possible (Danilewicz et al., 2008; Kreitman et al., 2016b), but under real wine conditions, other reactions that consume Fe(III) are likely to dominate, e.g., oxidation of catechol groups (Fig. 1B), which would generate thiol-scavenging quinones from *o*-diphenols (Nikolantonaki and Waterhouse, 2012). Alternatively, Fe(III) in wines could play an important synergistic role in accelerating copper-catalyzed sulfhydryl oxidation by regenerating [Cu(II)] species (Fig. 3) (Kreitman et al., 2016b). The role of iron in favoring either of these pathways is not yet clear.

3.2.3 Reactions of sulfhydryls with other transition metals

Manganese, typically present in wines at concentrations that are comparable to iron (i.e., average of 1 – 2 mg/L) (Martin et al., 2012), has been suggested to play an important role in non-enzymatic wine oxidation. Recent work by Danilewicz suggests that Mn(II) can react with Fe(III) complexes under wine conditions to generate highly oxidizing Mn(III), which will rapidly oxidize *o*-diphenols to quinones (Danilewicz, 2016a). This may help explain why manganese additions increase acetaldehyde production in wine (Cacho et al., 1995). Direct reactions between manganese and sulfhydryls in wine have yet to be reported, but based on work in model systems, sulfhydryls appear to be more susceptible to direct oxidation by Mn(III) than Fe(III) (Bagiyan et al., 2003). Finally, MnS impurities in stainless steel tanks have been suggested as a potential source of H_2S in wines, as discussed in more detail in Section 6.2.

Zinc concentrations in wine are comparable to that of copper and average between 0.3 – 0.7 mg/L, but can exceed 1 mg/L (Martin et al., 2012). The binding of H_2S and MeSH to Zn(II) has been demonstrated in model wine solutions and beer (Walker, 1995; Franco-Luesma and Ferreira, 2014). Unlike the other transition metals discussed, Zn(II) does not redox cycle and is unlikely to affect the rate of oxidation reactions in wine. Table 3 shows that complexation of Zn(II) with H_2S is comparable to Cu(II) (Luther and Rickard, 2005), and similar to Cu (II), Zn(II) may form a hydrated Zn_3S_3 ring structure under aqueous conditions that may react to yield larger clusters, e.g. $Zn_4S_6^{4-}$ (Luther et al., 1999). However, unlike the reaction of sulfhydryls with Cu(II), which involves an electron transfer, sulfhydryls and Zn(II) undergo a simple substitution reaction (Luther et al., 1999, 2002).

Reactions of other first row transition metals, including chromium, cobalt, and nickel, with sulfhydryls have not been studied under wine conditions. Because these metals are generally present at concentrations below 0.1 mg/L (Tariba, 2011), they are assumed to be of low importance.

3.3 Release of sulfhydryls from metal-sulfhydryl complexes

As described in Section 3.2, the reaction of oxidized transition metals (particularly Cu(II)) with sulfhydryl groups can lead to disulfides, diorganopolysulfanes and/or metal-sulfhydryl complexes. The fate of disulfides is discussed in more detail in Section 4. The importance of metal-sulfhydryl interactions as a major latent source of H_2S and MeSH has only recently been appreciated (Ugliano et al., 2011; Ugliano, 2013; Viviers et al., 2013). Release can be induced by dilution of the wine and addition of 35% brine (Franco-Luesma and Ferreira, 2014). Franco-Luesma and Ferreira defined the *total* concentrations of H_2S, MeSH, and other sulfhydryls as the amount measured following brine addition, in contrast to the *free* concentrations, which were detectable by headspace measurements with no adjustment of the sample. The authors originally defined the difference between total and free sulfhydryls as the *bonded* concentration. In more recent publications these and other authors have referred to either the bound or the total concentration as the *"brine-releasable" (BR)* fraction (Chen et al., 2017; Vela et al., 2017). In this review, we will use the term "brine-releasable" to specifically refer to the complexed forms, since the initially free sulfhydryls would not be affected by brine addition and thus should not be considered brine-releasable. This brine-releasable fraction respectively accounted for 94% and 47% of the sum of free and brine-releasable H_2S and MeSH in commercial wines, and lesser amounts of higher-molecular weight sulfhydryls (Franco-Luesma and Ferreira, 2016b). Remarkably, the ability of high concentrations of Cl^- to liberate copper-complexed H_2S from beer (using HCl in place of NaCl) appears to have been noted in 1964 (Jansen, 1964), but completely ignored in the enology community until recently. Chloride anions can ligate, stabilize, and solubilize Cu (I) to generate the corresponding $CuCl_3^{2-}$ and $CuCl_4^{3-}$ complexes (McConnell and Davidson, 1950; Gilbert et al., 1997), effectively displacing organic thiols (Gilbert et al., 1997). Similarly, chloride can cause dissociation of bulk metal sulfide minerals by displacing sulfide (Watling, 2006).

Complete release of sulfhydryls appears to require both high dilution as well as high brine concentrations. Most work has used a 50-fold dilution with 35% brine, and minimum dilution of 1:7 appears to be necessary to achieve > 80% recovery of complexed H_2S. A dilution of 1:3 resulted in < 25% recovery. Based on this data, methods that employ NaCl addition in place of brine dilution, e.g. a method described from the Australian Wine Research Institute in which 2 g of NaCl is added to 10 mL of wine without dilution, are expected to measure primarily free sulfhydryl forms, although minor contribution from bound forms cannot be ruled out (Table 2, footnote).

Although several transition metals can reversibly complex H_2S, complete binding (and complete release by brine addition) is only observed following addition of Cu(II) (Franco-Luesma and Ferreira, 2014). Furthermore, the concentration of brine-releasable H_2S and other sulfhydryls in wine is better correlated with copper concentration than with other transition metals (Ferreira et al., 2014) and may reflect the effect of brine on the chemistry of copper complexes. As described in Section 3.2.1, soluble Cu_xS_y clusters can exist in wine and other aqueous systems without precipitating. Taken together, these observations suggest that covellite-like Cu_xS_y clusters are the major source of brine-releasable H_2S in wine.

The reversible formation of soluble copper-sulfhydryl complexes may explain the counterintuitive observation that elevated copper concentrations in a finished wine can be associated with a greater increase in H_2S and MeSH during post-bottling storage (Ugliano, 2013; Smith et al., 2015). The formation of metal-sulfhydryl complexes in wine may protect the sulfhydryl group from oxidative loss through other mechanisms, e.g., quinone-thiol reactions (see Section 2.1). However, an important question is whether the brine-releasable sulfhydryl pool is releasable during normal wine storage conditions. Results are limited at this point, but for 20 white, rosé, and red wines stored under anoxic conditions for 379 d at room temperature, free H_2S increased at the end of storage for all wines, but the sum of free and brine-releasable H_2S concentration remained unchanged in all but one wine (Franco-Luesma and Ferreira, 2016a). An overall increase of free MeSH occurred in all wines; however, the sum of free and brine-releasable MeSH was observed to increase during 379 d storage (Franco-Luesma

and Ferreira, 2016a). Thus, there is strong evidence for brine-releasable H_2S being a major source of free H_2S during storage, but evidence is not as strong for brine-releasable MeSH.

The covellite-like Cu_xS_y clusters initially formed following Cu(II) addition are likely to be unstable during wine storage. Covellite is one of several copper-sulfide minerals described in the geochemical literature that differ in Cu:S stoichiometry in addition to physical appearance. Examples include covellite (CuS), anilite ($Cu_{1.75}S$), djurleite ($Cu_{1.96}S$), and chalcocite (Cu_2S). As shown in the E_h-pH diagram in Fig. 4, all of the forms can exist in the range of wine pH (i.e., pH 3–4), with the more copper-rich forms favored at lower redox potential (more negative E_h). The value of redox potential measurements in wine is a contentious topic. Lower redox potential is observed in more anoxic wines, and has been correlated with a greater proportion of free H_2S as compared to the sum of brine-releasable and free H_2S forms. However, as discussed in Section 7.2, the major redox process occurring during redox measurements appears to be the (irreversible) oxidation of ethanol by O_2; thus, wine redox potentials are largely a proxy for dissolved O_2 (Danilewicz, 2012). Regardless, wines contain low mM concentrations of bisulfite (HSO_3^-), a relatively strong reducing agent ($E_{pH\ 3.5} \sim -0.7$ V). Thus, it appears thermodynamically plausible that the stoichiometry of Cu_xS_y clusters will change from an initial value of $CuS_{1.4}$ (Kreitman et al., 2016a) to clusters with a higher copper-sulfur molar ratios, (e.g., $Cu(I)_{1.75}S$, $Cu(I)_{1.96}S$, $Cu(I)_2S$ or even Cu(0)) during reductive storage (Fig. 4). This conversion would result in a release of H_2S. More generally, this transformation could apply to a copper-sulfhydryl aggregate of arbitrary stoichiometry ($Cu_x[S/SR/SR']_y$) which would release sulfhydryls during storage to form a different aggregate ($Cu_{x'}[S/SR/SR']_{y'}$) with a lower copper:sulfhydryl molar ratio (i.e. $\frac{y}{x} > \frac{y'}{x'}$) (Fig. 3).

