

# Effects of Five Metals on the Evolution of Hydrogen Sulfide, Methanethiol, and Dimethyl Sulfide during Anaerobic Storage of Chardonnay and Shiraz Wines

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**S** Supporting Information

**ABSTRACT:** The synergistic effects of Cu, Fe, Mn, Zn, and Al on the evolution of different volatile sulfur compounds (VSCs) in a Chardonnay and a Shiraz wine have been investigated. The evolution of H<sub>2</sub>S, MeSH, and DMS were influenced by metal addition, and in some instances, a combination of metals was responsible for the largest variation in the concentration of VSCs. The metals and metal combinations associated with significant changes in VSC concentrations in both Chardonnay and Shiraz samples after anaerobic storage were Cu, Fe, Zn, Al, Cu\*Fe, Cu\*Mn\*Al, and Cu\*Zn\*Al for H<sub>2</sub>S; Cu, Zn, Fe\*Mn, and Cu\*Fe\*Mn for MeSH; and Al and Zn\*Al for DMS. The effect of Cu addition on the evolution of VSCs has previously been shown; however, this investigation has demonstrated that metals other than Cu could also be involved in the catalytic release of VSCs and that the interactions and combinations of metals are important. In some instances, the metal effect was reversed, associated with significant decreases during high oxygen conditions and with significant increases during low oxygen conditions.

**KEYWORDS:** volatile sulfur compounds, reductive aromas, hydrogen sulfide, methanethiol, dimethyl sulfide, wine, copper, iron, manganese, aluminum, zinc, anaerobic, oxygen

## INTRODUCTION

Wine is not an inert system but one that is continuously changing. The most obvious changes take place during fermentation and the early parts of the winemaking process, but the subtle changes that take place after bottling and during storage are just as critical in the establishment of the final product. It is well-known that the amount of oxygen a wine is exposed to postbottling influences the aroma, color and mouthfeel of the wine.<sup>1–10</sup> Wines exposed to very low levels of oxygen during fermentation and postbottling can develop “reductive” aromas that are associated with the presence of volatile sulfur compounds (VSCs).<sup>11</sup> VSCs naturally occur in wines in low concentrations, but their contribution to the overall flavor and aroma of the wine can be significant. Typical “reduced” odors can be attributed to hydrogen sulfide (H<sub>2</sub>S) and methanethiol (MeSH), which are characterized by aromas of rotten egg, sewage, and rubber.<sup>12,13</sup> When present in high concentrations, dimethyl sulfide (DMS) is known to impart aromas associated with canned corn, asparagus, or vegetal aromas,<sup>11</sup> although in low concentrations, it can enhance the bouquet.<sup>14,15</sup> Similarly, H<sub>2</sub>S can add complexity to the wine aroma when present in low concentrations at which it is not perceived as a fault.<sup>1,12,16,17</sup> A recent study has shown that a correlation exists between lower consumer preference and the presence of certain VSCs, in particular, MeSH.<sup>1</sup>

The formation of VSCs in wine and food can be explained by a variety of chemical and biochemical mechanisms, although many of these mechanisms are not well-defined.<sup>11,18,19</sup> The main source of wine VSCs are the yeast metabolism, involving the degradation of organic sulfur compounds, (i.e., sulfur-containing amino acids), sulfur-containing pesticides, and the formation of VSCs from precursor molecules.<sup>11,19</sup> It is also

known that in some instances, photochemical and thermal reactions are responsible for the formation of VSCs during storage.<sup>11</sup> Not all the factors involved in the chemical formation of VSCs postbottling have been fully elucidated,<sup>5</sup> but recent literature has demonstrated the increase of H<sub>2</sub>S, MeSH, and DMS during bottle maturation and that lower postbottling oxygen exposure resulted in an increase in H<sub>2</sub>S and MeSH concentration.<sup>1,3,4</sup>

VSCs such as H<sub>2</sub>S and MeSH can be managed to a certain degree during winemaking. VSCs can be treated by the addition of lees combined with gentle aeration because yeast cell walls seem to have the ability to bind various VSCs and remove them from wine.<sup>20</sup> High concentrations of H<sub>2</sub>S can also be lowered by aerating the wine, although this practice carries a few risks. For example, white wines are susceptible to oxidative browning, and in red wines, dormant acetic acid bacteria could be activated.<sup>20</sup> Even when sulfidic odors are present in the wine glass, they seem to be diminished by mild aeration in the glass, but higher molecular weight sulfur compounds are more difficult to treat.<sup>20</sup> Copper sulfate treatment can be used immediately after fermentation to reduce the concentration of unwanted thiols (i.e., H<sub>2</sub>S, MeSH); however, Cu fining does not remove disulfides, thioacetates, or cyclic sulfur compounds, potentially associated with off-odors in wine.<sup>18</sup> An unwanted side effect of Cu treatment is the risk of Cu reacting with the varietal thiols, which could lead to a decrease in the intensity of wine aroma and a loss of varietal character.<sup>1</sup>

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Table 1. Metal Concentrations in Base Wines, Spiked Samples and Average Metal Concentrations for Australian Wines

		Al mg/L	Cu mg/L	Fe mg/L	Mn mg/L	Zn mg/L
Chardonnay	base metal concentrations	0.7	0.2	1.0	2.0	1.0
	spiked metal concentrations	9.1 <sup>a,c</sup>	3.0 <sup>a,b,c</sup>	7.6 <sup>a,c</sup>	16.3 <sup>b</sup>	14.6 <sup>b</sup>
Shiraz	base metal concentrations	0.5	0.3	2.0	2.0	1.2
	spiked metal concentrations	9.3 <sup>a,c</sup>	3.1 <sup>a,b,c</sup>	8.5 <sup>a,c</sup>	15.2 <sup>b</sup>	15.1 <sup>b</sup>
Australian wine <sup>d</sup> n = 880 <sup>e</sup>	median	0.41	0.15	0.88	0.97	0.54
	max	1.79	1.89	5.68	3.61	2.72
	min	0.00	0.00	0.00	0.19	0.01
	legal limit for metals in wine	8.00 <sup>f</sup>	1.00 <sup>f</sup>	10.00 <sup>g</sup>		5.00 <sup>f</sup>

<sup>a</sup>Concentrations at which metals are reported to have a detrimental effect on wine's organoleptic properties: Cu,<sup>24,52</sup> Fe,<sup>24,50</sup> Al.<sup>24</sup> <sup>b</sup>Approximately 10 times the concentration of the metal in the base wine. <sup>c</sup>Concentrations of metals seen in wines produced around the world.<sup>25</sup> <sup>d</sup>As adapted from Martin et al.<sup>26</sup> <sup>e</sup>Number of wine samples analyzed.<sup>26</sup> <sup>f</sup>Requirements for the export of Australian wine to the European Union.<sup>64</sup> <sup>g</sup>Maximum allowed concentration for South African wines.

