Australian Government

Australian Grape and Wine Authority

The Australian Wine Research Institute

Formation and fate of positive and negative sulfur compounds



FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

Project Number: **AWRI 3.5.3**

Principal Investigator: Dr Paul Smith

Research Organisation:

The Australian Wine Research Institute

Date: **22 September 2017**

Project 3.5.3 - Formation and fate of positive and negative sulfur compounds

Abstract

Low molecular weight sulfur compounds (LMWSCs) can contribute both positive and negative sensory attributes to wines. Positive aromas include 'passionfruit', 'tropical' and 'blackcurrant' and negative characters include 'rotten egg', 'rubber', 'sewage' and 'canned corn'. This project focused on increasing understanding of the formation and fate of sulfur compounds responsible for these sensory attributes and applying this knowledge to develop practical strategies to manipulate and modulate LMWSCs during winemaking. A further aspect was to ensure that positive and negative compounds do not undergo any further unwanted changes in tank or bottle, once a wine has a desirable LMWSC profile.

As part of this project, new advanced analytical methods were developed to enable characterisation of the very challenging and reactive LMWSCs at part per billion (ppb) concentrations and below. Another very significant breakthrough was the identification of precursors to several key LMWSCs. The work also showed that the addition of copper significantly influenced the evolution of LMWSCs. Importantly, the effects of copper differed depending on dissolved oxygen concentration post-bottling and also as a function of the residual copper concentration. In addition, copper effects were further influenced by pH and SO_2 and it was demonstrated that post-bottling formation of H_2S, MeSH, DMS, and CS_2 is significantly impacted by both copper additions and wine pH. Beyond copper, the project showed that the formation of LMWSCs from their precursors in wine is influenced by the presence of other metal ions that naturally occur in wine, especially when present in high concentrations. Data on factors modulating the chemical formation of LMWSCs in wine was augmented by the discovery of significant differences among wine yeast in their ability to release both positive and negative LMWSCs. Taken together, this information will help winemakers to make informed choices regarding yeast strain and winemaking conditions to suit their wine style, potentially reducing the formation of negative LMWSCs in a winery environment.

Executive summary

Low molecular weight sulfur compounds (LMWSCs) can contribute both positive and negative sensory attributes to wines. Positive characteristics can include 'passionfruit', 'tropical' and 'blackcurrant' but the negative characteristics include 'rotten egg', 'rubber', 'sewage' and 'canned corn'. Their control in a winery environment is an important avenue to increasing the value of wine by either increasing positive sensory attributes or decreasing those deemed to be negative. Their occurrence can be influenced by factors including yeast selection and fermentation conditions; the nature and quantity of precursor compounds; the availability or absence of oxygen at different points of the winemaking process; and availability and speciation of transition metal ions such as copper. Broadly, this project focused on increased understanding of these factors; this knowledge was then used to develop practical strategies to manipulate and modulate LMWSCs during winemaking and to ensure that once a wine has produced the expressed LMWSCs, positive or negative, they do not undergo any further unwanted changes in tank or bottle.

More specifically, the project aimed to develop an in-depth understanding of the role of compounds suspected to be the main precursors of the sensorially important LMWSCs, with a focus on negative sensory characters deriving from hydrogen sulfide (H_2S), methanethiol (MeSH), ethanethiol (EtSH), dimethylsulfide (DMS), phenylmethanethiol (BnSH) and the generally positive varietal thiol sensory characteristics of 3-sulfanylhexan-1-ol (3-MH) and 3-sulfanylhexyl acetate (3-MHA). The project sought to understand the metabolic and chemical pathways that lead to formation of these thiol compounds and the chemical and environmental switches that lead to otherwise innocuous sulfur-based compounds being converted to those that have a significant sensory impact.

As part of the enabling technology required for this project, new advanced analytical methods were developed to enable characterisation of these very challenging and reactive LMWSCs. A new HPLC-

MS method for the analysis of thiols and disulfides in wine was developed and validated which quantifies a wider range of LMWSCs than previous methods. In addition, to support investigations of sulfur-containing amino acids as LMWSC precursors, an amino acid assay method was developed in collaboration with the AWRI Metabolomics group for cysteine (Cys), methionine (Met) and glutathione (GSH).

A very significant breakthrough from this project was the identification of precursors to several key LMWSCs. At the outset of this project, it was suspected that the presence of certain compounds such as Cys, GSH, Met, disulfides, and thioacetates such as methyl thioacetate (MeSAc) and ethyl thioacetate (EtSAc) were contributing factors in determining H_2S, MeSH, EtSH, and DMS concentrations in wines post-bottling. However, these hypotheses had yet to be tested in real wines. This study demonstrated that the direct desulfurisation of Cys, Met, or GSH did not pose an obvious risk of H_2S or MeSH formation in wine post-bottling. However, the presence of MeSAc, or disulfides such as DMDS, significantly increased the risk of MeSH formation, with up to a 20% MeSH yield and a 70% MeSH yield obtained from MeSAc and DMDS, respectively, as measured in wines over a 12-month storage period. Additionally, model studies do not support the theory that BnSH forms from benzaldehyde reacting with H_2S, leaving unanswered the question about how BnSH and the associated 'struck flint' character are formed. Based on these findings it is recommended that thioacetates and disulfides are monitored to assess potential risk of post-bottling LMWSC release, in addition to the 'total packaged oxygen' (TPO) concentration of wine.

It was shown that the addition of copper significantly influenced the evolution of LMWSCs, but the effects of copper differed depending on dissolved oxygen concentration post-bottling and with the residual copper concentration. As expected, at higher oxygen concentrations, some metals such as copper, significantly reduced the concentration of the thiols in the wine tested. During wine maturation, once the oxygen concentration decreased to non-detectable, the effect of copper was reversed, with the presence of copper now being associated with a significant increase in MeSH concentration, regardless of the presence or absence of other metals. Higher residual copper concentrations also resulted in significantly higher H_2S concentrations in wine, but interestingly, these differences did not become evident until 12 months post-bottling. This work highlights the copper concentration dependency of H_2S formation and the weakness of traditional benchtop trials used to determine copper additions, where only the immediate effects are assessed and not the long-term impacts in-bottle resulting from residual copper.

In addition, copper effects can be influenced by pH and SO_2 and this study has demonstrated that the post-bottling formation of H_2S, MeSH, DMS, and CS_2 is significantly impacted by both copper additions and wine pH. For some 'reductive' aroma compounds, the interaction between pH and copper treatment was an important factor in determining their final concentrations in Chardonnay and Shiraz wines post-bottling. The current results established that both wine pH and copper additions have significant impacts on H_2S, MeSH, and DMS concentrations in wines post-bottling. Specifically, less H_2S and MeSH were produced through copper-catalysed reactions in wines at a lower pH than in wines at a higher pH level. It should be noted also that different effects were observed in red wines and white wines, likely due to the differing nature of the wines' matrix components. Investigations also showed that the presence of SO_2 plays a fundamental role in the modulation of H_2S profiles through copper interactions in both red and white wine post-bottling.

One possibility for managing copper effects is to bind copper to chelating compounds that completely remove it from the wine matrix or chemically isolate it so that it can no longer participate in the formation of reductive characters. The results from this project suggest that differing metal chelation environments can be established using additives and that these can have a significant effect on H_2S generation. It is possible to optimise copper's beneficial effects through the timing of its addition and in terms of limiting residual copper concentrations it is more desirable to add copper at 0° Brix (during the final phase of active fermentation) rather than at the end (post-

active ferment) which is a common industry practise. This timing ensures that yeast and solids adsorb copper and limit the residual copper concentration in a final wine.