Although thermodynamically plausible, the pathway by which copper sulfide clusters could undergo reduction to release H_2S during wine storage is not established, and the stoichiometry of the final products is also unclear. Polyphenols

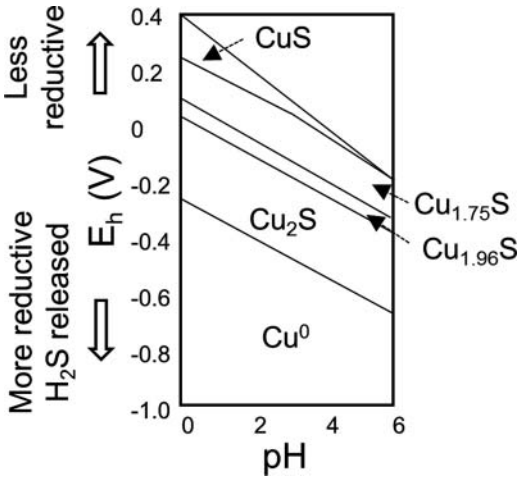


Figure 4. Pourbaix diagram for covellite (CuS) and other copper sulfide forms, adapted from Woods, et al. (Woods *et al.*, 1987). Akin to a phase diagram, the Pourbaix diagram depicts the thermodynamically-favored mineral form for a given E_h-pH combination, with the boundary lines representing transitions between two mineral forms. Based on thermodynamics, at typical wine pH of 3–4, copper sulfide species formed following copper fining are expected to release H_2S in a more reductive environment (lower E_h).

(Franco-Luesma and Ferreira, 2016a) and major wine thiols (Ferreira et al., 2017), e.g., Cys and GSH, have been proposed to facilitate release of free H_2S and other reducing agents in wine could also presumably participate in release mechanisms, including ascorbic acid and bisulfite. Ascorbic acid, as well as tris(2-carboxyethyl)phosphine (TCEP), were shown to release H_2S from copper sulfide complexes, albeit with lower efficiency than brine dilution (Chen et al., 2017). This observation is intriguing because of the common use of ascorbic acid as a putative test for disulfides (Zoecklein et al., 1999) and the potential for copper sulfhydryls to interfere with this test are discussed in Section 7.2.

The ability of copper to form reversible complexes with H_2S may also explain the observation that yeast will produce significantly more H_2S during fermentation when copper spray residues are present at elevated concentrations in the must (Eschenbruch, 1974). For example, increasing copper content from 0.98 to 3.91 mg/L resulted in an increase in final H_2S from 8.5 to 52.7 μg/L (Eschenbruch and Kleynhans, 1974). Although biochemical explanations for the phenomena have been presented (Eschenbruch, 1974), another possibility is that the added copper resulted in formation of copper-sulfhydryl complexes during fermentation. The analytical method employed relied on initial acidification of the wine with HCl, and then stripping H_2S from the wine with inert gas into a zinc acetate receiver flask (Brenner et al., 1953). The use of concentrated HCl would presumably have caused the same result as brine dilution (i.e., release of H_2S due to Cl^- complexation of copper), and the apparent interferences of copper-sulfhydryl complexes in this method were noted by brewing chemists (Jansen, 1964).

In addition to copper sulfides, other metal sulfide clusters could potentially release H_2S or MeSH following brine addition. In particular, zinc can form reversible complexes with sulfhydryls (Franco-Luesma and Ferreira, 2014), and an increase in the release of H_2S during anoxic storage of white and red wine in the presence of Zn(II) (Viviers et al., 2013) supports this notion. However, a survey of wines submitted to accelerated aging studies showed that zinc was negatively correlated with H_2S production (Franco-Luesma and Ferreira, 2016b), although this may be due to the fact that zinc deficiency results in greater H_2S production by yeast during fermentation, and thus higher amounts of latent H_2S in the wine prior to bottling (Gauci et al., 2009).

Finally, although a large proportion of latent H_2S and MeSH can be credited to brine-releasable copper sulfides, accelerated aging showed that in certain wines, up to 42% and 76% of H_2S and MeSH, respectively, are formed through other pathways (Franco-Luesma and Ferreira, 2016b). Likely additional latent precursors are discussed in Sections 4 and 5.

4. Precursors with S-S groups: Disulfides, polysulfanes, and (di)organopolysulfanes

4.1 Occurrence in wine

In organic chemistry, disulfides are well known to form free sulfhydryl compounds through reversible redox reactions (Carey and Giuliano, 2011). Disulfides discussed in enology

texts typically have much higher sensory thresholds than their corresponding thiols, and have been suggested to serve as sulfhydryl precursors in wine; e.g., DMDS (threshold = 30 μg/L) has been proposed to be reduced during storage to form MeSH (threshold = 1 μg/L), although a mechanism was not discussed (Limmer, 2005). Several sources, including well known wine production texts, advise winemakers to avoid excessive aeration of wines with reduced aromas to avoid generation of disulfides from thiols, as disulfides are not removed by the practice of copper fining (indeed, they can be formed during this process, see Section 4.2) and could serve as a latent source of thiols (Bobet et al., 1990; Zoecklein et al., 1999; Ribéreau-Gayon et al., 2006). Analogously, polysulfanes and (di)organopolysufanes have been documented in model systems (see Table 1) (Nedjma and Hoffmann, 1996), and could potentially serve as a latent source of both H_2S and thiols (Vela et al., 2018).

Although disulfides (particularly DMDS and DEDS) are often mentioned as both latent precursors of sulfhydryls and off-aroma compounds in their own right (Bobet et al., 1990; Zoecklein et al., 1999; Ribéreau-Gayon, et al., 2006), the overwhelming majority of surveys of commercial wines (including some involving wines described as having reductive sensory characters) show that DMDS and DEDS are rarely observed in real products (Mestres et al., 1997, 2000; Sala et al., 2000; Belancic Majcenovic et al., 2002; Nguyen et al., 2010; Siebert et al., 2010; Ugliano et al., 2012; Bekker, Day, et al., 2016). DMDS and DEDS can be formed during alcoholic fermentation under extraordinary conditions, e.g., in the presence of certain sulfur-containing pesticide residues, or with mutant yeasts that produce high levels of H_2S (Rauhut and Kurbel, 1994; Kinzurik et al., 2016). Even under controlled conditions, it has proven challenging to produce these precursors in real wines. For example, oxidation and storage (for 60 days) of EtSH-spiked wines resulted in ~90% loss of this thiol but with low yields (~10%) of the corresponding disulfide, DEDS (Belancic Majcenovic et al., 2002). Similarly, oxidation of wines containing MeSH results in sulfhydryl loss without concurrent formation of DMDS whereas conversely, MeSH can increase during bottle storage without concomitant loss of DMDS. However, the low prevalence of DMDS and DEDS in wines casts doubt on their importance as sulfhydryl precursors during wine storage (Ugliano, 2013; Ferreira et al., 2014).

The absence of DMDS and DEDS in real wines contrasts with work performed in model systems, e.g., aerial oxidation of EtSH in simple model wine yields DEDS (Bobet et al., 1990). One possibility is that oxidation of sulfhydryls in real wines favors formation of quinone-thiol adducts (Fig. 1B) (Nikolantonaki et al., 2010, 2012), which are not expected to serve as latent sulfhydryl precursors. Additionally, oxidation of volatile sulfhydryls could result in higher molecular weight *asymmetric* disulfides capable of releasing H_2S, MeSH, or other volatile thiols. These disulfides would be overlooked by typical analytical methods used for studying sulfurous off aromas (often, GC coupled to a selective detector such as a sulfur chemiluminescence detector (SCD)), which are capable of measuring only volatile species. These asymmetric disulfides, along with polysulfanes and (di)organopolysulfanes, may be generated in the presence of Cu(II) in wine or hydroalcoholic solution (Nedjma and Hoffmann, 1996; Sarrazin et al., 2010; Kreitman et al., 2016a, 2017). Alternatively, (di)organopolysulfanes could be formed by degradation of S(0) pesticide residues (Jastrzembski et al., 2017). These species may then undergo sulfitolysis to yield alkylthiosulfates capable of forming volatile thiols (Bobet et al., 1990; Arapitsas et al., 2016), as discussed in more detail in the next section.

4.2 Mechanisms for S-S bond formation in wine

Several mechanisms well-known in the general literature for disulfide formation appear unlikely to occur in real wine. For example, although sulfhydryls cannot be directly oxidized by O_2 to disulfides due to Pauli's exclusion principle, they can be oxidized by two-electron oxidants such as H_2O_2 to yield a sulfenic acid (RSOH), which can subsequently condense with thiols to form disulfides (Nagy, 2013) (and another equivalent of water). However, the initial reaction of thiols with H_2O_2 is relatively slow under wine conditions and thiols will likely be outcompeted by oxidation of bisulfite and/or Fe(II) (in the Fenton reaction, see Figure. 1A) (McArdle and Hoffmann, 1983). Alternatively, S-S groups could be formed via oxidation of sulfhydryls through radical-mediated reactions, e.g. following reaction with oxidized transition metals. In the absence of radical scavengers, the thiyl radical may dimerize to form disulfides (de Almeida et al., 2013; Kreitman et al., 2013) or react with a thiol to form the disulfide anion radical, which further reacts with oxygen to yield a disulfide and peroxyl radical (Schäfer et al., 1978; Danilewicz et al., 2008; Nagy, 2013). However, in wine, thiyl radicals should be scavenged by catechol and pyrogallol moieties (Kreitman et al., 2013), or else form adducts with α,β-unsaturated compounds like hydroxycinnamic acids (Bouzanquet et al., 2012).