Metal ions are naturally present in grapes and wine and when present in trace concentrations—generally parts per million concentrations, but some ultratrace elements are present in parts per billion levels—they are essential cofactors in vitamins and enzymes that are important to the fermentation process.<sup>20,21</sup> When metals exceed these trace amounts, it may indicate contamination through human activity, that is, the use of pesticides or fertilizer, machinery in the winery, or use of fining agents such as bentonite and copper fining.<sup>20</sup> The following metals, in decreasing order, have the ability to catalyze oxidation–reduction (redox) reactions: W, Zn, Cu, Co, Fe, Ni, and Mn.<sup>22</sup> When the concentrations in which these metals are found in wine are taken into account, the only metals that are likely to have a catalytic role of functional significance are Fe, Zn, Cu, and Mn.<sup>22</sup> The role of Al in limiting oxygen consumption has been shown,<sup>22,23</sup> and it is possible that the ability of wine compounds to chelate Al<sup>3+</sup> reduces their sensitivity to oxidation.<sup>22</sup> Furthermore, several of the metals mentioned above (Al, Cu, Fe, Mn, Ni, Zn) can contribute to haze formation and undesirable sensory properties.<sup>24</sup> The concentrations of metals in wine vary widely across the world.<sup>25</sup> Metal concentrations in wine are regulated by national laws and organizations, such as the European Union and the International Organization of Vine and Wine (OIV), which have established maximum acceptable limits for trace elements. It is recommended that metal concentrations in wine should be kept as low as possible.<sup>24</sup> The average concentrations for the above-mentioned five metals that are commonly found in Australian wine,<sup>26</sup> as well as legal limits as recommended by the European Union, are summarized in Table 1.

The role of metal ions, specifically Fe and Cu, in wine oxidation was first studied by Jean Ribéreau-Gayon in 1931.<sup>27,28</sup> This nonenzymatic oxidation process is initiated by the metal-catalyzed reduction of oxygen.<sup>29–32</sup> Danilewicz showed that the consumption of oxygen is mediated by Fe, and the process is enhanced by Cu.<sup>28,33,34</sup> These transition metals reduce oxygen to hydrogen peroxide, which is then further reduced to hydroxyl radicals through Fenton reactions.<sup>32,34</sup> Hydroxyl radicals are extremely reactive species, able to react non-selectively with a wide variety of wine components.<sup>29,30</sup> The reaction between hydroxyl radicals and ethanol, yielding 1-hydroxyethyl radicals, is known to be one of the main radical reactions taking place in wine.<sup>32,35</sup>

The effects of certain metal ions in alcoholic beverages on the evolution of VSCs have previously been investigated. Nedja et al.<sup>36</sup> demonstrated that in the presence of Cu, H<sub>2</sub>S can react

with MeSH and ethanethiol (EtSH) to form symmetrical and unsymmetrical trisulfides. Walker et al.<sup>37</sup> showed that when Cu (1.6 mg/L) was added to beer, H<sub>2</sub>S and EtSH displayed significantly reduced concentrations. However, Zn, Fe<sup>3+</sup>, Mn<sup>2+</sup>, Ni, Pb<sup>2+</sup>, and Sn<sup>2+</sup> had no effects on H<sub>2</sub>S, MeSH, EtSH, methyl thioacetate (MeSAC), or DMS concentrations when these metals were added at 1.6 mg/L. Only when added at a concentration of 1 g/L did Zn, Fe<sup>3+</sup>, and Pb<sup>2+</sup> bind reversibly to H<sub>2</sub>S and EtSH.<sup>37</sup> The ability of Cu to increase H<sub>2</sub>S concentration in Sauvignon Blanc wines during anaerobic storage was demonstrated by Ugliano et al.<sup>1</sup> Other metal ions can also be involved in the development of VSCs in wines, as has been seen in cases when the evolution of H<sub>2</sub>S was linked to the use of agrochemicals that contained Mn and Zn.<sup>38</sup>

Because of yeast metabolism, heat treatment, light exposure, and other nonenzymatic reactions, a wide range of sulfur compounds are formed during fermentation, maturation, and bottle-aging<sup>20</sup> however, not all the precursors to H<sub>2</sub>S, MeSH, and DMS have been identified.<sup>5</sup> Some sulfur-containing nonvolatile precursor molecules are present in milligrams per liter concentrations, and VSCs start to become problematic at micrograms per liter concentrations. For example, sulfur-containing amino acids are present in wine in relatively high concentrations and are thought to act as precursors to VSCs. Studies have shown that cysteine can generate H<sub>2</sub>S in the presence of a dicarbonyl compound in wine-like systems.<sup>39</sup>  $\alpha$ -Amino acids can also undergo cleavage in the presence of pyridoxal and polyvalent metal ions, acting as catalysts in a variety of reactions. These reactions have been studied in aqueous solutions; for example, transamination as catalyzed by Al<sup>3+</sup>, Fe<sup>3+</sup> and Cu<sup>2+</sup>;<sup>40</sup> racemization as catalyzed by Al<sup>3+</sup>, Fe<sup>3+</sup>, and Cu<sup>2+</sup>;<sup>41</sup> and desulfhydration from cysteine, S-methyl cysteine, and methionine, as catalyzed by Al<sup>3+</sup>, Fe<sup>3+</sup>, Fe<sup>2+</sup>, Sn<sup>4+</sup>, and Sn<sup>2+</sup> at pH 5.8.<sup>42</sup> Gruenwedel et al.<sup>42</sup> studied the catalyzed elimination of H<sub>2</sub>S and MeSH from cysteine, S-methyl cysteine, and methionine in nitrogen atmosphere at 100 °C at pH 5.8 and at 100 °C at pH 6.2 in aqueous solutions. They found that for cysteine, the catalytic activity of the metal ions was as follows: Al<sup>3+</sup>  $\approx$  Fe<sup>3+</sup> > Fe<sup>2+</sup>  $\gg$  Sn<sup>4+</sup>  $\approx$  Sn<sup>2+</sup>, with only a 30% yield of H<sub>2</sub>S for the metals with the highest catalytic activity. In comparison, S-methyl cysteine was almost completely converted to MeSH in the presence of pyridoxal and Fe<sup>3+</sup> over 24 h, with the order of catalytic activity Fe<sup>3+</sup>  $\approx$  Fe<sup>2+</sup>  $\gg$  Sn<sup>4+</sup>  $\approx$  Sn<sup>2+</sup>. The metal-catalyzed removal from a  $\gamma$ -substituted amino acid (i.e., methionine) proceeded much

more slowly than the removal from a  $\beta$ -substituted amino acid.<sup>42</sup>

Multiple pathways exist for the loss of thiol compounds, including the loss of thiols through reactions with quinones.<sup>6,43</sup> It was recently demonstrated that the reactivity of thiols seems to be dependent on the  $pK_a$  of the sulfhydryl group and the structural characteristics and steric accessibility of those sulfhydryl groups during reactions with quinones.<sup>6</sup> In the reactions between aroma active thiols (i.e., 3-sulfanylhexas-1-ol, furan-2-ylmethanethiol, 4-methyl-4-sulfanylpentan-2-one) and phenolics (+)-catechin and (-)-epicatechin, it was demonstrated that Fe plays an important catalytic role by promoting the formation of quinones, which participate in addition reactions with thiols.<sup>6,44–46</sup> Blank et al.<sup>47</sup> also demonstrated that in coffee, the degradation of 2-furfurylthiol proceeds via Fenton-like reactions involving both carbon- and sulfur-centered free radical intermediates.