The project has shown that the formation of LMWSCs from their precursors in wine is influenced by the presence of not only copper, but also by other metal ions that naturally occur in wine when present in sufficiently high concentrations. These metals include manganese, aluminium, iron and zinc, and it is recommended that producers monitor these and keep metal concentrations as low as possible in wine, to minimise the post-bottling evolution of wine LMWSCs.

As part of this project the AWRI collaborated with a project led by Dr Andrew Clark at Charles Sturt University (CSU). The results from the collaboration demonstrate that major non-volatile matrix components (red wine tannin, white wine protein, white wine polysaccharide, red wine polyphenol, white wine polyphenol) had limited impact on the form of copper present in wine, most likely because the major form of copper in most wines is as copper(I) sulfide (Cu_2S), rather than as complexes with matrix components as suggested by previous studies.

In further research to understand the chemical forms of copper in wine, differences in particle size and concentration of copper-tartrate complexes suggested that the various types of copper-tartrate complexes produced at varying pH levels may affect the binding sites of copper that are available to either catalyse the formation of LMWSCs such as H_2S, or quench the thiols produced to form copper sulfide complexes. This may explain some of the pH-related effects observed in these experiments.

This project has also discovered significant differences among wine yeast in their ability to release both positive and negative LMWSCs. Small-scale fermentation experiments with a large number of yeast strains have shown the great diversity in the production of LMWSC by yeast. This analytical information, combined with genomic data on these yeast strains, has allowed the identification of several yeast markers associated with the formation of important LMWSCs, such as the 'tropical' thiols 3-MH and 4-MMP and H_2S, from their precursors. This information will help winemakers to make informed yeast strain choices to suit their wine style, and potentially reduce the formation of negative LMWSCs in a winery environment.

Background

Volatile sulfur compounds (LMWSCs) can contribute both positive and negative attributes to wines, and their control in a winery environment is an important avenue to increasing the value of wine by either increasing positive sensory attributes or decreasing those deemed to be negative. Their occurrence can be influenced by factors including yeast selection and fermentation conditions, the nature and quantity of precursor compounds; the availability or absence of oxygen at different points of the winemaking process; and availability and speciation of transition metal ions such as copper.

The project sought to develop an in depth understanding of:

- the role that compounds suggested as the main precursors to of the sensorially important LMWSCs, with focus on hydrogen sulfide (H_2S), methanethiol (MeSH), ethanethiol (EtSH), dimethylsulfide (DMS), phenylmethanethiol (BnSH), 3-sulfanylhexan-1-ol (3-MH) and 3-sulfanylhexyl acetate (3-MHA);
- the metabolic and chemical pathways that lead to their formation
- the chemical and environmental switches which lead to otherwise innocuous sulfur based compounds being converted to those that have a significant sensorial impact.

This framework of understanding is required to develop practical strategies in winemaking to manipulate and modulate LMWSCs during the winemaking process. A further aspect was to ensure that positive and negative compounds do not undergo any further unwanted changes in tank or bottle, once a wine has a desirable LMWSC profile.

Highlights

Precursors to LMWSCs identified

Key precursors to several key LMWSCs were identified. It was demonstrated that the direct desulfurisation of Cys, Met, or GSH did not pose a risk of H_2S or MeSH formation in wines post-bottling. The presence of Cys and GSH was associated with smaller increases in H_2S concentrations compared to the increases in H_2S concentration seen with the presence of copper (acting on yet to be identified precursors) in Shiraz wines. For MeSH and EtSH, important precursor compounds were their corresponding disulfides and thioacetates, such as dimethyldisulfide (DMDS), methylthioacetate (MeSAc), and ethylthioacetate (EtSAc). These precursor compounds produced large concentrations of MeSH and EtSH, respectively, of between 30% to 70% yields of the thiols as measured in wines over a twelve-month storage period. External factors such as copper addition to wine, and the pH of the wine were important factors that determined 'reductive' aroma accumulation in wines post-bottling.

The effect of copper differs depending on dissolved oxygen concentration post-bottling and residual copper concentration

The addition of copper was found to significantly influence the evolution of LMWSCs. Oxygen concentration played a significant role in copper's effect on the formation of LMWSCs in wine. Initially, at high oxygen concentrations, the presence of some metals such as copper, significantly reduced the concentration of the thiols in the wine tested. During wine maturation, the oxygen concentration decreased to 0 ppb after four months of anaerobic storage and the effect of copper was reversed, with the presence of copper now being associated with a significant increase in MeSH concentration, regardless of the presence or absence of other metals.

Managing copper effects

It is possible to manage copper through the timing of its addition. To limit residual copper concentrations in wine it is more desirable to add copper at 0°Brix (during the final phase of active fermentation) rather than at the end (post-active ferment) which is a common industry practice. This ensures the yeast and solids adsorb copper and limit the residual copper in the final wine.

Impacts of metals on reduced aroma formation

The formation of LMWSCs from their precursors in wine was found to be influenced by the presence of not only copper, but also other metal ions that naturally occur in wine, when they are present in high concentrations. Winemakers looking to reduce the risk of aromas from LMWSCs can consider taking steps to minimise metal concentrations in wine.

Understanding differences among wine yeast strains in their ability to release volatile sulfur compounds (LMWSCs)

New understanding has been gained of the differences among wine yeast in their ability to release both positive and negative LMWSC. Small-scale fermentation experiments with a large number of yeast strains showed the great diversity in the production of LMWSCs by yeast. This analytical information, combined with genomic data on these yeast strains, has allowed the identification of several yeast markers that modulate the formation of important LMWSCs, such as the 'tropical' thiols 3-MH and 4-MMP and H_2S, from their precursors.

Objectives

The core objective of the project is to develop successful strategies to manipulate and modulate key LMWSCs (i.e. H_2S, MeSH, EtSH, DMS, BnSH, 3-MH and 3-MHA) in a commercially practical winemaking context. This was achieved through the investigation of the core precursors of the sensorially important LMWSCs; the metabolic and chemical pathways that lead to their formation and the chemical and environmental switches which lead to their sensorial expression or repression.

Specifically, this involved the development of an understanding of:

- the origin of LMWSCs (H_2S, MeSH, DMS, BnSH, 3-MH and 3-MHA)
- the role of transition metal ions, in particular copper, in the release of LMWSCs via their interactions with other wine components
- the impact of the quantity and timing of copper and sulfur dioxide (SO_2) additions when producing wine to ensure the optimum sensorial outcome and eliminate the development of negative LMWSCs both in tank and in bottle
- the influence of yeast strain on LMWSC formation (in collaboration with AWRI Projects 3.2.2 and 3.2.3), by observation of levels produced in model fermentations, and through qualitative assays of key enzymatic activities;
- which yeast genes encode enzymes that mediate formation of H_2S from Cys (Cys sulfhydrylase or desulfhydrase), 3-MH from Cys and GSH conjugates, and MeSH from Met (Met γ-lyase or methioninase); and how yeast genetic determinants and/or fermentation conditions regulate the accumulation of S-reserve compounds (Cys, GSH) and LMWSCs, which may be released via autolysis into the wine once fermentation is finished, and contribute to the subsequent chemical formation of LMWSCs.