A more plausible mechanism for S-S group formation in wine involves the participation of transition metals, particularly copper, with Cu(II)-facilitated oxidation of sulfhydryls in model and real wine resulting in putative formation of disulfides, polysulfanes, and (di)organopolysulfanes (Kreitman et al., 2016a, 2017). This pathway is thoroughly discussed in Section 3.2 and beyond previously cited studies, support for this pathway is also provided by work on a model wine containing yeast lees reported to have 640 mg/kg copper. Incubation of MeSH under oxidizing conditions in this system resulted in production of DMDS, which could be further increased through addition of $CuSO_4$ (Vasserot et al., 2003). The binding of sulfhydryl compounds by lees can be suppressed by addition of EDTA, as can production of DMDS (Palacios et al., 1997; Vasserot et al., 2003), further implicating the role of Cu(II) in S-S bond formation.

In real wines containing excess thiols (e.g., GSH, Cys, etc.), the formation of asymmetric disulfides, polysulfanes, or (di) organopolysulfanes would be statistically favored in the presence of Cu(II), rather than increases in symmetric disulfides (DMDS and DEDS), with this possibility mentioned in passing by some authors (Belancic Majcenovic et al., 2002; Vasserot et al., 2003). In support of this, a recent report ostensibly showed that oxidation of GSH, Cys and MeSH in wine containing Cu(II) could generate asymmetric disulfides, e.g.,

glutathionyl methyl disulfide (Kreitman et al., 2017), and earlier work using 20% ethanol solutions showed that H_2S, MeSH and EtSH will react in the presence of Cu(II) to form mixed disulfides and trisulfanes (Nedjma and Hoffmann, 1996).

In addition to oxidation of free sulfhydryls by transition-metal mediated reactions, wine compounds with S-S groups could also be formed from elemental sulfur (i.e., S(0)) pesticide residues. S(0) is well known to generate H_2S during fermentation (Acree et al., 1972; Schutz and Kunkee, 1977), but recent work has also demonstrated that wines fermented in the presence of S(0) can continue to form H_2S during storage (Jastrzembski et al., 2017). However, the latent H_2S precursors in finished wines are unlikely to be residues of S(0), due to its poor solubility in aqueous systems (~5 μg/L in H_2O at 25 °C) (Boulegue, 1978) and the fact that settling and racking can remove 95% of S(0) (Kwasniewski et al., 2014). During fermentation, S(0) can be reduced by GSH to yield diorganopolysulfanes and GSSG. The diorganopolysulfanes can be transported into the yeast cell where they undergo further reduction by GSH to eventually produce H_2S (Sato et al., 2011). However, some fraction of diorganopolysulfanes may survive and serve as latent precursors, and a recent study putatively identified diglutathionyl trisulfide at higher concentrations in a red wine fermented in the presence of S(0) as compared to a control (Jastrzembski et al., 2017). Potentially, dioorganopolysulfanes could be produced *de novo* during fermentation (and not just via S(0)), as has been reported in mammalian cells, although this possibility has been largely unexplored in yeast (Huang et al., 2017).

Two other pathways for formation of S-S compounds in alcoholic beverages have been described. First, Maillard-type reactions with α-dicarbonyls (discussed in Section 6), such as between ascorbic acid degradation products and Cys, have been reported to yield S-S compounds (Yu et al., 2012), although the relevance of these reactions under normal wine conditions is not established. Second, spiking experiments in beer indicate that both methional (3-methylthiopropanal) and methionol (3-methylthiopropanol) can form DMTS during storage when added to newly fermented beer (Gijs et al., 2000). Methional concentrations in non-oxidized wines are typically undetectable (Escudero et al., 2000), and the spiking concentrations of methional (101 μg/L) in the beer study would only be expected in oxidized wines (9.45-140 μg/L) (Escudero et al., 2000). However, the methionol concentration (124 μg/L) used in the beer spiking study to cause an increase of DMTS (from 49 to 287 ng/L) was well within the concentration range observed in wines.

4.3 Mechanisms for release of sulfhydryls from S-S containing compounds

4.3.1 Thiol-disulfide exchange

Thiol-disulfide exchange (also called interchange) reactions involve nucleophilic substitution of a free thiolate (e.g., RS^-) for a thiolate leaving group from a disulfide (e.g., $R''S^-$). The reaction follows a concerted S_N2 mechanism with a trisulfide-like transition state complex and delocalized negative charge (Fig. 5, top) (Fava et al., 1957; Fernandes and Ramos, 2004; Bach et al., 2008; Liang and Fernández, 2009; Nagy, 2013).

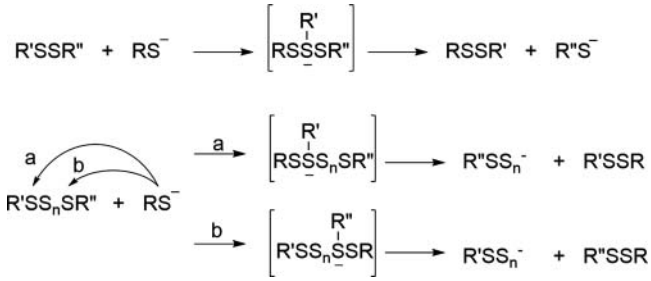


Figure 5. S_N2 reaction mechanism of thiol-disulfide exchange (top) and thiol-diorganopolysulfane exchange (middle and bottom, n ≥ 1). For thiol-disulfide exchange, the reaction proceeds by nucleophilic addition of a thiolate (RS^-) to a disulfide (R'SSR") to form a trisulfide-like transition state, which then generates a new disulfide (RSSR') and a corresponding thiolate ($R''S^-$). An analogous reaction occurs for thiol-diorganopolysulfane exchange, with the possibilty of releasing different disulfides depending on the position of attack.

Thiol-disulfide exchange reactions are biologically important, as they play key roles in intracellular redox homeostasis, antioxidant defense and cell signaling *in vivo* (Winterbourn and Hampton, 2008). For monothiol-disulfide systems within the pH range observed in wine, the distribution of species at equilibrium is near random (Singh and Whitesides, 1993). Therefore, from a strictly thermodynamic perspective, exchange reactions involving higher molar concentrations of nucleophiles such as GSH or Cys to release MeSH or other lower molecular weight thiols from disulfides should be feasible, assuming the wine did not start at a thiol-disulfide equilibrium. An analogous mechanism can be invoked for thiol-diorganopolysulfane exchange under synthetic conditions (and presumably thiol-polysulfane or thiol-organopolysulfane exchange), although substitution is favored both kinetically and thermodynamically at the terminal sulfur positions (Fig. 5, middle and bottom) (Steudel, 2002). Thus, thiol-diorganopolysulfane exchange should generate a thiol and an organopolysulfide, and should not release H_2S from the central sulfur atoms of the diorganopolysulfane unless further reactions (e.g. sulfitolysis, Section 4.3.2) are also involved.

Because the exchange involves a thiolate rather than thiol, the reaction proceeds most rapidly when pH > pK_a of the thiol (or in other words, when thiol pK_a is lower for a given pH when comparing different thiols). However, because of the opposing effect where pK_a is directly correlated with the nucleophilicity of the thiol, reaction rates are roughly similar among primary thiols when pH << pK_a. For example, the thiol pK_a for 2-mercaptoethanol is one unit lower than for propanethiol (9.6 vs. 10.5), but at pH 7 the ~10-fold greater thiolate concentration of 2-mercaptoethanol is partially negated by the greater nucleophilicity of propanethiolate, resulting in rate constants for reactions with disulfides within a factor of two (Singh and Whitesides, 1993). At wine pH, similar statements can be made about thiol leaving groups, i.e., for disulfides composed of two primary thiols, there should only be small differences in the preference for the leaving group. The preferred organopolysulfide (RSS_n^-) leaving group during thiol-diorganopolysulfane exchange (Figure 5) will presumably follow similar considerations, i.e., a modest preference for the organopolysulfide with the lower pK_a. As a caveat, these

statements would not hold in situations where pH ~ pK_a for one of the thiols, in which case the thiol with the lower pK_a is favored as a leaving group.

For exchanges involving typical monothiols including GSH, second-order rate constants at pH 7 and room temperature are approximately 10 M^{-1} min^{-1}. These rates will have a first-order dependence on thiolate concentration (Singh and Whitesides, 1993), and assuming a combined GSH + Cys concentration of 0.1 mM, a pseudo-first order half-life of 1.3 years at pH 4 and 13 years at pH 3 would be expected. This exchange pathway would thus represent a slow but plausible mechanism for release of thiols from disulfides.

4.3.2 Sulfitolysis of disulfides and thiosulfate hydrolysis

Free thiols may also be released from disulfides via sulfitolysis. Similar to thiol-disulfide exchange, sulfitolysis of disulfides proceeds through an S_N2 reaction (Fig. 6) (Kice, 1968). Subsequently, the alkylthiosulfate may undergo acid-catalyzed scission to yield an additional thiol (Kice et al., 1966). Sulfitolysis of Cys and GSH disulfides and other biochemically-relevant disulfides are well studied due to their importance to proteins (Cecil and McPhee, 1959; Thannhauser et al., 1984; Gonzalez and Damodaran, 1990). In literature reports, sulfitolysis is typically performed at neutral or higher pH to favor the sulfite (SO_3^{2-}) species. The contribution of bisulfite (HSO_3^-) to sulfitolysis is assumed to be negligible (Cecil and McPhee, 1959). At pH < 9 and 25 °C, second-order rate constants of 140–180 and 720–1120 M^{-1} min^{-1} were reported for sulfitolysis of GSH disulfide (GSSG) and Cys disulfide (cystine), respectively (Cecil and McPhee, 1955). Assuming an excess of free SO_2 and using a value of 7.2 for the pK_a of SO_3^{2-}, a pseudo first order half-life of about 48 days is predicted for GSSG in a typical wine (free SO_2 = 0.5 mM, pH = 3.4). This value is in strikingly good agreement with numbers reported recently for GSSG (1.3 mM) in a model wine system (free SO_2 = 13 mM, pH = 3.4) (Arapitsas et al., 2016). Under those circumstances, a 30% decrease in GSSG was observed after 24 hours, which would be equivalent to a half-life of 52 days at free SO_2 = 0.5 mM.