There are many possible precursors to VSCs in wine, making it important to understand not only the formation of VSCs from precursor sources but also the mechanism (switches) involved in the release of VSCs from various precursor molecules. Investigating the role of metal ions as catalysts as well as the synergistic effects of the metals during their catalytic action in the formation of VSCs is crucial to gain a better understanding of the chemical processes governing the formation of postbottling “reductive” aromas. In this study, we have investigated the formation of VSCs as catalyzed by five metals (Cu, Fe, Mn, Zn, and Al) normally present in wine and that are known for their catalytic ability.

## MATERIALS AND METHODS

**Materials.** Dimethyl sulfide (DMS, 99.8%), ethylmethyl sulfide (EMS, 99.0%), sodium hydrosulfide hydrate ( $\text{NaSH}_3 \cdot x\text{H}_2\text{O}$ , 71%), and sodium thiomethoxide ( $\text{NaSMe}$ , 95%) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Reference standards for  $\text{H}_2\text{S}$  and  $\text{MeSH}$  were prepared from their sodium salts, and all standards were dissolved in cold water (4 °C) and used immediately. Potassium hydrogen tartrate (Fluka) was supplied by Sigma-Aldrich, and tartaric acid and sodium chloride were obtained from Merck (Frenchs Forest, NSW, Australia). Ethanol (99.5%, Rowe Scientific) was redistilled in-house prior to use, and water was obtained from a Milli-Q purification system (Millipore, North Ryde, NSW, Australia). DMS and EMS stock solutions were prepared volumetrically in redistilled ethanol and stored at -20 °C. Containers of  $\text{NaSH}_3 \cdot x\text{H}_2\text{O}$  and  $\text{NaSMe}$  were sparged with nitrogen and stored in a desiccator at room temperature.

L-Methionine, iron(III) sulfate hydrate, and manganese(II) sulfate monohydrate were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Zinc(II) sulfate heptahydrate was obtained from Standard Laboratories, (Melbourne, VIC, Australia), and copper(II) sulfate pentahydrate and aluminum(III) potassium sulfate dodecahydrate were purchased from Ajax Chemicals (Sydney, NSW, Australia). Stock solutions of methionine and metals were prepared volumetrically in water obtained from a Milli-Q purification system.

**Wine Samples.** Chardonnay wine from the 2011 vintage and Shiraz wine from the 2009 vintage, both produced in South Eastern Australia, were obtained from local wineries. Analyses of the chemical compositions of the two base wines were conducted by The Australian Wine Research Institute (AWRI) Analytical Service (Adelaide, Australia) and are as follows: pH 3.53, 2.8 g/L residual sugars, 12.71% (v/v) alcohol, 0.26 g/L

volatile acidity (as acetic acid), 5.8 g/L titratable acidity (as tartaric acid), 39 mg/L free  $\text{SO}_2$ , and 170 mg/L total  $\text{SO}_2$  for the Chardonnay wine; pH 3.49, 4.3 g/L residual sugars, 13.74% (v/v) alcohol, 0.46 g/L volatile acidity (as acetic acid), 6.1 g/L titratable acidity (as tartaric acid), 36 mg/L free  $\text{SO}_2$  and 85 mg/L total  $\text{SO}_2$  for the Shiraz wine.

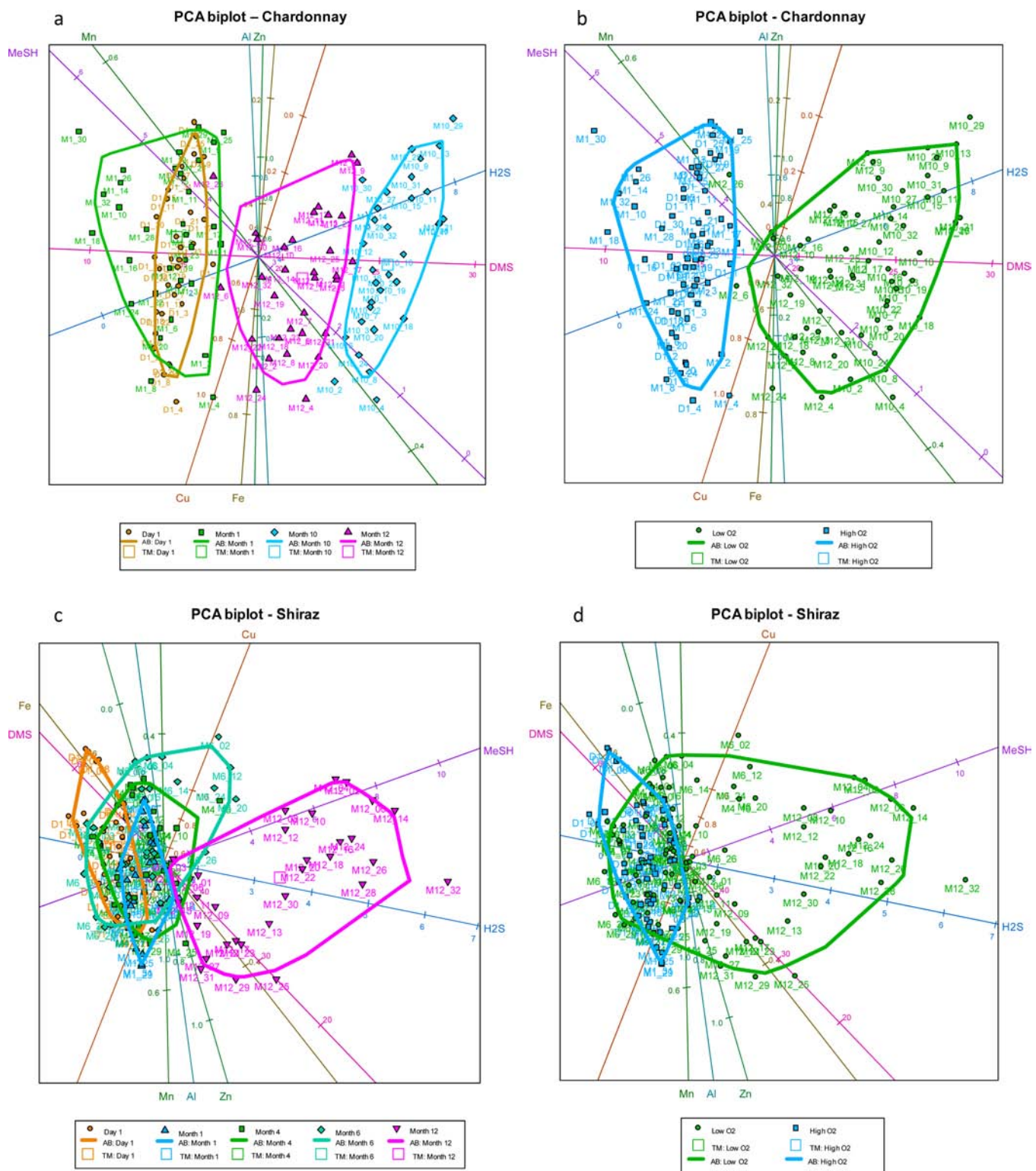
**Chemical Analyses. Gas Chromatography Coupled to Sulfur Chemiluminescence Detection.** The base wines were analyzed for their VSC profiles using an Agilent 355 sulfur chemiluminescence detector (SCD) coupled to an Agilent 6890A gas chromatograph (Forest Hill, VIC, Australia). The system was equipped with a Gerstel multipurpose sampler (MPS 2XL, Lasersan Australasia, Robina, QLD, Australia). Instrument control and data analysis were performed with Agilent GC ChemStation software, rev. B.04.02 [96] and Maestro software integrated version 1.3.9.13/3.5. The gas chromatograph was fitted with a 15 m  $\times$  0.25 mm Varian Wax FactorFour VFWAXms fused-silica capillary column, 0.50  $\mu\text{m}$  film thickness (Varian, Mulgrave, VIC, Australia) connected with a fused-silica universal straight connector (Grace Davison Discovery Sciences) to a 60 m  $\times$  0.25 mm Varian VB-5 fused-silica capillary column, 0.50  $\mu\text{m}$  film thickness (Varian, Mulgrave, VIC, Australia) with a 2 m  $\times$  0.53 mm retention gap. Helium (Air Liquide ultrahigh purity) was used as a carrier gas. Experimental and analytical parameters were as described by Siebert et al.<sup>48</sup>