Methods

Gas chromatography (GC) coupled to sulfur chemiluminescence detection was used to analyse low molecular weight sulfur compounds (LMWSC). Wines were analysed for their LMWSC profiles using an Agilent 355 SCD sulfur chemiluminescence detector coupled to an Agilent 6890A gas chromatograph as described by Siebert et al. (2010). The varietal thiol 3-MH was quantified as the pentafluorobenzyl derivative using a stable isotope dilution assay, by means of solid phase microextraction (SPME) coupled with GC-MS as described by Ugliano et al. (2011).

Wines and stock solutions were analysed for their metal concentrations by Flinders Analytical, Flinders University (Adelaide, Australia) using an Agilent 7500 ICP-MS (Agilent Technologies, Tokyo, Japan) as described in Thiel et al. (2004).

The amino acid concentrations of the wines were determined by the AWRI SA Metabolomics Facility using the method described by Ovalles et al. (2005) that was adapted for wine analysis.

The LMWSCs present in the liquid phase of the wines were analysed using the method described by Seiwert and Karst (2007) that was adapted for wine analysis.

The reaction between benzaldehyde and BnSH was monitored using high performance liquid chromatography (HPLC) performed on an Agilent 1100 liquid chromatograph (LC) with diode array detection (DAD) (Agilent, Australia) using a Phenomenex Synergi C18 Hydro column (4 µm particle size, 80Å, 150 mm, 2.1 mm ID) (Lane Cove, NSW, Australia). The mobile phase was 50% acetonitrile in MilliQ water and the flow rate was 0.25mL/min.

Results and discussion

The origin of LMWSCs (H_2S, MeSH, DMS, BnSH, 3-MH and 3-MHA)

As part of this project, a comprehensive review of the sources of volatile sulfur compounds in wine was compiled and published (Smith et al. 2015).

New methods developed to enable monitoring of sulfur compounds

A new HPLC-MS method for the analysis of thiols and disulfides in wine was developed and validated which quantifies a wider range of LMWSCs than previous methods. The new method also allows for a comparison between the LMWSCs present in the headspace of wine and the LMWSCs present in the liquid phase to gain better insight into the fate of LMWSCs in wine and to possibly predict the way a wine will age in bottle. To assess a potential role of sulfur-containing amino acids as LMWSC precursors, an amino acid assay was developed with the SA Metabolomics Facility and samples are now being assessed to quantify the sulfur-containing amino acids in wine, Cys and Met and the tri-peptide, GSH. The new methods were applied to determine the formation and fate of H_2S and other LMWSCs throughout the winemaking process and during wine bottle storage.

What are the main precursors of the sensorially important LMWSCs?

At the outset of this project, it was suspected that the presence of certain compounds such as Cys, GSH, Met, disulfides, and thioacetates such as MeSAc and EtSAc were contributing factors in determining H_2S, MeSH, EtSH, and DMS concentrations in wines post-bottling. However, these hypotheses had not been tested in real wines.

Experiments were performed to determine the ability of sulfur containing amino-acids such as Cys, Met, and the tripeptide GSH; and other LMWSCs to act as precursors to H_2S and MeSH in real wines (Figure 1) (Bekker et al. 2017). Cysteine and GSH did not act as sensorially relevant precursors to H_2S. Their presence was associated with small increases of H_2S concentrations, but this could also be an indirect effect where the reducing characteristics of Cys and GSH promoted an environment that favoured H_2S formation and not necessarily a direct desulfurisation of the thiol moiety of Cys or GSH. In these experiments, the presence of copper remained the main factor that contributed to significant increases in H_2S concentrations from yet to be identified precursor compounds. Cysteine and GSH were associated with lower H_2S formation from copper-catalysed reactions in Shiraz wines. Cysteine and GSH may inhibit H_2S formation from copper-catalysed reactions; or possibly form mixed disulfides or polysulfanes through the reaction of Cys/GSH with H_2S.

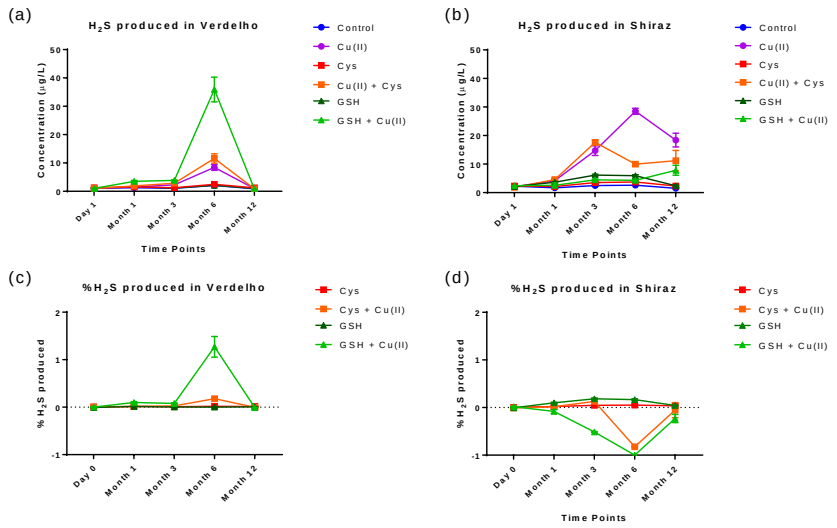


Figure 1. Hydrogen sulfide (H_2S) produced from cysteine (Cys) and glutathione (GSH), with and without the addition of copper, in Verdelho and Shiraz wines.

Notably, DMDS and MeSAc did contribute significantly to MeSH concentrations in the wines post-bottling on a sensorially relevant scale (Figure 2). In all cases, the presence of copper had significant effects on the ability of the precursors to produce H_2S and MeSH, as well as the rate of H_2S and MeSH formation. Wine pH also significantly affected MeSH formation, with higher release of MeSH at lower pH, most likely due to increased rates of acid catalysis (discussed subsequently).

The accumulation of LMWSCs in white wines and red wines were also different, with significantly less H_2S, MeSH, and EtSH accumulating in red wines that in white wines. This suggests that these thiols may have interacted with wine compounds that inhibited their accumulation in the red wines. However, the risk remains that under changing wine conditions any thiols incorporated into other molecular forms may again be released.

This study demonstrated that Cys, Met, or GSH did not pose a risk of H_2S or MeSH formation from direct desulfurisation in wines post-bottling. However, the presence of MeSAc, or a disulfide such as DMDS, posed a significant risk of MeSH formation, with up to a 20% MeSH yield and a 70% MeSH yield obtained from MeSAc and DMDS, respectively, as measured in wines over a twelve-month storage period.