Sulfitolysis of lower molecular weight or mixed disulfides to yield free volatile thiols like MeSH is not well studied. Bobet et al. investigated the release of EtSH from DEDS over a pH range of 3.5–7 and found the kinetics to be well predicted based on SO_3^{2-} concentration, which accords with previous work that it is the only species to participate in sulfitolysis (Bobet et al., 1990). However, the sulfitolysis rate constant of DEDS (2.3 M^{-1} min^{-1}) was two orders of magnitude slower than previous reports for GSSG, leading to predicted half-lives on the order of decades for a disulfide such as DEDS in a typical wine. The reason for the considerably slower kinetics (~100-fold) for sulfitolysis of DEDS as compared to GSSG or cystine at wine pH may be due to the lower pK_a (and thus better leaving group potential) for aminothiols like GSH and Cys, which are typically 1–2 units lower than for alkylthiols (Tajc et al., 2004). However, as discussed in the next paragraph, pK_a differences have relatively small effects on sulfitolysis of disulfides (van Rensburg and Swanepoel, 1967), and are thus unlikely to fully explain the discrepancy between GSSG and DEDS kinetics.

Although symmetrical disulfide precursors (DEDS, DMDS) of volatile sulfhydryls are generally not detected in wine to any great extent, asymmetric disulfides of MeSH and other more abundant wine thiols (e.g. GSH, cysteine) could be expected to form in the presence of Cu(II) and other oxidized transition metals. Sulfitolysis of mixed disulfides would be expected to favor attack on a less hindered thiyl group (Ichimura et al., 1983) to form the corresponding alkylthiosulfate, with concurrent release of the more hindered thiyl group as a free thiol. Indeed, tertiary thiols are released at a rate that is more than 100-fold greater from asymmetric disulfides containing both tertiary and primary thiyls (van Rensburg and Swanepoel, 1967). Conversely, disulfides consisting of different primary thiyl groups (cysteamine, Cys, and their derivatives) showed only a minor variation (less than a factor of two) in the rate of sulfitolysis (van Rensburg and Swanepoel, 1967). The kinetics of sulfitolysis of asymmetric disulfides like those detected in wine, e.g., glutathionyl methyl disulfide (Kreitman et al., 2017) have yet to be reported, but presumably would favor release of GSH over MeSH due to the lower pK_a of the former. Hypothetically, similar mechanisms could also result in release of free sulfhydryls from (di)organopolysulfanes, although this and other aspects remain to be explored. Considering the lack of information, comparative measurements of sulfitolysis kinetics for multiple symmetric and asymmetric disulfides (or other S-S species) under wine-like conditions would also be of interest.

The potential for alkylthiosulfates formed via sulfitolysis (Fig. 6) to release free sulfhydryls during wine storage via acid-catalyzed conditions is not established, but existing data from model systems suggest that kinetics may be too slow to be relevant. For example, the half-life of ethanethiosulfate in 1 M HCl at 77 °C is approximately 1 month (Kice, 1963), and acid hydrolysis rate constants among different thiosulfates (in acidified dioxane-water systems) were reported to be largely independent of the thiosulfate (Kice et al., 1966). These reports suggest there would be negligible formation of free sulfhydryls for ordinary wine storage conditions and times, which would explain the observation of accumulated thiosulfates in aged wines (Arapitsas et al., 2016). Interestingly, sulfitolysis of DEDS in 10% ethanol at pH 7.2 was reported to yield two equivalents of EtSH (Bobet et al., 1990), and kinetic data indicated that release of the thiol from the thiosulfate was faster than the initial

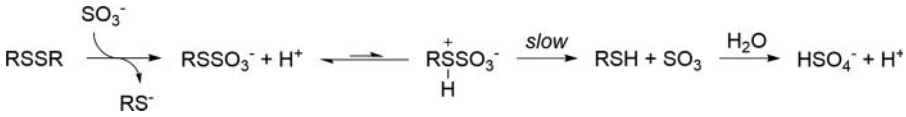


Figure 6. Sulfitolysis of a disulfide (Cecil and McPhee, 1959) followed by acid-catalyzed cleavage of an organic thiosulfate (Bunte salt) (Kice *et al.*, 1966), leading overall to the release of two equivalents of thiol.

sulfitolysis step. Because this observation occurred under alkaline conditions, it is not clear if the result can be extrapolated to wine-like pH.

As a final caveat, studies on sulfitolysis in real wine systems are made more challenging due to difficulties in accurately determining the free SO_2 concentration, as traditional methods can overestimate free SO_2 by a factor of four or more (Coelho et al., 2015). A further complication is that pK_a values are dependent on both ethanol concentration and ionic strength, but correction factors for the pK_a of SO_3^{2-} are not available, although they do exist for HSO_3^- (Usseglio Tomasset and Bosia, 1984). Taken together, caution should be used with comparing modest differences in kinetics between real and model systems.

4.3.3 Metal-catalyzed disulfide scission

As discussed earlier, base-catalyzed thiol-disulfide exchange reactions are rather slow, with a half-life on the order of 1–10 years at room temperature (Section 4.3.1). However, Bekker and colleagues recently demonstrated that addition of Cu(II) can increase the rate of disulfide scission, e.g., after 1 month, MeSH release from added DMDS increased from < 20% to > 70% (Bekker et al., 2018). This observation suggests that scission of a disulfide (and by extension, diorganopolysulfanes) can be metal-catalyzed under wine conditions, and also suggests that some fraction (referred to by the authors as "unknown precursors") capable of releasing H_2S in the presence of Cu(II) may be in part composed of diorganopolysulfanes (Bekker et al., 2018). Two possible mechanisms for metal-catalyzed scission are shown in Figure 7, neither of which has been well studied under wine-like conditions:

i. An oxidized metal could bind to one sulfhydryl component of the S-S functionality, enabling the displacement of the other sulfur moiety by nucleophilic attack (Kice, 1968). This could facilitate S-S cleavage by thiols, or by other wine nucleophiles including bisulfite, ascorbic acid, and perhaps polyphenolic compounds.
ii. A reduced metal could bind to a thiol(ate), increasing the nucleophilicity of the thiol ((Boerzel et al., 2003; Chen et al., 2013) and references therein). This pathway would be facilitated if the metal is simultaneously bound to an electron withdrawing group (Garusinghe et al., 2015).

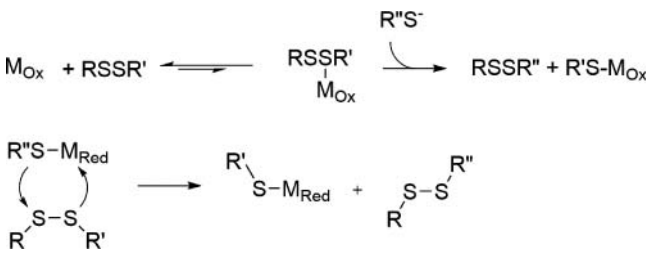


Figure 7. Hypothetical pathways through which metals could facilitate thiol-disulfide exchange. (Top) Binding of the disulfide (RSSR') by an electrophilic oxidized transition metal (M_{Ox}) may facilitate nucleophilic substitution by a thiol (R"SH). (Bottom) Complexation of a thiol by a reduced transition metal (M_{Red}) could enhance that thiol's nucleophilicity, accelerating the rate of exchange with a disulfide. The thiolate could also be replaced by another nucleophile.

4.3.4 Ascorbic acid

The chemistry of L-ascorbic acid (vitamin C) in relation to wine has been recently reviewed (Bradshaw et al., 2011; Barril et al., 2016). Most ascorbic acid present in grapes is lost during fermentation, but ascorbic acid additions to wine are legal in many countries. Although it does not rapidly react with H_2O_2, ascorbic acid can readily reduce quinones, such that its presence in wine results in lower consumption of SO_2 for a given amount of O_2 exposure (Danilewicz, 2016c). Thus, ascorbic acid may indirectly facilitate the formation or retention of volatile sulfhydryls through preservation of SO_2.

A second reason for the use of ascorbic acid in the winery is its putative ability to reduce disulfides to thiols. Ascorbic acid additions are part of a bench test to identify the species responsible for sulfurous off-aromas in wines, as described in Section 7. In this bench test, the disappearance of sulfurous off-aromas following addition of Cu(II) solution and ascorbic acid (but not following addition of Cu(II) alone) is taken as evidence that a wine possesses malodorous disulfides, which can then be treated in the bulk tank by additions of Cu(II) and ascorbic acid (Zoecklein et al., 1999). As discussed in Section 4.1, volatile disulfides (e.g., DEDS, DMDS) can potentially be formed during fermentation or by metal-mediated oxidation reactions of wines containing high concentrations of low-molecular weight sulfhydryls; however, these volatile disulfides are rarely observed in wine above their sensory thresholds. Thus, it is unclear what is measured in an "ascorbic acid test" that requires both Cu(II) and ascorbic acid to remedy a malodor.