**Metal Analyses.** Base wines and stock solutions were analyzed for their metal concentrations by Flinders Analytical, Flinders University (Adelaide, Australia) using an Agilent 7500 cx inductively coupled plasma mass spectrometer (Agilent Technologies, Tokyo, Japan), as described in Thiel et al.<sup>49</sup> The concentrations of the five metals present in the Chardonnay and Shiraz base wines are given in Table 1.

**Amino Acid Determination.** The concentration of methionine was determined by the Australian Proteome Analysis Facility (Macquarie University, Sydney, Australia). The methionine concentration was 5.9 mg/L in the Chardonnay and 1.26 mg/L in the Shiraz.

**Oxygen Measurement.** Three colorless 20 mL crimp top vials (Chromacol, Part of Thermo Fisher Scientific Inc., Scoresby, VIC, Australia) fitted with PreSens Pst6 oxygen sensors (Presens, Regensburg, Germany) were filled with the base wine with no metal additions (Chardonnay,  $n = 3$ ; Shiraz,  $n = 3$ ). These samples were used to measure DO during storage of the wines. Oxygen measurements were carried out using a PreSens Fibox 3 trace v3 oxygen meter (Presens, Regensburg, Germany). These samples were stored under the same conditions as the spiked samples.

**Sample Preparation and Analyses.** The Chardonnay and Shiraz base wines were subsampled by placing 10 mL of wine into a 20 mL crimp top amber vial (Chromacol, Part of Thermo Fisher Scientific Inc., Scoresby, VIC, Australia). The metal effects were studied at two levels: the low level being the concentration of the metals present in the base wine, plus the metals were spiked to a higher level. Although the concentration for each metal in the spiked samples was higher than the average concentration range reported in the literature, they were at either concentrations that are reported to have a detrimental effect on wine's organoleptic properties,<sup>50,51</sup> at concentrations that have been seen in wines produced around the world,<sup>26,52,53</sup> or at  $\pm 10$  times the concentration they were present in the base wine (Table 1). Stock solutions were prepared in such a manner that the high level of each metal was



**Figure 1.** PCA biplots for VSC concentration in the wine as modulated by metal addition and grouped by time of analyses for (a) Chardonnay and (c) Shiraz wine samples, and grouped by low or high dissolved oxygen in the control for (b) Chardonnay and (d) Shiraz wine samples.

added by spiking 50  $\mu\text{L}$  of the appropriate stock solution to the 10 mL wine sample using a pipet. By adding only small amounts of stock solution, significant dilution effects were avoided. This protocol gave a total of 31 possible metal combinations and one control sample ( $n = 32$ ). Each treatment was prepared in triplicate, giving a total of 96 samples. A set of 96 samples was prepared for each time point of analyses for

both the Chardonnay and Shiraz wines, and each sample was used only once for GC–SCD analyses and then discarded. Methionine was added to each of the wine samples by adding 50  $\mu\text{L}$  of the prepared stock solution using a pipet to the 10 mL wine sample to give a total concentration of 15.93 mg/L in the Chardonnay and 12.6 mg/L Shiraz wines. This is a midrange methionine concentration, relative to concentrations reported

by Ough and Amerine.<sup>54</sup> The headspace of the wine samples was flushed with 98% N<sub>2</sub> gas for 30 s to remove headspace oxygen before the vials were sealed with a 20 mm magnetic crimp cap with 8 mm center and blue PTFE/silicon septa (118 mm) (Grace Davison Discovery Sciences, Rowville, VIC, Australia).

Each sample set for each time point was then stored at room temperature (22 °C) in 19 L post mix Cornelius kegs (Ambar technology, Alexandria, NSW, Australia). The lid of each keg was fitted with a PreSens Pst6 oxygen sensor (Presens, Regensburg, Germany) (Figure S1, Supporting Information). The kegs were flushed with N<sub>2</sub> (g) until they reached <1 ppb and maintained at a slight positive pressure of 1 psi N<sub>2</sub> (g) to prevent air from entering the keg storage systems. The kegs were continuously monitored and not allowed to exceed an oxygen measurement of 25 ppb. Kegs were flushed with N<sub>2</sub> (g) to <1 ppb if increases in oxygen concentration were observed (Table S1, Supporting Information).

Chardonnay wines were analyzed for VSC concentrations immediately after spiking with metals (day 1), and then following 1 month (month 1), 10 months (month 10), and 12 months (month 12) of storage. The same measurements were performed for the Shiraz wines immediately after spiking with metals (day 1), followed by analysis at 1 month (month 1), 4 months (month 4), 6 months (month 6), and 12 months (month 12) of storage.

**Statistical Analyses.** Calculated sample concentrations were normalized across all samples to produce comparable variables with zero means and unit standard deviation.<sup>55</sup> Design of experiment (DOE) and the analysis of the design matrix were carried out using The Unscrambler X (CAMO Software AS, Oslo, Norway). A two-level full factorial experiment was designed with 5 design variables (Cu, Fe, Mn, Zn, Al) present at two levels (concentration of the metal in the base wine, and spiked concentration). Three response variables were measured (H<sub>2</sub>S, MeSH, and DMS), and the number of design points was 32. The results of the full factorial design were analyzed using a linear model with interactions.<sup>56</sup> Analyses of variance (ANOVA), boxplots, and principal component analyses (PCA) biplots were carried out using R (Vienna, Austria), as described in Aldrich,<sup>57</sup> Gardner-Lubbe et al.,<sup>58</sup> Grower et al.<sup>59</sup> and Nel et al.<sup>60</sup>

## RESULTS AND DISCUSSION

**Dissolved Oxygen.** All samples were prepared in the presence of air, and after each sample was spiked with the metal treatment, it was flushed with 98% N<sub>2</sub> gas for 30 s to remove the oxygen from the vial's headspace and capped and stored in an anaerobic atmosphere. During this experimental setup, oxygen was introduced into the samples and the DO measurements on day 1 for the Chardonnay control samples were on average ( $\pm$ STDEV)  $1.11 \pm 0.342$  mg/L, and the DO measurements for the Shiraz control samples were  $1.43 \pm 0.354$  mg/L (Figure S2, Supporting Information). The DO decreased steadily during the early stages of the experiment (day 1 to month 1), and the introduced oxygen was completely consumed after 4 months of anaerobic storage (Figure S2, Supporting Information).