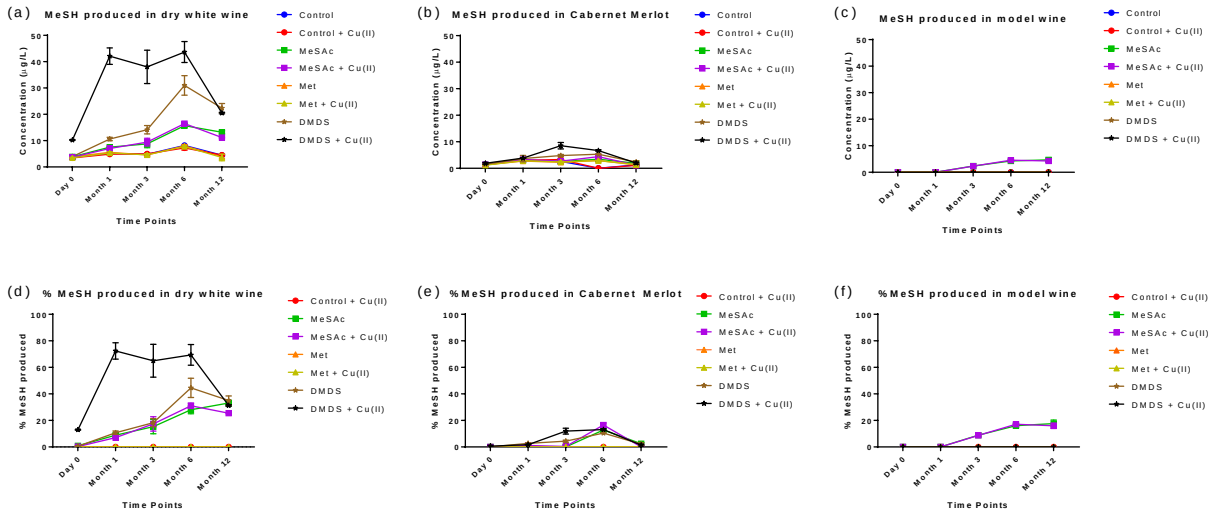


Figure 2. Methanethiol (MeSH) produced from methylthioacetate (MeSAc), methionine (Met), and dimethyldisulfide (DMDS), with and without the addition of copper, in dry white wine, Cabernet Merlot, and model wines.

The reactivity of benzaldehyde with H_2S was studied in model wine systems over a nine-month period. Benzaldehyde and H_2S were added to model wines, with benzaldehyde at a concentration of 9.42 µM and the H_2S concentration varied (9.42 µM, 94.2 µM, 0.942 µM) to give molar ratios of 1:1, 1:10, and 10:1 of benzaldehyde to H_2S. Model wines were either treated with 0.5 mg/L of copper and 4.0 mg/L of iron, or treated with no metals. Samples were duplicated with one set prepared with the total exclusion of oxygen and the second set prepared in an environment rich in oxygen. After nine months of storage no BnSH could be detected using HPLC analysis, or by LCMS analysis with a detection limit of 0.040 µM for BnSH. As such, these studies do not support the theory that BnSH forms from benzaldehyde reacting with H_2S.

Management of LMWSCs can be effectively performed using oxygen during fermentation

The final report chapter on Project 3.3.2 outlines in detail the effects on LMWSCs of using oxygen during fermentation (Bekker et al. 2016c). In summary, conditional on the dose and timing, oxygen can be very effective at limiting the accumulation of LMWSCs during fermentation and for significant periods of storage time after treatment.

The role of transition metal ions, in particular copper, in the release of LMWSCs via their interactions with other wine components

A series of experiments aimed to generate detailed information about how 'reductive' sulfur aromas form post-bottling, and the potential catalytic role of copper and other metals in such reactions. There are many possible precursors to LMWSCs in wine, making it important to not only understand the chemical nature of precursor sources but also the mechanisms or chemical switches that are involved in the release of LMWSCs from their various precursor compounds. 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 LMWSCs, is crucial to gain a better understanding of the chemical processes governing the formation of post-bottling 'reductive' aromas.

As part of the current management strategy to deal with 'reductive' aromas, copper is often added to wine prior to bottling to treat unpleasant 'sulfidic' aromas such as 'rotten egg'. It is thought that the copper ions react with H_2S to produce an insoluble solid (copper sulfide), which results in the removal of both copper and H_2S from the wine. However, previous work at the AWRI (Ugliano et al. 2011) established that copper additions at bottling can promote the accumulation of H_2S at later stages of bottle ageing.

It was found that the addition of metals to wine significantly influenced the evolution of LMWSCs. Oxygen concentration also played a significant role in the effect of the metals on LMWSC formation. Initially, at high oxygen concentrations, some metals such as copper, significantly reduced the concentration of thiols in the wine tested. During wine maturation, the oxygen concentration decreased to 0 ppb after four months of anaerobic storage and the effect of copper was reversed with the presence of copper now being associated with a significant increase in MeSH concentration, regardless of the presence or absence of other metals (Viviers et al. 2013).

A series of boxplots in Figure 3 describe the evolution of MeSH in Shiraz samples (n = 96) with or without added copper. The boxplots graphically display differences among MeSH concentration in the samples; the median (white line); the mean (star) with the red area depicting the 95% confidence interval for the mean; as well as outliers (black dots). At Day 1 (a) no MeSH was present in samples with or without added copper, but, after one month of storage, the scavenging ability of copper can be observed in the significantly decreased MeSH concentration in all samples with added copper (Figure 3b). However, as the oxygen concentration in the wine decreased, the MeSH concentration slowly increased to nearly the same levels in both samples with or without added copper (Month 4, Figure 3c). After 6 to 12 months of anaerobic storage, the MeSH concentration had significantly increased in all samples with added copper and reached concentrations above MeSH's odour threshold value of 1.8 µg/L (Siebert et al. 2010) (Figure 3d and 3e). This shows that the formation of MeSH is not only influenced by the presence of metals, but that the oxygen concentration in wine also significantly affects the evolution these compounds.

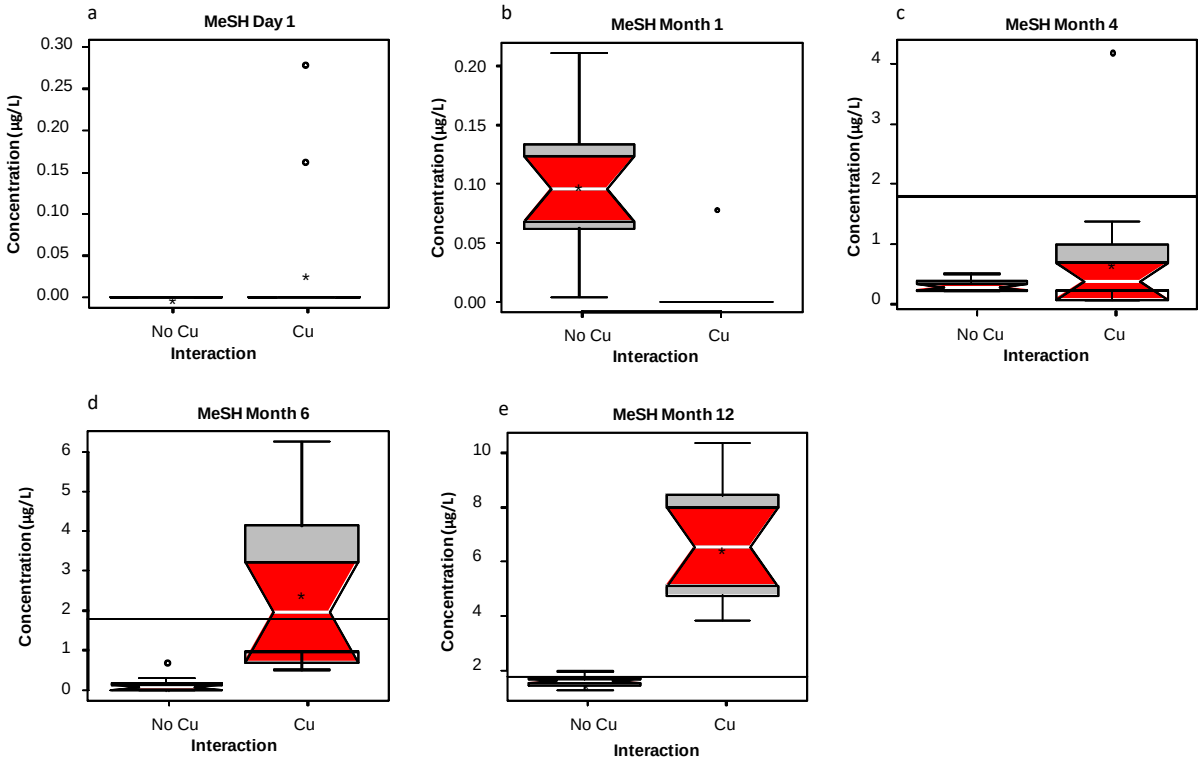


Figure 3. Notched boxplots of the MeSH concentration (µg/L) in Shiraz wine samples during storage show initially a significant decrease (b) and later significant increases (d) and (e) due to copper addition. The line parallel to the x-axis in (c), (d) and (e) indicates the odour threshold value for MeSH at 1.8 µg/L.