Surprisingly, little is known concerning the reaction mechanism of ascorbic acid with disulfides, or even the plausibility of the reaction during typical wine storage conditions. Reaction rate constants for biologically relevant disulfides (e.g., GSSG) and ascorbic acid range from ~3–5 $\times$ 10^{-5} M^{-1} s^{-1} at physiological pH (7.4), although values at wine relevant pH were not reported (Giustarini et al., 2008). Studies on reactions of ascorbic acid with nitrosothiols (RSNO), which should react analogously to disulfides, suggest a substitution involving the mono-anion of ascorbic acid (Williams, 1999). Based on the pK_a of ascorbic acid (4.25) (Bradshaw et al., 2011), one would predict a half-life for disulfides of approximately 8 months assuming an initial ascorbic acid concentration of 1 mM. These kinetics are compatible with release of thiols during long-term wine storage, and one textbook recommends waiting months following ascorbic additions to allow for conversion of disulfides to thiols (Zoecklein et al., 1999). However, the pathway seems less plausible for release of thiols during the "ascorbic acid test", which reportedly requires only minutes following addition of ascorbic acid at 1 g/L (~5 mM) (Zoecklein et al., 1999).

Although kinetic data suggest that ascorbic acid could reduce disulfides over long term wine storage, the thermodynamic data is less favorable – the reduction potential of dehydroascorbic acid (DHA) is significantly more positive than a typical disulfide (0.3 V vs. -0.1 V at pH 3.5). One possibility is that following its formation, DHA could form adducts with bisulfite or irreversibly decay to form other products (Danilewicz, 2016c), which would drive consumption of ascorbic acid and favor disulfide reduction. Also, even if the overall disulfide

(or (diorgano)polysulfane) vs. thiol ratio stays the same, ascorbic acid addition may facilitate exchange of S-S and thiol bonds, which could result in release of volatile sulfhydryls if the wine is not initially at equilibrium. Ascorbic acid will also rapidly reduce Cu(II) to Cu(I) (Srogl and Voltrova, 2009), and Cu(I) could potentially facilitate thiol-disulfide exchange (Section 4.3.3). However, as previously discussed in Section 3.2, Cu (II) is readily reduced by thiols in wine, and the majority of copper in wines likely exists in Cu(I)-sulfhydryl complexes, and this role for ascorbic acid seems less likely.

5. *S*-alkyl thioacetates as precursors

S-Alkylthioacetates such as MeSAc and EtSAc have been proposed to serve as latent sources of thiols in both wine and beer (Leppänen et al., 1980). Because both MeSAc and EtSAc have unpleasant sulfurous odors, these thioacetates have also been suggested to contribute directly to reduced aromas in wines (Rauhut et al., 1998). However, as shown in Table 1 the sensory thresholds of MeSAc and EtSAc (50 and 10 μg/L, respectively) are an order of magnitude higher than their corresponding thiols (2 and 1 μg/L, respectively), and surveys of typical concentrations in both faulted and sound wines suggest that direct sensory impact of thioacetates should usually be minimal. For example, one survey reported detectable but sub-threshold concentrations of MeSAc (3-18 μg/L) in 29 out of 40 commercial red wines with sulfurous off-aromas, with undetectable concentrations in the remaining wines (Siebert et al., 2010). MeSAc concentrations in white wines, and EtSAc concentrations in both red and white wines, were largely below instrumental detection limits ($<$ 1 μg/L) (Siebert et al., 2010). Similar sub-threshold concentrations (typically, $<$ 20 μg/L for MeSH, and lower concentrations for EtSAc) are reported in other surveys (Leppänen et al., 1980; Mestres et al., 2000; Fang and Qian, 2005), with the exception of a study of German wines which reported a single Pinot noir with MeSAc = 115 μg/L and EtSAc = 56 μg/L (Rauhut et al., 1998). These concentrations were presumably much higher just after fermentation than after storage, as a consequence of hydrolysis (as described later). Tracking "typical" *S*-alkylthioacetate concentrations from fermentation through aging in a range of wines would be of interest but does not currently appear in the literature.

S-Alkylthioacetates in wine are assumed to be formed during primary alcoholic fermentation via enzymatic acetylation of thiols by acetyl-CoA (Fig. 8), analogous to formation of acetate esters from alcohols (Walker and Simpson, 1993). MeSH, the precursor of MeSAc during fermentation, can be formed by enzymatic catabolism of methionine by yeast (Perpète et al., 2006). EtSH, the precursor of EtSAc, has been speculated to form via reaction of H_2S with either acetaldehyde or ethanol during fermentation, although this has not yet been demonstrated in model solutions or wine (Rauhut, 2009). However, formation of both EtSH and EtSAc is increased in fermentations that produce high levels of H_2S, e.g., in musts containing S(0) pesticide residues (Rauhut and Kurbel, 1994) or by yeast mutants with defective S-amino acid pathways (Kinzurik et al., 2016). The end of fermentation likely represents the maximum concentration of these thioacetates, for reasons discussed later in this section.

Conversion of *S*-alkylthioacetates to more potent free thiols is expected to proceed by hydrolysis (Leppänen et al., 1980). The hydrolysis rate is not expected to depend on wine oxygen exposure, in contrast to some of the other putative precursor reactions, although any thiols released could be lost due to oxidation reactions (e.g., through reaction with quinones). Acetyl-CoA and related thioesters are of biochemical interest, and the hydrolysis of MeSAc has been studied over a pH range of 1–14 in aqueous buffers (Bracher et al., 2011). At pH extremes, hydrolysis can proceed through either alkaline or acid-catalyzed hydrolysis. Over the pH range of wine (3–4), water is the nucleophile, and hydrolysis is reported to be largely pH independent ($k_w = 3.8 \times 10^{-8}$ s at 25 °C), which would equate to a half-life of about 7 months. The minor effect of pH was demonstrated in a recent study of MeSAc and EtSAc spiked red, white, and model wines. The authors observed only a modest increase (less than a factor of 2) in thioacetate degradation when decreasing pH from 3.4–3.7 to 3.0 (Bekker et al., 2018); by comparison decreasing pH from 3.58 to 2.95 increased *O*-acetate ester hydrolysis by a factor of 3 or more (Ramey and Ough, 1980).

The typical MeSAc concentration of a young wine (~10 μg/L) would be sufficient to generate ~5 μg/L of free MeSH during a 1–2 year storage period. This MeSH concentration is comparable to the increase in MeSH observed during 50 °C anoxic storage of wines that could not be explained by the presence of brine-releasable complexes (see Section 3.3) (Franco-Luesma and Ferreira, 2016a), suggesting that MeSAc is a plausible MeSH precursor in wines.

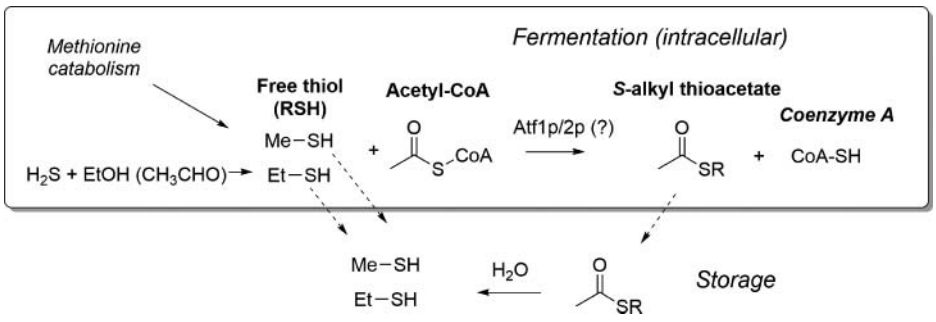


Figure 8. Pathway for enzymatic formation of *S*-alkyl thioacetates from free thiols (RSH) during alcoholic fermentation (top) followed by *S*-alkylthioacetate hydrolysis during wine storage to regenerate RSH (bottom). RSH can putatively be formed during fermentation by catabolic degradation of methionine to yield MeSH, or by reaction of H_2S with either ethanol (EtOH) or acetaldehyde (CH_3CHO) to yield EtSH. RSH can then be acetylated to *S*-alkyl thioacetates, potentially by similar acetyltransferase enzymes (Atf1p/2p) as those involved in production of *O*-acetate esters.

Recent work by Bekker *et al.* showed that spiking Chardonnay with MeSAc (50 μg/L) and EtSAc (60 μg/L) resulted in the release of 18% and 39% in molar equivalents of MeSH and EtSH, respectively, after 16 months of anoxic storage. The loss of MeSAc and EtSAc in red wine was comparable to that of the Chardonnay, but the accumulation of MeSH and EtSH was lower, likely due to further reaction of the thiols with wine components. A similar explanation can be invoked to rationalize the concurrent decrease in MeSAc and MeSH observed in a separate wine storage study (He et al., 2013). More perplexing are studies showing no change or slight increases in thioacetates during storage (Ugliano et al., 2012) (Bekker, Day, et al., 2016). Formation of thioesters is thermodynamically unfavorable (delta G = -30 kJ/mol) and spontaneous acetylation of MeSH by acetic acid is implausible. Potentially, unknown latent sources capable of maintaining a steady state of thioacetates at low μg/L concentrations exist in wines. An alternative explanation is that typical GC analyses with sulfur selective detectors have an interfering compound at the same retention time as MeSAc or EtSAc.

Alternate pathways to release free sulfhydryls from *S*-alkylthioacetates could occur, although their relevance under wine conditions is questionable. For example, thiol-thioester exchange, in which a thiol adds to the carbonyl group of the thioester to release the previously acetylated thiol, is faster than the analogous transesterification reaction involving an alcohol and *O*-ester (Van Vranken and Weiss, 2012). However, under low pH conditions like those in wine, thiol-thioester exchange will be slower than thioester hydrolysis due to the low concentration of thiolate – one recent report involving MeSAc and 2-sulfhydrylethanesulfonate (1 mM) as a representative thiol reported that hydrolysis would be the dominant pathway at pH < 4.5 (Bracher et al., 2011).