**Volatile Sulfur Compounds.** Overall, the VSCs under investigation displayed significant changes in concentration over the course of the experiment for both the Chardonnay samples (H<sub>2</sub>S, MeSH, and DMS *p* values <  $2.2 \times 10^{-16}$ ) as well as in the Shiraz samples (H<sub>2</sub>S *p* values <  $2.2 \times 10^{-16}$ , MeSH *p*

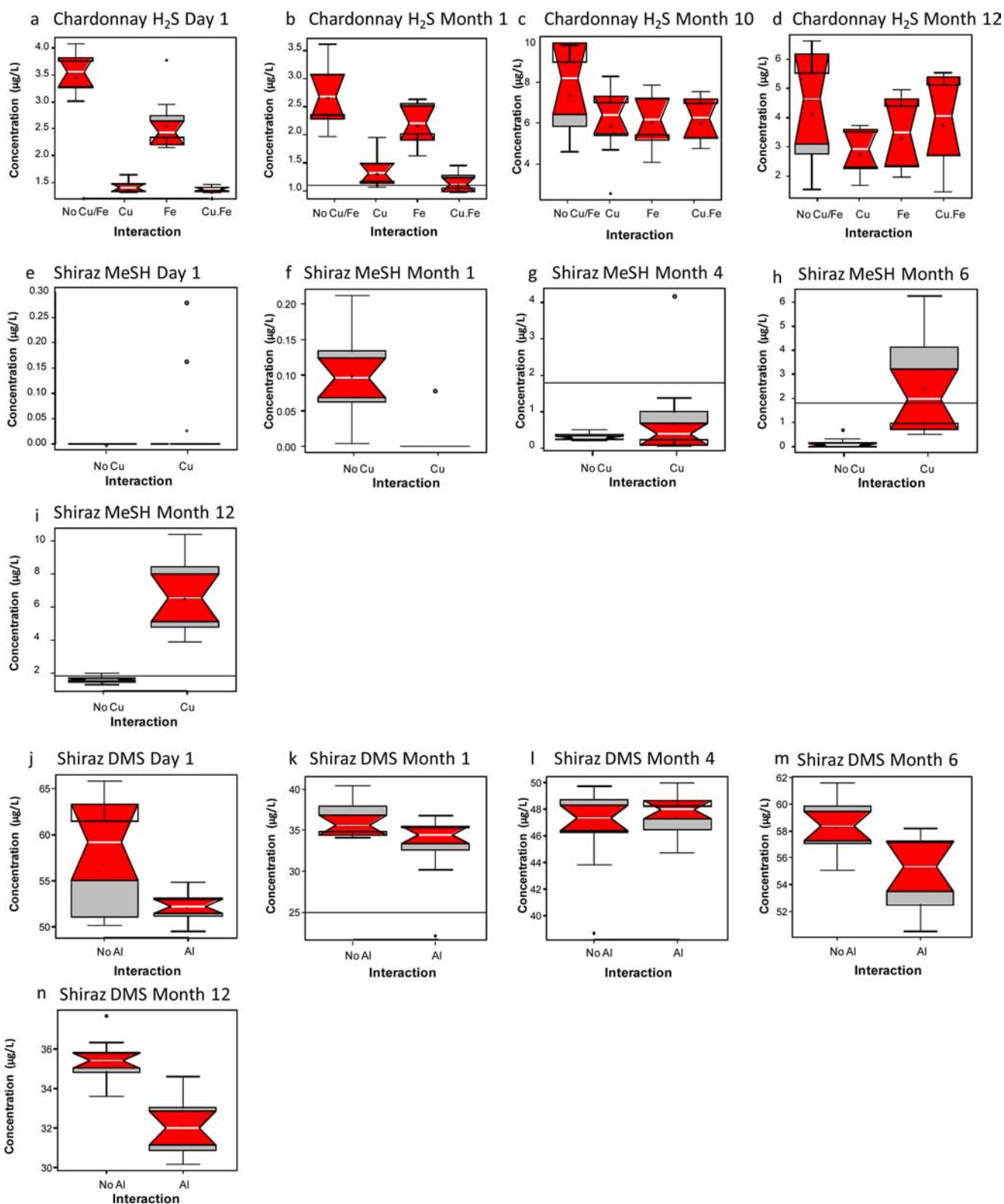
value =  $1.3 \times 10^{-05}$ , and DMS *p* value = 0.005). The changes in VSC concentrations are summarized in the PCA biplots that provide the optimal two-dimensional representation of the data matrix (Figure 1). The quality of a biplot is an overall measure of the accuracy of the two-dimensional approximation of the data matrix.<sup>59</sup> In Figure 1a and b, the quality of display is 40%, and the quality of display in Figure 1c and d is 38%. Axis predictivities are calculated and indicate the degree of accuracy in the predictions made from the biplot axes, and these predictivity values range from 0 to 1, with a value of 1 representing the best predictivity.<sup>58</sup> The axes with the highest predictivities for the Chardonnay samples (Figure 1a and b) are H<sub>2</sub>S (0.849) and MeSH (0.834), and the axis with the highest predictivity for the Shiraz samples (Figure 1c and d) is MeSH (0.867). In Figure 1b and d, the samples are grouped by the measured DO concentrations shown in Figure S2 (Supporting Information). The high oxygen groups in Figure 1b and d correspond to samples measured at day 1 and month 1 for Chardonnay samples and at day 1 to month 4 for Shiraz samples. The low oxygen groups correspond to samples measured at months 10 and 12 for the Chardonnay samples and months 6 and 12 for the Shiraz samples. The major changes observed in the VSC concentrations in the Chardonnay samples over time are overall increases in H<sub>2</sub>S and DMS concentration (Figure 1a and 1b). At month 12, the H<sub>2</sub>S concentrations were higher than at month 10, but the measured H<sub>2</sub>S concentrations for the treated samples were still higher than that of the control. For the Shiraz samples, the major changes were increases in H<sub>2</sub>S and MeSH concentrations (Figure 1c and 1d). It has previously been shown that H<sub>2</sub>S, MeSH and DMS concentrations in wines have a tendency to increase during bottle maturation and that the greatest increases in concentrations for H<sub>2</sub>S and MeSH are seen in samples with low oxygen exposure, as described by Ugliano et al.<sup>1,3,4</sup> and Lopes et al.<sup>4</sup>