Residual copper concentrations are influenced by timing of copper additions and affect reduced aroma formation.

Another study investigated the dose effect of copper on Chardonnay wine LMWSCs. The combination of an early (post-ferment, on solids) treatment and a late (pre-bottling) treatment

resulted in higher residual copper concentration (0.55 mg/L residual copper) compared with just an early treatment (0.17 mg/L residual copper). These higher residual copper concentrations resulted in significantly higher H_2S concentrations in the wine, but interestingly, these differences did not become evident until the 12-month time point, with earlier time points showing essentially equivalent results for both treatments (Figure 4). This work highlights the copper concentration dependency of H_2S formation and the weakness of traditional benchtop trials used to determine copper additions, where only the immediate effects are assessed and not the long-term impacts in-bottle resulting from residual copper.

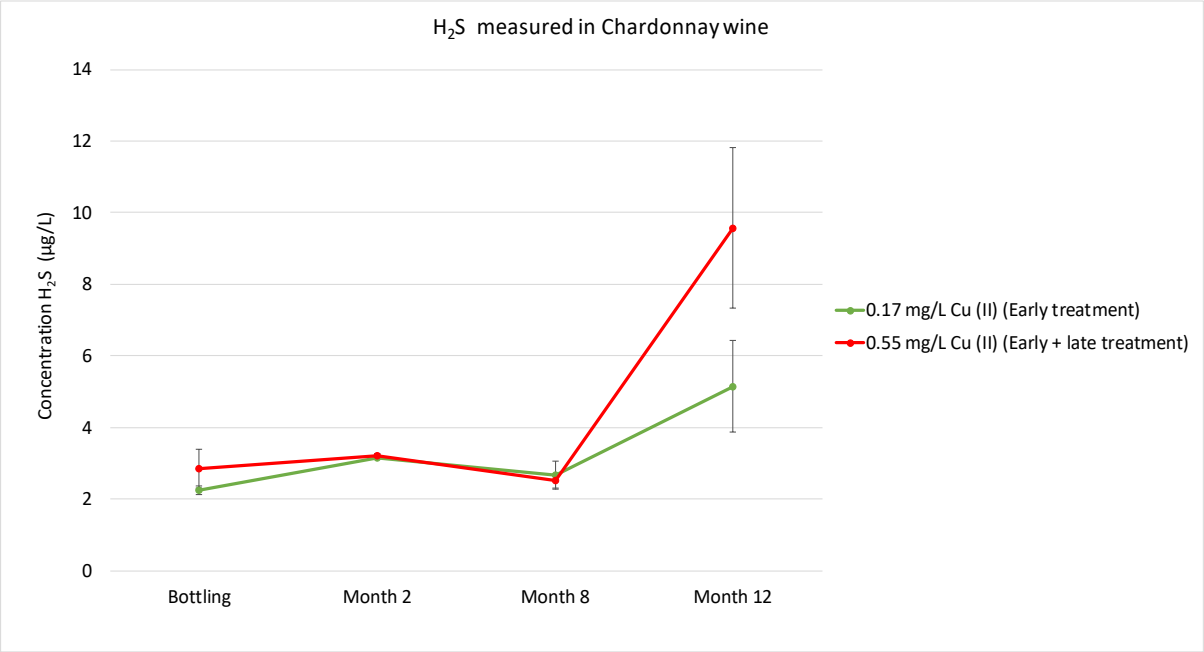


Figure 4. The effects of timing of copper addition on the evolution of hydrogen sulfide (H_2S) as measured over 12 months post-bottling.

To more deeply investigate the effects of a wider range of metals on the formation of the 'reduced' aroma compounds MeSH, H_2S and DMS during bottle ageing, a large experiment was designed in which five metals (copper, iron, manganese, zinc, aluminium) were added to Chardonnay and Shiraz wine samples in all possible combinations (resulting in 31 treatments and 1 control for each wine). The metals were present at either their native level in the base wine (the base wines selected exhibited relatively low metal levels), or a high level spiked to approximately ten times that native concentration. The concentrations of LMWSCs were analysed over a 12-month period. At bottling, the wines contained oxygen at around the recommended level of 1 mg/L (Chardonnay 1.11 ± 0.34 mg/L; Shiraz 1.43 ± 0.35 mg/L) and after four months of anaerobic storage the dissolved oxygen (DO) of both Chardonnay and Shiraz samples decreased to undetectable levels.

Significant changes in volatile sulfur compounds were observed over the 12-month period, with the Chardonnay samples showing increases in H_2S and DMS, and the Shiraz samples showing increases in H_2S and MeSH (Figure 5).