6. Other potential precursors of sulfhydryls

6.1 S-amino acids

The potential for S-amino acids and related compounds to serve as precursors of H_2S and MeSH through enzymatic degradation during fermentation (Waterhouse et al., 2016b) or through photodegradation (Grant-Preece et al., 2017) is well established. The metal-catalyzed release of H_2S and MeSH from S-amino acids has also been proposed as a source of VSC during storage by Ugliano and colleagues (Ugliano et al., 2011). Metal-catalyzed de-sulfurization has been previously reported in model food systems – however, these observations were made at much higher temperature and pH (100 °C, pH ~6) (Gruenwedel and Patnaik, 1971). An alternate route to formation of H_2S or MeSH via S-amino acid degradation involves reaction of an amino acid with an α-dicarbonyl to yield "Strecker aldehydes" and other degradation products, including free sulfhydryls in the case of S-amino acids (Belitz et al., 2009). With respect to dicarbonyl species that exist in wine, authors have shown that S-amino acids can react with *o*-quinones (Rizzi, 2006; Grant-Preece et al., 2013), ascorbic acid degradation products (which include several dicarbonyls) or microbially-derived dicarbonyls (diacetyl and 2,3-pentanedione) (Bradshaw et al., 2011; Yu et al., 2012; Grant-Preece et al., 2013). Release of free sulfhydryls from S-amino acids in a pH 3.5 model wine has been reported, in which dicarbonyls such as glyoxal and methylglyoxal seemingly react with Cys to yield H_2S and with Met to generate free MeSH (Pripis-Nicolau et al., 2000). Significant degradation of Cys occurred within an hour at 25 °C, but the quantitative impact is not readily understood because the H_2S and MeSH yields were not reported. Although plausible under oxidizing conditions, the relevance of Strecker reactions to real wines under anoxic conditions is still in doubt, especially since quinones react more rapidly with stronger nucleophiles like bisulfite or GSH (Nikolantonaki and Waterhouse, 2012).

The extent to which metal catalyzed S-amino acid degradation can occur in wine was recently investigated by Bekker and colleagues by spiking Cys, GSH, and Met into wines. In the absence of added Cu(II), only small increases in H_2S were observed for Cys (0.02% – 0.05% molar equivalent) and GSH spikes (0.02% – 0.18%) in real wines after 12 months. Assuming a total Cys and GSH concentration of 0.1 mM, this would result in an increase of <5 μg/L due to their degradation. The addition of Cu(II) alone to either a red or white wine also increased H_2S by up to 11-fold after 12 months, which the authors credited to "unknown precursors" – as discussed in Section 4.3, this may be evidence of metal-catalyzed diorganopolysulfane scission. When Cu(II) was added along with either Cys or GSH to the white wine, H_2S further increased by 0.18% or 1.3% molar equivalents, respectively, after six months, which the authors interpreted as evidence of metal-catalyzed GSH/Cys degradation. Interestingly, the increase in H_2S was not observed in the white wine after 12 months, and addition of Cu(II) and either sulfhydryl precursor to a red wine resulted in lower H_2S after 12 months than Cu(II) alone, which the authors attribute to additional reactions with wine components. The effect of Met spikes on MeSH release was minimal (increase of <0.12% molar equivalent of Met), both in the presence and absence of Cu(II) (Bekker et al., 2018). The concentration of Met in wine is reported to be 0.01 mM or less (Kutlán and Molnár-Perl, 2003), indicating it is unlikely to be an important VSC precursor.

One complication with accepting spiking studies as evidence that GSH/Cys can serve as precursors of H_2S (either in the presence or absence of Cu(II) additions) is that these sulfhydryls could also facilitate release of H_2S from other precursors, e.g., through thiol-polysulfane exchange (see Section 4.3) or by disrupting copper sulfhydryl complexes (Section 3). Furthermore, GSH/Cys could protect any H_2S released by reacting with thiol-scavenging quinones or other oxidation products (see Section 2), such as those released by Cu(II) from the unknown precursors mentioned by Bekker (Bekker et al., 2018). Further studies using ^{34}S-labeled analogues would help clarify if GSH or Cys are capable of directly forming H_2S post-bottling.

6.2 Sulfide impurities from steel tanks

The direct contribution of metal tanks or fittings to H_2S levels in wine was well-researched by early enologists. For example, in a 1937 study, pieces of steel (non-stainless), along with cast-

iron, tin, and some aluminum alloys, were reported to generate H_2S aromas following exposure to wine (Mrak et al., 1937). An Australian study from 25 years later reported that addition of metal (20 cm^2 of iron, zinc, or tin) to 200 mL of wine resulted in up to 0.8 mg/L H_2S in wine with high initial SO_2 levels (Rankine, 1963). A plausible explanation for this phenomenon is the solvation of sulfide impurities, particularly the slightly soluble MnS in the case of stainless steel tanks (Lea et al., 2003). This reference also states that the problem can be prevented by washing with citric acid prior to wine storage; hypothetically, this strategy facilitates removal of manganese or other transition metals through chelation. Although these claims are made without reference to the primary literature, they are plausible – as discussed in Section 3.1, MnS has a relatively high solubility as compared to other transition metal sulfides, and MnS impurities are well-recognized causes of pitting in stainless steel tanks (Frankel, 1998). Dissolution of sulfide impurities could potentially be an important contributor to H_2S during tank storage, although in modern wineries, wines are unlikely to be exposed to large amounts of metals other than stainless steel, and the impact will be mitigated by the large volume-to-surface-area ratio of wine tanks. However, this issue could be of concern to research-scale operations, e.g., studies that rely on small tanks or kegs for wine storage, or that filter small quantities of wine through commercial-scale equipment, especially if the equipment is not pre-conditioned through citric washes or other treatments.

6.3 Sulfur dioxide and sulfate

Wines typically contain *ca.* 1.5 mM sulfate (SO_4^{2-}) (Leske et al., 1997) in addition to 1 mM total SO_2 (predominantly in the form of HSO_3^-) (Peterson et al., 2000). Enzymatic reduction of SO_4^{2-} and SO_2 to S^{2-} through the sulfate reduction sequence pathway during fermentation is critical for biosynthesis of S-amino acids (Waterhouse et al., 2016a). One paper has proposed – without evidence – that similar non-enzymatic reactions can occur spontaneously in wine in the presence of transition metals, phenols, ascorbic acid or other catalysts (Lopes et al., 2009). Similarly, the aforementioned study on formation of H_2S upon contact of metal squares with wine (Section 6.2) put forward the explanation that H_2S was evolved following attack of the metal by wine acids, which would produce H_2 gas capable of reducing SO_2 (Rankine, 1963). As mentioned earlier, this may instead be due to dissolution of metal sulfide impurities rather than *de novo* conversion. To our knowledge, the conversion of either SO_4^{2-} and SO_2 to H_2S in either model or real wine systems has not been convincingly demonstrated, and a recent study investigating a Cu(II) + SO_2 model wine system observed no increase in H_2S during long term storage (Bekker, Smith, et al., 2016). In real wines, increases in H_2S resulting from SO_2 additions are more likely to arise indirectly from other roles of SO_2 as opposed to SO_2 serving as a direct H_2S precursor; e.g., SO_2 could react with quinones formed during oxidation thereby preventing loss of any H_2S formed, or could release sulfhydryls from disulfides via sulfitolysis.

7. Summary: Applications to winemaking, and future directions

There is clear evidence from the recent literature that wines stored under anoxic conditions can show an increase in H_2S and MeSH during storage (refer to Table 2), and that the occurrence of these malodorous compounds is associated with "reduced", sulfurous off-aromas (Siebert et al., 2010). Potential precursors of H_2S and MeSH that likely satisfy the criteria described in Section 1 (sufficient concentration and appropriate degradation rates) are listed in Table 4. Of these, the role of copper-sulfhydryl complexes seems best established – comparison of release from high-temperature storage tests and brine dilution (both described in more detail in Section 7.1.1) indicates that approximately 60−90% of H_2S release and 20−50% of MeSH release could be attributed to these complexes (Franco-Luesma and Ferreira, 2016b). Two other latent VSC classes likely account for the balance: asymmetric disulfides and other S-S containing species (e.g. polysulfanes, Section 4), and *S*-alkyl thioacetates (Section 5). Some recent evidence exists for S-amino acids and related aminothiols (specifically GSH and Cys) serving as H_2S precursors through a metal-catalyzed pathway (Section 6.1), but further work is needed to confirm that these compounds are releasing H_2S rather than exerting a protective effect. Evidence for the contribution of other potential latent sulfhydryl compounds including quinone-thiol complexes (Section 2) and SO_2 or sulfate (Section 6.3) in real wine

Table 4. Summary of precursor classes most likely responsible for appearance of free sulfhydryls during wine storage, and conditions that can be used to induce release of free sulfhydryls (Bracher *et al.*, 2011; Franco-Luesma and Ferreira, 2014, 2016b; Chen *et al.*, 2017; Kreitman *et al.*, 2017). Test conditions were in the range of normal wine pH unless otherwise specified.