The most remarkable results of the current study, however, were the effects observed due to metal additions (i.e., Mn, Zn and Al) that have not previously been considered in the context of wine VSCs as well as the interactions among the five metals. In this study, the metal concentrations used were higher than the average concentration range reported in the literature, and metals need not necessarily be present at high concentrations to induce significant effects. The effect of Cu on VSC evolution has been demonstrated at lower concentrations of 1.6 mg/L<sup>37</sup> and even 0.3 mg/L.<sup>1</sup> Multivariate data analysis methods were used to gain meaningful interpretations of the effects of the five variables (Cu, Fe, Mn, Zn, Al) and the interactions among the variables on the evolution of H<sub>2</sub>S, MeSH, and DMS. Statistical DOE is based on a mathematical theory that makes it possible to investigate all variables simultaneously and obtain reliable conclusions about all individual effects.<sup>56</sup> This mathematical basis ensures minimal impact of experimental error on the final results, provided that all experimental results are interpreted simultaneously, and not sequentially, as in the classical approach of changing one variable at a time.<sup>56</sup> The advantages of DOE are that the individual effects of each potential variable and the interactions among the variables can be studied independently from each other from a single set of designed experiments.<sup>56</sup> Furthermore, the results are analyzed with a model that makes it possible to predict what would happen for any experiment within a given range.<sup>56</sup> In the current study, the range would be the presence or absence of metals at high

Table 2. Metals Inducing Significant Influence on VSCs Formation in Wine<sup>a</sup>

Chardonnay											
H <sub>2</sub> S	p value								comments <sup>b</sup>		
	day 1		month 1		month 10		month 12				
Cu	0.001	- - -	0.001	- - -	0.009	- -	0.537	NS	a		
Fe	0.026	-	0.045	-	0.017	-	0.909	NS	a		
Zn	0.393	NS	0.139	NS	0.001	+++	0.600	NS	a		
Al	0.306	NS	0.376	NS	0.002	++	0.135	NS	a		
Cu*Fe	0.032	-	0.265	NS	0.011	-	0.278	NS	a		
Fe*Al	0.587	NS	0.762	NS	0.014	+	0.800	NS			
Zn*Al	0.882	NS	0.149	NS	0.005	++	0.678	NS			
Cu*Mn*Al	0.418	NS	0.908	NS	0.018	-	0.664	NS	a		
Cu*Zn*Al	0.562	NS	0.912	NS	0.013	+	0.913	NS	a		
Fe*Zn*Al	0.727	NS	0.215	NS	0.036	+	0.732	NS			
Mn*Zn*Al	0.222	NS	0.101	NS	0.033	+	0.279	NS			
MeSH											
MeSH	p value								comments <sup>b</sup>		
	day 1		month 1		month 10		month 12				
Cu	0.002	- -	0.586	NS	0.288	NS	0.055	(0.1)+	b,d		
Zn	0.636	NS	0.096	(0.1)+	0.017	+	0.519	NS	b		
Fe*Mn	0.919	NS	0.711	NS	0.019	-	0.792	NS	b		
Cu*Fe*Mn	0.586	NS	0.695	NS	0.022	-	0.545	NS	b		
DMS											
DMS	p value								comments <sup>b</sup>		
	day 1		month 1		month 10		month 12				
Al	0.062	(0.1)+	0.186	NS	0.020	-	0.312	NS	c		
Zn*Al	0.045	+	0.491	NS	0.060	(0.1) -	0.589	NS	c		
Mn*Zn*Al	0.048	+	0.891	NS	0.245	NS	0.574	NS			
Shiraz											
H <sub>2</sub> S	p value								comments <sup>b</sup>		
	day 1		month 1		month 4		month 6	month 12			
Cu	0.000	- - -	0.157	NS	0.277	NS	0.681	NS	0.010	++	a,d
Fe	0.000	- - -	0.522	NS	0.641	NS	0.968	NS	0.580	NS	a
Mn	0.392	NS	0.036	-	0.440	NS	0.718	NS	0.486	NS	
Zn	0.004	++	0.030	-	0.070	(0.1)+	0.671	NS	0.354	NS	a
Al	0.014	+	0.014	-	0.053	(0.1)+	0.544	NS	0.154	NS	a
Cu*Fe	0.000	- - -	0.844	NS	0.138	NS	0.327	NS	0.010	++	a,d
Cu*Zn	0.014	-	0.900	NS	0.313	NS	0.834	NS	0.526	NS	
Fe*Zn	0.043	-	0.732	NS	0.998	NS	0.804	NS	0.429	NS	
Mn*Al	0.006	- -	0.105	NS	0.165	NS	0.742	NS	0.097	(0.1)+	d
Cu*Fe*Zn	0.114	NS	0.756	NS	0.890	NS	0.687	NS	0.015	+	
Cu*Mn*Al	0.007	- -	0.532	NS	0.110	NS	0.900	NS	0.324	NS	a
Cu*Zn*Al	0.056	(0.1) -	0.495	NS	0.904	NS	0.313	NS	0.050	+	a,d
Fe*Mn*Al	0.039	-	0.874	NS	0.158	NS	0.684	NS	0.065	(0.1)+	
MeSH											
MeSH	p value								comments <sup>b</sup>		
	day 1		month 1		month 4		month 6	month 12			
Cu	0.067	(0.1) -	0.007	- -	0.015	+	0.068	(0.1)+	0.002	++	b,d
Zn	0.067	(0.1) -	0.884	NS	0.031	+	0.603	NS	0.570	NS	b
Cu*Zn	0.067	(0.1) -	0.654	NS	0.017	+	0.583	NS	0.520	NS	
Fe*Mn	0.474	NS	0.317	NS	0.015	+	0.908	NS	0.342	NS	b
Zn*Al	0.067	(0.1) -	0.316	NS	0.047	+	0.605	NS	0.344	NS	
Cu*Fe*Mn	0.474	NS	0.626	NS	0.015	+	0.949	NS	0.329	NS	b
Fe*Mn*Zn	0.474	NS	0.529	NS	0.035	+	0.625	NS	0.707	NS	
Cu*Fe*Mn*Zn	0.474	NS	0.960	NS	0.029	+	0.681	NS	0.672	NS	
Cu*Fe*Mn*Zn*Al	0.078	(0.1) -	1.000	NS	0.013	+	0.882	NS	0.783	NS	
DMS											
DMS	p value								comments <sup>b</sup>		
	day 1		month 1		month 4		month 6	month 12			
Mn	0.048	-	0.081	(0.1) -	0.730	NS	0.129	NS	0.079	(0.1) -	
Zn	0.006	- -	0.808	NS	0.402	NS	0.410	NS	0.082	(0.1) -	
Al	0.004	- -	0.027	-	0.637	NS	0.048	-	0.003	- -	c
Zn*Al	0.004	- -	0.920	NS	0.283	NS	0.176	NS	0.089	(0.1) -	c
Fe*Mn*Zn	0.430	NS	0.039	-	0.770	NS	0.635	NS	0.401	NS	