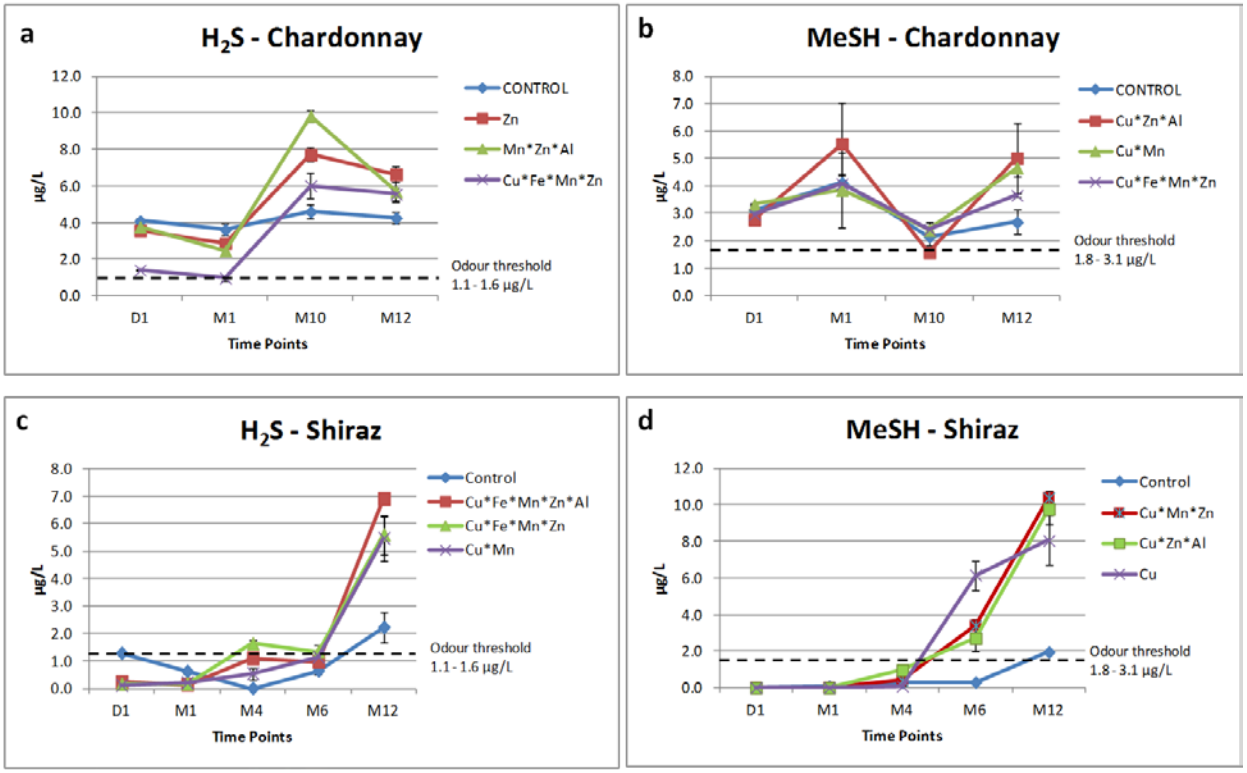


Figure 5. The impact of metals and metal combinations on the concentrations of H_2S and MeSH in Chardonnay and Shiraz wines over time

The most remarkable results of this current study, however, were the effects observed due to additions of metals (i.e. manganese, zinc, and aluminium) that have not previously been considered in the context of wine LMWSCs, as well as the interactions between the five metals. Not all metals and metal combinations had significant effects on the formation of H_2S throughout the experiment, and in some instances the metals were only associated with significant effects at one analysis time point. To distinguish between the different significant metal effects, multivariate statistical methods were used. For example, the Chardonnay samples that displayed the largest decrease in H_2S concentration at Day 1 and Month 1 were all samples that were treated with copper, and it seemed that copper was the only metal associated with significant decreasing effects. But by making use of multivariate statistical analysis it was possible to distinguish between the significant effect of copper on the evolution of H_2S at Day 1, as well as the significant effects of copper*iron, that was also associated with significant decreasing effects on H_2S concentrations at Day 1 in the Chardonnay samples. This reaction between thiols and metals is routinely used in copper fining trials to reduce the impact of unwanted thiols in wines (Ugliano et al. 2009). If both the Chardonnay and Shiraz samples are considered, only 7 of the 31 metal treatments significantly affected the evolution of H_2S in both wines, and they were copper, iron, zinc, aluminium, copper*iron, copper*manganese*aluminium, and copper*zinc*aluminium. Three of the metal treatments, zinc, manganese*zinc*aluminium, and copper*iron*manganese*zinc were associated with the largest increases in H_2S concentrations in the Chardonnay samples at Month 10. Using multivariate statistical analyses it could be concluded that the increases in H_2S concentrations are due to the significant effect of either zinc, aluminium, zinc*aluminium, or manganese*zinc*aluminium in these metal combinations.

There were more metals and metal combinations that were associated with significant effects in the Shiraz samples than in the Chardonnay samples, with four metal treatments associated with significant effects in the Chardonnay samples, compared to the nine metal treatments that were associated with significant effects on MeSH concentration in the Shiraz samples. This could be due to higher concentrations of polyphenols and anthocyanins present in the red wine samples which are

likely involved in the redox cycling of the metal ions. Three examples of metal treatments associated with some of the largest increases in MeSH concentration in the Shiraz samples are shown in Figure 5d. The increased MeSH concentration in samples with added copper*manganese*zinc, copper*zinc*aluminium, and copper were driven by the significant effect of copper, and not due to the other metals (Figure 5d).

Overall, fewer metals produced significant effects on DMS evolution, and the metal treatments were mostly associated with an overall decrease in DMS concentration. The only metal treatments associated with significant effects on DMS concentration in both Chardonnay and Shiraz samples were aluminium and zinc*aluminium. The decreasing effects of the metals could possibly be due to metals inhibiting the formation of DMS from its precursor molecules already present in the wine, or due to the catalytic degradation of DMS.

This study showed that metals can act singly or in combination to greatly influence evolution of undesirable 'reduced' aromas. Winemakers that wish to lower the risk of such characters should therefore take steps to minimise metal concentrations in wine. From these results, it is clear that the formation of LMWSCs from their precursors in wine is not only influenced by the presence of metals, but oxygen concentration in wine also significantly interacts with metals in the evolution of LMWSCs.

The impacts on LMWSC formation due to interactions between copper and pH

Many factors affect the formation of LMWSCs in wines post-bottling, including early oxygen treatment during fermentation (Bekker et al. 2016), the presence of the precursor compounds in wine, elevated residual copper concentrations post-bottling, and anaerobic storage conditions (Ugliano et al. 2011, Viviers et al. 2013). Wine pH also has the potential to influence the chemical reactions that are related to the formation or degradation and loss of flavour compounds. This can be through its effects on precursor compounds or through its effects on catalytic compounds that facilitate the release of LMWSCs from their precursor compounds or through loss mechanisms. Although the role of copper in the formation of H_2S and MeSH has recently been established (Ugliano et al. 2011, Viviers et al. 2013), the ability of other ubiquitous winemaking variables, including pH, to modulate the formation of LMWSCs had not yet been studied.

As part of this project, the effects of wine pH, and the effects of interaction between pH and copper, on the LMWSCs associated with 'reductive' aroma formation in wines post-bottling were assessed (Bekker et al. 2016d). A Chardonnay and a Shiraz wine were selected to determine whether wine pH had significant effects on the formation of 'reductive' aroma formation post-bottling. Each wine was divided into two portions. The pH of one portion of wine was not adjusted (Chardonnay pH 3.46, Shiraz pH 3.72) and the pH of the second portion of the wine was adjusted using tartaric acid to pH 3.0 for both the Chardonnay and the Shiraz wines. To investigate the interaction between residual copper and wine pH, all wines were also either treated with copper to give a final concentration of 0.5 mg/L residual copper or received no added copper (0 mg/L residual copper).

The LMWSC profiles of all wines were monitored of the course of six-months post-treatment using a gas-chromatograph coupled to a sulfur chemiluminescence detector as described by Siebert et al. 2010. All experiments were conducted in an oxygen-free environment and all wines were also stored in an oxygen-free environment (Bekker et al. 2016).

The effects of wine pH on H_2S and MeSH formation in Chardonnay and Shiraz wines were only observed in samples that were also treated with copper. In samples without added copper, pH had no effect on the amount of H_2S and MeSH produced in the Chardonnay or Shiraz wines post-bottling (Bekker et al. 2016a).

In samples with added copper, however, significantly less H_2S was produced in Chardonnay wines when the pH levels of the wines were adjusted to pH 3.00 relative to Chardonnay samples with added copper at the unadjusted pH level of pH 3.46 (Figure 6a). On average 51% less H_2S was

produced in Chardonnay wines with added copper at pH 3.00, which shows that wine pH significantly impacts H_2S formation caused by elevated residual copper concentrations. The effects of pH and copper interactions on H_2S formation in Shiraz wines were not as pronounced as in the Chardonnay wines, with significant decreasing effects of lower pH levels only measured directly after treatment and again after a month post-treatment.