		Prospective conditions inducing >50% release					
Latent sulfhydryl source	Species Released	NaCl brine	50 °C, 2–3 wks, no O_2	TCEP[a]	Ascorbic acid	pH 0, 4 h, 25 °C	Cu(I) chelators
Copper–sulfhydryl complexes	H_2S, thiols	Yes	Yes	Yes[b]	Yes[b]	Unlikely	Yes[b]
Asymmetric disulfides	Thiols	Unlikely	Possible	Yes	Likely	Unlikely	No Unlikely
Polysulfanes[c]	H_2S						
(Di)organo-polysulfanes[c]	H_2S, thiols						
S-alkyl thioacetates	Thiols	Unlikely	Possible	Unlikely	Unlikely	Likely	Unlikely

[a]TCEP: tris(2-carboxyethyl)phosphine
[b]at pH 3.6
[c]May exist as alkylthiosulfate (Bunte salt) intermediates, Section 4

conditions is lacking – although all are likely to be present at sufficient concentrations, their ability to release sulfhydryls under standard wine storage conditions is not established. The possibility of metal-sulfide extraction (particularly MnS) from steel tanks into wine (Section 6.2) is probably of little consequence in modern winemaking, although it could lead to artifacts in research-scale winemaking.

7.1 Accelerated tests for sulfhydryl precursors

Wines require stabilization to prevent the formation of hazes and deposits and winemakers use a range of bench tests that simulate storage conditions to evaluate the stability of their wines (Zoecklein et al., 1999; Jackson, 2008). For example, so-called "contact tests", in which wine is cooled in the presence of potassium bitartrate seed crystals, are often used to predict the likelihood of potassium bitartrate instability (i.e., testing for cold stability). Conversely, heating tests are used to predict the formation of protein hazes due to denaturation of heat-unstable grape proteins (i.e., testing for heat stability). Analogous tests for latent sulfhydryl compounds would be helpful to winemakers to identify problematic wines, or (preferably) determine appropriate intervention strategies. Simple approaches to releasing sulfhydryls would also be useful to researchers, since many of the latent forms (particularly polysulfanes and copper-sulfhydryl complexes) could include an indeterminate number of specific compounds, which would be challenging to quantify. Several recent publications have explored using selective tests to release sulfhydryls from particular precursor classes, as well as general tests designed to release all precursors and/or simulate release during wine storage.

7.1.1 Selective tests

As discussed in Section 3.3, addition of concentrated NaCl brine to wine (9:1 or greater ratio of brine to wine) results in complete release of sulfhydryls from copper-sulfhydryl complexes in both real and model wines (Franco-Luesma and Ferreira, 2014; Chen et al., 2017). This effect is presumably due to the ability of Cl^- to displace sulfhydryls (Gilbert et al., 1997; Watling, 2006) from Cu(I) and form stable complexes, e.g., $CuCl_3^{2-}$ and $CuCl_4^{3-}$ (McConnell and Davidson, 1950; Gilbert et al., 1997). When coupled with an appropriate detection technique (e.g., GC-SCD, or gas detection tubes), brine dilution should be effective as a selective test for the presence of copper-sulfhydryls.

One drawback of the brine addition approach is that it results in sample dilution, and thus may not be appropriate for use in informal "sniff tests" (i.e., sensory assessment) if the dilution factor leads to subthreshold concentrations of released VSCs. An alternate approach to selectively measuring copper-complexed sulfhydryls involves the use of copper chelators. Addition of EDTA, a chelator capable of binding Cu(II), was ineffective, but addition of ~1 mM neocuproine, a strong chelator of Cu(I), to wines resulted in 50–60% recovery of spiked copper-complexed H_2S, with slightly higher recovery in model wines (Chen et al., 2017). This recovery was less than for brine dilution, but would still be appropriate for qualitative testing. However, a separate study using 1 mM bathocuproine disulfonic acid (BCDA, another strong Cu(I) chelator) showed negligible H_2S recovery from copper-sulfhydryl complexes in model wine (Kreitman et al., 2017). As a caveat, the neocuproine method resulted in a pH shift to ~6 due to the use of antacid tablets to generate CO_2 gas, which purged the H_2S from solution, as compared to the bathocuproine study, which occurred at normal wine pH, although it is not obvious how this may have affected the outcome.

There is not currently an approach to selectively release sulfhydryls from disulfides and related compounds without simultaneously degrading copper-sulfhydryl complexes. As discussed in Section 4.3.4, wine texts advise winemakers to use ascorbic acid to convert disulfides to free sulfhydryls (Zoecklein et al., 1999). Ascorbic acid additions are expected to reduce not only the symmetric disulfides mentioned in the texts, but also other related species (e.g., asymmetric (mixed) disulfides, polysulfanes, diorganopolysufanes, Bunte salts). Furthermore, ascorbic acid additions appear to recover the majority of H_2S from copper-sulfhydryl complexes, at least when tested at pH 6 (Chen et al., 2017). Similarly, tris(2-carboxyethyl)phosphine (TCEP) is known to reduce S-S bonds (Burns et al., 1991), but recent work demonstrated that TCEP is capable of releasing sulfhydryls from copper-sulfhydryl complexes too (Chen et al., 2017; Kreitman et al., 2017). Selective tests for *S*-alkyl thioacetates have not been described, but the hydrolysis rate constant for MeSAc is reported to increase ~300-fold at pH 0 (to $k = 1 \times 10^{-5}$ s^{-1}), giving a half-life of just under 4 h (Bracher et al., 2011), which may be exploitable in some manner.

7.1.2 General tests

Two types of general accelerated aging tests could be useful: either a test that predicts the final release of sulfhydryls after a specified time, or a test that measures the total pool of all precursors that could be released given indefinite storage time. In the former category, a recent study proposes that anoxic storage at elevated temperature (50 °C) can be used to simulate longer term reductive storage (Franco-Luesma and Ferreira, 2016b). The approach is analogous to shelf-life studies of other foods and beverages that rely on elevated temperatures and short times to approximate long-term storage (Taoukis and Giannakourou, 2004). A study of 21 Spanish wines reported that final H_2S and MeSH concentrations in wines stored for 379 d at 25 °C were well-correlated with the increase in H_2S and MeSH in those same wines stored at 50 °C for 12.5 days ($R^2 = 0.83$ for H_2S and 0.85 for MeSH). The average amounts of H_2S and MeSH in wines stored at room temperature were 57% and 90%, respectively, of the concentrations observed in the 50 °C wines. One challenge with evaluating these data sets is that sulfhydryl concentrations in wines following 12.5 d storage at 50 °C were not published, although data from 50 °C storage for 21 d (instead of 12.5 d) are available. Interestingly, it is evident from the aforementioned data that sulfhydryl release is strongly temperature-dependent, since increasing the temperature by 25 °C resulted in a 30-fold increase in release rate (i.e., 379 d vs. 12.5 d to achieve similar sulfhydryl increases).

Because a 12.5 day "accelerated reduction assay" is still rather slow as compared to other winery bench tests which can

be completed within a day's time, e.g., cold and heat stability tests, other authors have suggested using TCEP as a component of a general test, since TCEP is capable of releasing free sulfhydryls from both copper-sulfhydryl complexes and disulfides (and presumably related forms, Section 7.1.1). Under anoxic conditions, recoveries of >90% for H_2S in Cu(II) treated model wines (copper-sulfhydryls) and ~75% for MeSH and EtSH in air-saturated model wines (disulfides or putative (diorgano)polysulfanes) were reported using 1 mM TCEP in the presence of 1 mM Cys and 1 mM of BCDA (Kreitman et al., 2017). The role of Cys was putatively to serve as a sacrificial thiol and react with any BCDA-Cu(II) present to prevent oxidative losses of other thiols. On the other hand, Cys had a minimal effect on real wines, presumably as the wines would already contain Cys, GSH and related sulfhydryls at 0.05-0.1 mM concentrations. TCEP would not release MeSH or other thiols from *S*-alkyl thioacetates but an assay involving a cocktail of both a strong acid along with TCEP could presumably achieve a more holistic outcome for VSC release. In summary, there is still only limited data to validate proposed general tests (TCEP or brine) for latent sulfhydryl precursors and sulfhydryl formation during wine storage and more work is required to derive a suitable test for winemakers.

7.2 Approaches to remediation of malodorous sulfhydryls and preventing their reappearance in the winery

There are two general approaches to remediating wines with reduced aromas in the winery – aeration (often via splash-racking or sparging), and addition of Cu(II) salts (Jackson, 2008). Based on the work summarized in this review, addition of Cu(II) has historically been expected to result in loss of H_2S and MeSH and the formation copper-sulfhydryl complexes that can be removed. However, several recent studies have shown that only a negligible amount of added copper is lost after filtration, racking or centrifugation (Clark, Grant-Preece, et al., 2015; Vela et al., 2017). Furthermore, Cu(II) addition may result in formation of asymmetric disulfides, polysulfanes, and/or (di)organopolysulfanes from H_2S and MeSH, and these species could also serve as latent forms of sulfhydryls during bottle storage (see Sections 3.2 and 4.2, and Fig. 3). In comparison, addition of O_2 should result in loss of H_2S and MeSH through formation of quinone-sulfhydryl adducts, which are believed to be stable during wine storage (see Section 2).

Because Cu(II) results in formation of latent VSC precursors, aeration would appear to be a better strategy than Cu(II) addition for remediation of sulfurous off-aromas, but this conclusion is not supported by two recent studies. In work using a Shiraz wine, aeration (performed by splash-racking) had no significant effect on VSCs (including H_2S, MeSH, and EtSH) as compared to a control at any time point for up to 12 months storage (Bekker, Day, et al., 2016). Because no effect on VSCs was observed immediately following treatment, it is possible that other nucleophiles like bisulfite or GSH were able to react with major oxidation products (particularly quinones) in place of the VSCs. (see Section 2). In contrast, a study investigating the effects of microoxygenation did report a significant decrease of H_2S and MeSH with increasing O_2 doses. These VSCs could be regenerated by accelerated reductive aging for 2 or 7 weeks at 50 °C, so the authors proposed that (diorgano)polysulfanes must have formed and not quinone-sulfhydryl adducts. However, as previously discussed (Section 2), this result does not necessarily discount the formation of quinone-sulfhydryl adducts, whose stability at elevated temperatures has not been established.