**Figure 4.** Notched boxplots summarizing selected VSC concentrations ( $\mu\text{g/L}$ ) in Chardonnay and Shiraz samples. The effects of the addition of Cu, Fe, Cu\*Fe, or no Cu/Fe on the evolution of H<sub>2</sub>S ( $\mu\text{g/L}$ ) in Chardonnay samples are shown in a–d. The effect of Cu additions on MeSH concentrations in Shiraz samples are shown in e–i. The influence of Al addition on DMS concentrations ( $\mu\text{g/L}$ ) in Shiraz samples is shown in j–n). Odor threshold values for H<sub>2</sub>S at 1.1  $\mu\text{g/L}$  are indicated by the line parallel to the x-axis in part b, the parallel line in g–i for MeSH at 1.8  $\mu\text{g/L}$ , and the line parallel to the x-axis for DMS at 25  $\mu\text{g/L}$  in k.<sup>12</sup>

on the evolution of H<sub>2</sub>S at day 1 but also to distinguish between the significant effects of the metal interactions such as Cu\*Fe and Cu alone, for example. Three examples of metals and metal

combinations that were associated with some of the largest increases in H<sub>2</sub>S concentration in the Chardonnay samples after 12 months of storage (Table S3, Supporting Information) are



shown in Figure 2a. Increases in H<sub>2</sub>S concentrations were seen for the metal treatments Zn, Mn\*Zn\*Al, and Cu\*Fe\*Mn\*Zn in the Chardonnay samples at month 10. Using multivariate statistical analyses, it was concluded that the increase in H<sub>2</sub>S concentrations was due to the significant effect of either Zn, Al, Zn\*Al, or Mn\*Zn\*Al in these metal combinations. The importance of Al as a catalyst involved in the evolution of H<sub>2</sub>S was previously seen by Gruenwedel et al.<sup>42</sup>

At day 1, some of the spiked metals were associated with a significant reduction in the H<sub>2</sub>S concentration under initial package conditions when the wine had not yet consumed all the introduced dissolved and headspace oxygen (Table 2, Figure 3, Supporting Information Figure S3). This reaction between thiols and metals is routinely utilized in copper fining trials to reduce the impact of unwanted thiols in wines, with the copper reacting irreversibly with thiols, forming odorless copper compounds that precipitate readily.<sup>37,61,62</sup> It is recommended that copper fining should be performed at least a month before bottling, allowing for the elimination of the precipitated copper complexes from the wine through racking and filtration.<sup>20</sup> However practical experience has shown that this is not always the case, with significant amounts of Cu remaining in wines post addition and filtering.

In this study, the effects of some of the added metals in the Shiraz samples were reversed after 4 months of anaerobic storage. Some metals (for example, Cu) that previously showed significant decreasing effects on the H<sub>2</sub>S concentrations were then associated with significant increases in H<sub>2</sub>S concentrations in the Shiraz samples (Figure 3, Supporting Information Figure S3, Table 2). In the Chardonnay samples, this interchangeable effect was not observed for H<sub>2</sub>S, in which case a systematic decrease in concentration was observed. This reversible effect of metals on H<sub>2</sub>S concentrations in the Shiraz samples is best illustrated in Figure 3a and b, where the effect of the addition of Cu, Fe, and the metal combination Cu\*Fe on the H<sub>2</sub>S concentration in the Shiraz wine samples is shown. At the first analysis time point, when samples were analyzed directly after metal spiking, it is clear that all three metal treatments suppressed H<sub>2</sub>S concentration. However, after 12 months of storage and after the control samples had consumed all available oxygen, several of the metals that were initially associated with a significant decrease in H<sub>2</sub>S concentration were then associated with a significant increase in H<sub>2</sub>S concentration. The changing effect of the addition of Cu, Fe, and the metal combination Cu\*Fe on the H<sub>2</sub>S concentration in Chardonnay wine samples is also depicted in Figure 4. Notched boxplots graphically display differences among VSC concentration in the samples, the median (white line), the mean (star) with the red area depicting the 95% confidence interval for the mean, and outliers (black dots). In Figure 4a–d, the effects of added Cu, Fe, and Cu\*Fe of Chardonnay samples are compared ( $n = 96$ ). All three treatments decreased H<sub>2</sub>S concentrations at day 1 and month 1 (Figure 4a, b). The suppression of H<sub>2</sub>S concentration due to the addition of Cu and the Cu\*Fe combination is much greater than that of Fe alone, although Fe also reduces the H<sub>2</sub>S concentration significantly. The suppressing effects of the metals on VSC concentrations are more pronounced during the early stages of the experiment (day 1 and month 1, Figure 4a, b) with the concentrations of H<sub>2</sub>S in all samples approaching the same concentration as the wine aged (months 10 and 12, Figure 4c, d). The metals that were associated with a significant decreasing effect on H<sub>2</sub>S concentration in the Shiraz samples during aerobic conditions but during anaerobic conditions

produced significant increases in H<sub>2</sub>S concentration were Cu, Cu\*Fe, Mn\*Al, Cu\*Zn\*Al, and Fe\*Mn\*Al (Table 2). From these results, it is clear that the effects of the metal ions can change with changing conditions in the wine, such as a change in DO concentrations due to the consumption of oxygen by the wine as it ages. If any residual copper, and possibly other metal ions, are present in the wine after bottling, a change in potential reaction pathways may occur. Nonstoichiometric copper additions during copper fining trials increase the risk that residual copper ions might act as catalysts in the release of low molecular weight sulfur compounds. A change in the reaction pathways could take place during anaerobic conditions, such as during storage or postpackaging, resulting in the release of sulfur ligands from the copper compounds.

In some instances, opposing or competing effects of the metal combinations on the evolution of VSCs were observed. For example, at month 10, the addition of Zn and Al to Chardonnay samples caused a significant increase in H<sub>2</sub>S concentrations, with average concentrations of  $7.70 \pm 0.327$  and  $8.64 \pm 0.664 \mu\text{g/L}$ , respectively (Table 2, Supporting Information Table S3, Figure S4). However, the inclusion of Cu in the Cu\*Zn and Cu\*Al metal combinations resulted in a decrease in H<sub>2</sub>S concentration, with average concentrations of  $6.18 \pm 2.26$  and  $6.62 \pm 1.66 \mu\text{g/L}$ , respectively (Supporting Information Table S3, Figure S4). The decreasing effect observed for Cu in the Chardonnay samples seemed to impede the increasing effect of Zn and Al on the formation of H<sub>2</sub>S in the Chardonnay samples. These impacts could be related to metal speciation and needs to be addressed in future studies.

**Metal Effect on MeSH.** Certain trends observed for H<sub>2</sub>S were also observed for MeSH; for example, the fact that the metal effect was not always constant in Chardonnay and Shiraz wine samples. For example, the Fe\*Mn metal combination showed a significant effect at only one time point of analysis as well as a varying effect in the Chardonnay and Shiraz samples. This metal combination induced a significant decrease in MeSH concentration at month 10 in Chardonnay samples and a significant increase in MeSH concentration at month 4 in Shiraz samples. Overall, the Shiraz samples were more responsive to the effects of the added metals than the Chardonnay samples, with four metal treatments associated with significant effects in the Chardonnay samples, compared with the nine metal treatments that were significant in the Shiraz samples (Table 2). One possible explanation for this phenomenon, as well as the varying effects seen in some instances in the Chardonnay and Shiraz samples, could be that the higher concentrations of polyphenols and anthocyanins present in the Shiraz samples are likely involved in the redox cycling of the metal ions. A wine's quinone profile could also influence the fate and formation of VSCs. Formed VSCs can react with quinones, which removes VSCs, and these reactions could be in competition with reactions involved in the formation of VSCs.<sup>5,32</sup>

Three examples of metals and metal combinations associated with some of the largest increases (Supporting Information Table S3) in MeSH concentration in the Shiraz samples after 12 months of storage are shown in Figure 2b. The increased MeSH concentration in samples with added Cu\*Mn\*Zn, Cu\*Zn\*Al, and Cu were driven by the significant effect of Cu and not due to the other metals (Table 2).