Methanethiol was not as affected by lower pH conditions. Lower pH was only associated with significant effects on MeSH concentration at Day 0 in the Chardonnay samples. Similarly, decreasing the pH of copper-treated Shiraz samples produced significantly less MeSH after one month of storage, and this effect was also measured three months post-treatment (Figure 6d). It is not clear why the interaction between pH and copper significantly impacted MeSH formation only at certain stages of the experiment. It could be that the lower pH is affecting the reaction rate of the formation of MeSH. The differences may be a reflection of the time needed for the wines to reach the same end-point MeSH concentration.

Dimethyl sulfide was significantly affected by the pH of the wines, with lower concentrations of DMS measured in both Chardonnay and Shiraz samples throughout the course of the six-month experiment in wines with a lower pH (Figure 6e, 6f). Six months' post-treatment there was 27% less DMS measured in Shiraz wines with a pH of 3.00 compared to Shiraz wines with a pH of 3.72. The effect of pH on DMS formation in wines post-bottling is remarkable given that DMS is a stable molecule that remains unaffected by oxygen treatment during fermentation (Bekker et al. 2016) and is unaffected by metals such as copper, iron, or manganese (Viviers et al. 2013). The decreased DMS measured in this study is most likely due to the precursor compounds of DMS becoming less prone to release DMS at a lower pH level. It is known that one of the main precursors to DMS (Segurel et al. 2005), namely S-methyl methionine (SMM), is stable in acid conditions but it rapidly decomposes at pH greater than 7.00 (Cantoni 1960). The decrease in pH from 3.72 to 3.00 most likely prevented the formation of DMS from SMM by stabilising SMM.

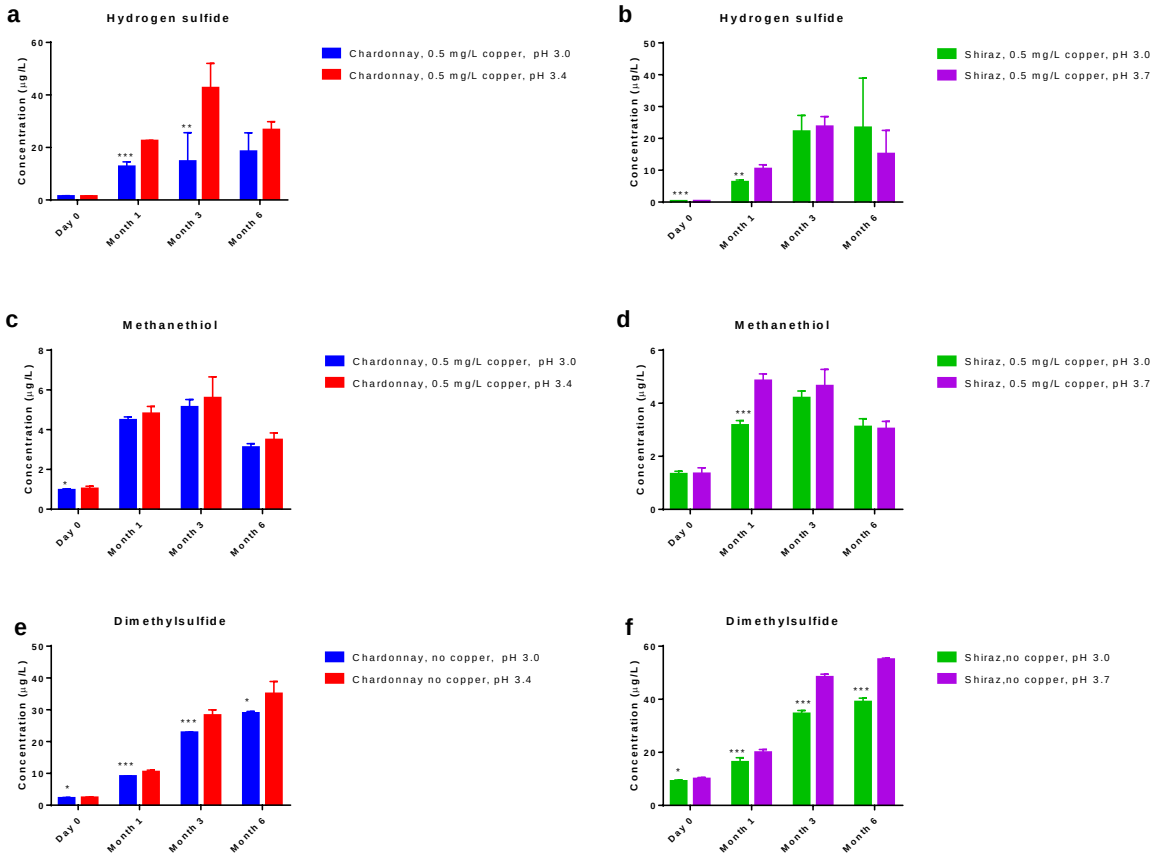


Figure 6. The effects of pH, and the interaction between pH and copper, on the formation of hydrogen sulfide [(a), (b)], methanethiol [(c), (d)], and dimethylsulfide [(e), (f)] in Chardonnay and Shiraz wines measured over the course of six months post-treatment. Significance of pH effect: P-value ≤ 0 (***), P-value ≤ 0.001 (**); P-value ≤ 0.01 (*).

This study demonstrated that the post-bottling formations of H_2S, MeSH, DMS, and CS_2 were significantly affected by both copper additions and wine pH. For some 'reductive' aroma compounds, the interaction between pH and copper treatment was an important factor in determining their final concentrations in the Chardonnay and Shiraz wines post-bottling. It is known that pH levels affect the rate of chemical reactions, and this will in turn impact the final concentration of LMWSCs present in wines. Winemakers have some flexibility in managing wine pH levels as well as the option to add copper to their wines through copper fining treatments. The current results have demonstrated that both wine pH and copper additions have significant impacts on H_2S, MeSH and DMS concentrations in wines post-bottling. In this study, less H_2S and MeSH were produced through copper-catalysed reactions in wines at a lower pH than in wines at a higher pH level (Bekker et al. 2016).

Use of chelating agents to isolate copper from the LMWSC formation process

While the risks of residual copper in packaged wine are becoming increasingly apparent, copper is still an important tool for winemakers dealing with volatile sulfur characters. For this reason it is important to identify ways to manage residual copper after it has been added to wine. One possibility is to bind copper to chelating compounds that completely remove it from the wine matrix or chemically isolate it so that it can no longer participate in the formation of reductive characters. To investigate this, a range of possible chelating agents were trialled in wine. A commercial polyphenol extract was found to suppress H_2S formation at the 12-month time point in comparison to controls (Figure 7). As seen in the trial on the timing of copper additions, this suppression did not

become evident until the 12-month time point, suggesting an interesting interaction of polyphenols, metals and other wine components occurring over time in bottle. Ethylenediaminetetraacetic acid (EDTA), a common chelation agent used in food production, also demonstrated significant effects both at equimolar concentrations (to copper and iron) and when present in excess. The results suggest that differing metal chelation environments can be present and that these can have a significant effect on the mechanism of H_2S generation.