The study on Shiraz also evaluated the effects of early and late Cu(II) treatments on VSC remediation (Bekker, Day, et al., 2016). Immediate decreases (40-90%) in MeSH and EtSH were observed following Cu(II) addition as compared to an untreated control, with significant but smaller effects for H_2S. MeSH and EtSH were observed to increase over the 12 month bottle storage period for the late Cu(II) treatment, potentially due to degradation of copper-sulfhydryl complexes (see Section 3.3). Curiously, the early Cu(II) treatment did not result in significantly higher copper than the untreated control for these research wines (Bekker, Day, et al., 2016), in contrast to other studies which reported almost complete retention of added copper (Clark, Grant-Preece, et al., 2015; Vela et al., 2017). The latter studies used commercial wines, which presumably had undergone greater clarification and stabilization than the Shiraz research wines, which were only racked once after malolactic fermentation. Young wines are expected to form heterogeneous precipitates during storage, particularly during cold-stabilization (Vernhet et al., 1999), which could result in co-precipitation of copper-sulfhydryls. Based on these results, a decision tree summarizing hypothetical situations involving O_2 vs. Cu(II) addition and sulfhydryls as a function of different wine compositions is shown in Fig. 9.

Wines with high levels of latent forms of sulfhydryls could also potentially be remediated, although there is a lack of literature on the topic. For example, copper-sulfhydryl precursors could potentially be removed using approaches described for removal of copper, e.g., non-polar adsorbents (Mira et al., 2007), cation-exchange resins (Benítez et al., 2002), or potassium ferrocyanide ("blue fining") (Ribereau-Gayon et al., 2006) but it is unclear if these approaches would co-remove metal-bound sulfhydryls. Similarly, although ascorbic acid in combination with Cu(II) is widely recommended for treating disulfides, there appears to be no formal evaluation of this treatment, nor whether it would result in formation of different sulfhydryl precursors (see Section 4.3.4).

Winemakers could also decrease VSC release by altering factors that affect the rate of precursor loss. For example, the release of sulfhydryls via hydrolysis of alkylthioacetates (e.g. MeSH from MeSAc) decreases with increasing pH (Section 5). However, this effect appears to be too modest to be useful – e.g., the amount of MeSH/EtSH formed from corresponding alkylthioacetates is no more than a factor of two lower at pH 3.4 – 3.7 han at pH 3.0 (Bekker et al., 2018) – and lower pH is generally more desirable for other reasons like wine color and microbial stability (Boulton et al., 1999).

A more interesting opportunity would be to control the relative balance of forward and reverse redox reactions involving VSCs. The appearance of VSCs is correlated with low O_2 conditions, and as shown in Fig. 3 and Fig. 4, two of the most likely VSC precursor classes (copper-sulfhydryl complexes and S-S containing compounds) can likely engage in reversible redox reactions during storage (Ferreira

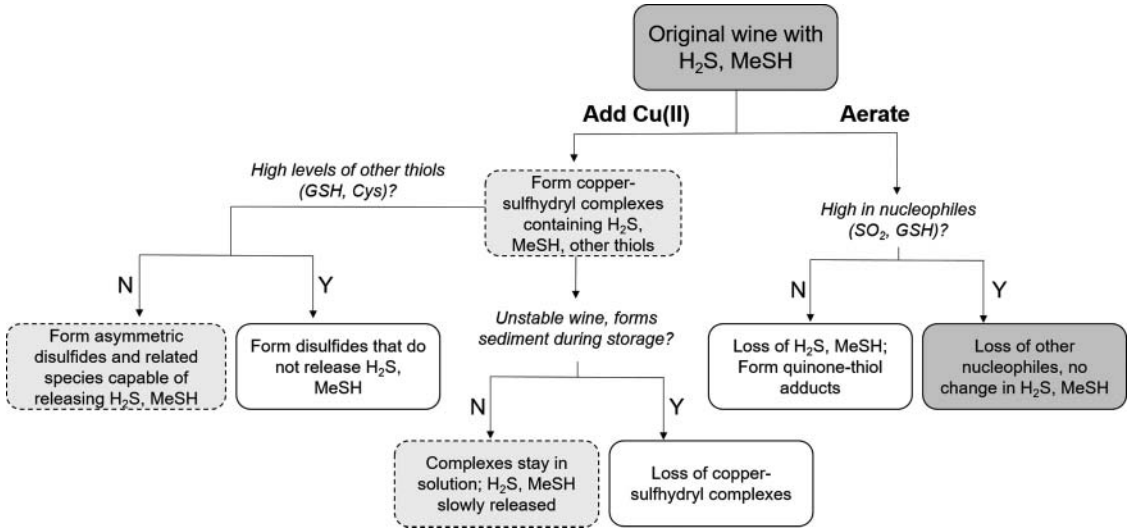


Figure 9. A decision tree to predict the hypothetical effects of Cu(II) addition or aeration on a wine containing H_2S and MeSH as a function of initial wine composition. Outcomes which lead to retention of H_2S and MeSH are in dark grey solid boxes; outcomes that lead to retention of latent H_2S sources are in light grey dashed boxes. Literature sources can be found in the text. The factors shown are not an exhaustive representation of these phenomena.

et al., 2017). Redox potential (measured by a Pt electrode) is known to decrease during fermentation and anoxic storage (Ribéreau-Gayon, et al., 2006), and a group has recently proposed that this redox potential can serve as a proxy for the relative balance of reduced vs. precursor forms of VSCs (Vela et al., 2018). Using three wines treated with varying levels of oxygen, the authors reported that redox potential was well correlated to the proportion of bound and free forms of VSCs (Vela et al., 2018). However, the value of electrode-based measurements of redox potential has been criticized as merely serving as a proxy for dissolved oxygen and the ratio of Fe(III):Fe(II) was suggested as a superior metric for estimating the redox state of a wine (Danilewicz, 2016b; Danilewicz, 2017), although VSCs and their precursors were not measured in these studies. Regardless of the redox-related parameter selected (redox electrode potential, Fe(III):Fe(II) ratio, or otherwise), it is plausible that "safe" values for this measurement could be identified that minimize the risk of VSCs (at one extreme) as well as oxidized aromas and/or microbial spoilage (at the other extreme). This information could then potentially be used to determine appropriate SO_2 additions at bottling and O_2 ingress rates during storage.

7.3. Closing thoughts

Recent work has established that in-bottle formation of H_2S, MeSH and related sulfhydryls can occur under anoxic conditions. Three precursor classes (Cu(I)-sulfhydryls, S-S compounds, *S*-alkylthioacetates) that likely contribute to this phenomenon have been identified. Both selective and general tests for the presence of sulfhydryl precursors have been proposed, although all would benefit from extensive and systematic validation. Certain S-amino acids and related compounds (Cys, GSH) may also serve as precursors of H_2S through a metal-mediated pathway, but reports are confounded because these compounds may also stabilize or release H_2S. The formation of certain precursors during winemaking is expected to depend not only on the concentration of sulfhydryls formed during fermentation, but also on the presence of copper and other metals. Recent conflicting results regarding the effects of copper addition and aeration on sulfhydryls suggests the need for more empirical studies on the best approaches to remediating wines with high sulfhydryls or sulfhydryl precursors – preferably, complemented by measurement of key wine nucleophiles (e.g. SO_2, GSH), residual copper, and latent sulfhydryl pools. Reports of parameters that indicate wine redox status (Pt electrode redox potential; Fe(III):Fe(II)) could be useful to predict or prevent VSC appearance, but further validation is necessary. Improved techniques for analyzing the different "passive" forms of sulfhydryl compounds will also be useful, particularly if larger copper-sulfhydryl aggregates exist. Recent work using nanoparticle analyzers is an interesting step in this direction, although the approach has so far been limited to model wines (Bekker, Mierczynska-Vasilev, et al., 2016).

Finally, we note that few reports on the appearance of sulfurous off aromas in wines during bottle storage existed prior to 2005, at which point seminal work on the effects of closure selection on wine sensory properties was published (Skouroumounis et al., 2005). The considerable number of recent literature reports on the latent sulfhydryl phenomenon (see Table 1) may therefore reflect an increased recognition of the issue, or else better analytical tools for VSC measurement (e.g., GC-SCD). However, this greater attention may also reflect an increased incidence of latent sulfhydryl appearance, resulting from improvements in wine packaging and/or changes in winemaking styles to avoid excess oxygen exposure (Goode, 2005; Ugliano, 2013), since sulfhydryls will only persist under anoxic conditions. From this perspective, the prevalence of both positive sulfhydryls (e.g., fruity-smelling 3SH) and malodorous sulfhydryls (such as MeSH, H_2S) in bottled wine can be perceived as markers of the progress of modern winemaking – a situation analogous to hypertension and Type II diabetes in developed countries ("diseases of affluence" (McKeown, 1988)) – which may provide some small level of comfort to winemakers struggling to deal with reduced wines.

Disclosure of interests

GYK, RJE, GLS, DWJ have no financial interests or benefits to disclose.

Funding

This work was supported by the Australian Government as a part of the Australian Research Council's Industrial Transformation Research Program under Project IC130100005; USDA National Institute of Food and Hatch Appropriations under Project PEN04565 and Accession 1002916; USDA Northeast Sustainable Agriculture Research and Extension (NESARE) Graduate Program under Award GNE14-082; and the Pennsylvania Department of Agriculture and the Pennsylvania Wine Marketing and Research Board under Grant 44155243.

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