In the Chardonnay and Shiraz samples, the same reversible effect with time was observed for MeSH as was seen for H<sub>2</sub>S. The addition of Cu had a significant effect on MeSH

concentration, with the presence of Cu during aerobic conditions (day 1 to month 1) associated with decreased MeSH concentrations, but after 12 months of storage and after all the oxygen in the control samples had been consumed by the wine, Cu was associated with increased MeSH concentrations (Table 2). The distributions of the MeSH concentrations ( $\mu\text{g/L}$ ) in Shiraz samples ( $n = 96$ ) with or without added Cu are graphically displayed in Figure 4e–i. At day 1 (Figure 4e), no MeSH was present in samples with or without added Cu, but after 1 month of storage, the Cu's affinity for interacting with thiols can be observed in the significantly reduced MeSH concentration in all samples with added Cu (Figure 4f); however, over the course of the experiment and as the introduced available dissolved oxygen diminished in the control samples, the MeSH concentration slowly increased to nearly the same levels in both samples with or without added Cu (month 4, Figure 4g). After 6–12 months of anaerobic storage, the MeSH concentration had significantly increased in all samples with added Cu to an average concentration  $>6 \mu\text{g/L}$ , more than 3 times the odor threshold value of  $1.8 \mu\text{g/L}$  for MeSH<sup>12</sup> (Figure 4h, i). This clearly shows the reversible nature of the Cu on the evolution of thiols. The effect of Cu on MeSH concentration over the course of 12 months is also illustrated in a series of biplots in Figure S3 (Supporting Information). This interchangeable metal effect could possibly be explained by a change in redox chemistry that occurred after the samples had consumed all available oxygen.

**Metal effect on DMS.** Metals and metal combinations that induced a significant effect on DMS concentration in both Chardonnay and Shiraz samples are highlighted in Table 2. The observed differences in DMS concentrations associated with the metal treatments are relatively small if the high concentrations of spiked metals are taken in consideration. Overall, fewer metals produced significant effects on DMS concentration, and the metals with significant effect on DMS were mostly associated with an overall decrease in DMS concentration. The effects of the metals on DMS were possibly due to metals inhibiting the formation of DMS from its precursors already present in the wine, or due to the catalytic degradation of DMS. The only metal and metal combination that induced a significant effect on DMS concentration in both Chardonnay and Shiraz samples were Al and Zn\*Al. In the Chardonnay samples, the Al and Zn\*Al were initially associated with a significant increase in DMS concentration, but after 10 months of anaerobic storage, these metals were associated with a significant decrease in DMS concentration. All the metals that were correlated with significant effects on DMS concentration in the Shiraz samples were associated with decreased DMS concentration (Table 2). In Figure 4j–n, the effects of added Al on Shiraz samples are shown. DMS concentrations were significantly decreased in all samples with added Al at four of the five analysis time points (Figure 4j, k, m, n). This is the first insight into the influence that these metal ions play in the evolution of DMS in wine. Previous investigations into the complexation of DMS with selected heavy metal ions ( $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Pb}^{2+}$ , and  $\text{Hg}^{2+}$ ) in aqueous solutions have shown that only  $\text{Hg}^{2+}$  bound significantly with DMS, with the complexation capacity influenced by pH and media.<sup>63</sup> Furthermore, the affinity of  $\text{Hg}^{2+}$  for DMS was greater at low pH (3.5–5.5) than at high pH (6–8).

In summary, we have investigated the evolution of  $\text{H}_2\text{S}$ , MeSH, and DMS as catalyzed by five metals (Cu, Fe, Mn, Zn, Al) at average reported concentrations for wines and at high

concentrations, and it was found that the addition of metals significantly influences the evolution of VSCs. At the start of the experiment, when oxygen was introduced during the experimental setup, certain metals significantly reduced the concentration of the thiols. During wine maturation, as the oxygen concentration decreased in the control samples, the effect induced by the metals was reversed, with their presence now being associated with significant increases in either  $\text{H}_2\text{S}$  or MeSH concentration. The metals and metal combinations that significantly affected  $\text{H}_2\text{S}$  concentration in both the Chardonnay and Shiraz samples were Cu, Fe, Zn, Al, Cu\*Fe, Cu\*Mn\*Al, and Cu\*Zn\*Al. Metals that significantly affected MeSH concentration in both Chardonnay and Shiraz samples were Cu, Zn, Fe\*Mn, and Cu\*Fe\*Mn. The evolution of DMS in both Chardonnay and Shiraz samples was significantly influenced by Al and Zn\*Al. The importance of the Cu\*Fe metal combination in polyphenol reactions is extensively studied, and the effects of metal ions, especially Cu, on the evolution of VSCs in alcoholic beverages has also been described.<sup>1,36,37</sup> This investigation has highlighted the role that other metals, such as Zn, Al and Mn, and their interactions could play in the evolution of  $\text{H}_2\text{S}$ , MeSH, and DMS during anaerobic storage postbottling. From these results, it is clear that the formation of VSCs from their precursors in wine is not only influenced by the presence of metals, but it is also likely that oxygen concentration in wine also significantly affects the metal effects on sulfur compounds. Walker et al.<sup>37</sup> and Ugliano et al.<sup>1</sup> showed that Cu had significant effects on VSC concentrations when used at lower concentrations of 1.6 and 0.3 mg/L, respectively. This demonstrates that it is not necessary for Cu to be present at high concentrations to induce a significant effect on VSC evolution. The current study highlights that metals can act singly or in combination to greatly influence the wine's VSC profile. The total metal composition and the concentrations in which these metals occur may vary considerably in real wines, and this would significantly influence their effects. It is therefore important to understand the effects of metals on VSC formation in wines postbottling and how oxygen management affects the end product.

## ■ ASSOCIATED CONTENT

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

ANOVA, analyses of variance; AVG, average; AWRI, The Australian Wine Research Institute; DMS, dimethyl sulfide; DO, dissolved oxygen; DOE, design of experiment; EMS, ethylmethyl sulfide; H<sub>2</sub>S, hydrogen sulfide; MeSH, methanethiol; NaSH<sub>3</sub>·xH<sub>2</sub>O, sodium hydrosulfide hydrate; NaSMe, sodium thiomethoxide; NS, not significant; OIV, International Organization of Vine and Wine; PCA, principal component analyses; redox, oxidation–reduction; STDEV, standard deviation; VSCs, volatile sulfur compounds

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