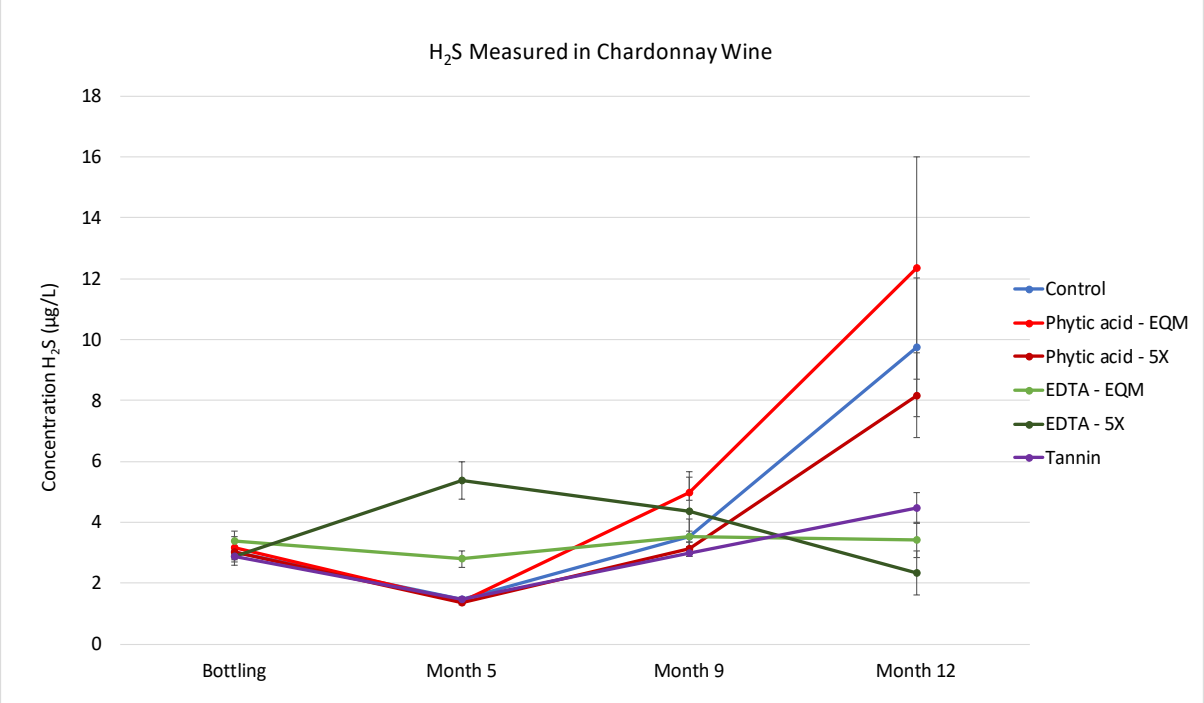


Figure 7. Effect of copper chelating agents on LMWSC accumulation over 12 months in a Chardonnay wine.

Managing copper addition(s)

Research was also performed on copper additions during ferments and the effects on the resulting wines (Reschke et al. 2015). The main purpose of this study was to determine what copper concentrations could be added at what stage of fermentation to minimise negative effects of sulfides on the resulting wines while limiting the residual copper in the final wine.

During laboratory-scale fermentation, copper was added at concentrations between 0 and 50 mg/L with the additions made at three time points: at the beginning of fermentation, at 0° Brix and after the fermentation was finished. The released hydrogen sulfide, residual copper concentrations, formation of volatile sulfur compounds, aroma profile and colour were determined where appropriate during, or at the end of fermentation. The results indicated that lower copper additions have similar impacts on wines as higher additions and that in terms of limiting residual copper concentrations it is more desirable to add copper at 0° Brix (during the final phase of active fermentation) rather than at the end (post-active ferment) which is a common industry practice. This ensures the yeast and solids are available to adsorb copper and limit the residual copper in the final wine. It should be noted that copper additions earlier in the fermentation process were also shown to pose greater disadvantages than the addition at 0° Brix. By adding copper at 0° Brix positive wine aromas can form in the log phase of culture growth and any reduced characters formed can be removed at this stage as well. Copper concentrations up to 5 mg/L can be added without significant

residual copper concentrations remaining in the wines and volatile sulfur containing compounds can be reduced by these levels of copper additions. Although the addition of copper has many advantages, the aromas of copper-treated wines can be affected negatively at the copper addition rates used in this study. All copper-treated wines reviewed in the informal sensory study showed a detectable aroma of 'nail polish remover'. Further study is needed to investigate the sources of the nail polish remover aroma.

Understanding the forms of copper in wine

It has been commonly thought that when sulfides in wine interact with copper, they simply precipitate out as copper sulfide and are removed from the wine through racking and filtration. Recent work by collaborators at Charles Sturt University (CSU) has shown, however, that this is not necessarily the case (Clark et al. 2015); in fact, copper sulfide remains in the wine. This finding, along with increased understanding of the risks of residual copper species in wine, has made it important to gain a more thorough understanding of the chemical nature and role of copper sulfides formed in wine.

Many wine compounds have the capacity to bind copper, including organic acids (such as tartaric acid), tannins, polyphenols, and LMWSCs. The binding of copper to these various compounds may affect the particle size of the copper-complexes, which in turn may affect the binding sites of copper that are available to catalyse the formation of LMWSCs. To investigate this, the impacts of different ratios of H_2S and copper in model wine were studied using nanoparticle tracking analysis (NTA). NTA affords the opportunity to monitor very small particles and their behaviours in liquids and was used to study the effects of variable pH concentrations in a model system containing copper, tartaric acid, and H_2S. (Bekker et al. 2016).

A decrease in the mean particle diameter was seen as the ratio of H_2S to copper increased. Furthermore, the copper-tartrate complex concentration increased with H_2S addition. At equimolar concentrations of H_2S and copper the particle size was smaller than in the other treatments, suggesting strong and uniform binding of H_2S and copper that prevented copper from further interacting with other compounds to produce larger particles. As the ratio of copper was increased in relation to H_2S, the particle size increased, suggesting that the unbound copper was available to interact with other compounds (in this case tartaric acid) to form larger particles. Further studies are needed to determine whether the smaller particle size is associated with lower catalytic ability, perhaps due to the binding sites of copper being occupied and prevented from further interaction with other wine compounds.

The impact of pH on complex formation was also investigated. Lower pH decreased copper-tartrate complex size and lower particle concentrations were measured when compared to copper-tartrate complexes produced at higher pH. The difference in particle size and concentration of copper-tartrate complexes suggests that the types of copper-tartrate complexes that are produced at varying pH levels may affect the binding sites of copper that are available to either catalyse the formation of LMWSCs such as H_2S, or quench the thiols produced to form copper sulfide complexes. As discussed previously, research in this project has also shown that wine pH, copper additions and the interaction between pH and copper significantly influence LMWSC formation in Chardonnay and Shiraz wines post-bottling.

A collaborative project led by CSU with the AWRI also investigated the speciation of the copper, by investigating a range of 52 commercially available wines. Using anodic stripping voltammetry Dr Andrew Clark and co-workers at CSU showed that only below very low levels (25 µg/L) of 'electrochemically labile' copper (able to react with H_2S) could free H_2S be liberated from the wine. While confirming the ability of copper to bind H_2S, the study also showed that in the vast majority of the wines tested, the copper was present in non-labile forms and was not available to inhibit the formation of free H_2S by reacting with it. This correlates with earlier findings that residual copper in

wine rarely serves to inhibit the formation of volatile sulfur compounds and in many cases appears to facilitate it.

To understand what the possible forms (chemical speciation) of the non-labile copper were, the collaboration between CSU and the AWRI focused on whether copper was bound to some of the major matrix compounds found in wine. The components of wine that can interact with Cu(II), Fe(II) and Fe(III) are numerous and vary widely in general type. They can consist of the wine macromolecules, including proteins, polysaccharides, tannins and their aggregate combinations, as well as monomeric species, including organic acids, phenolic compounds, and sulfur-containing compounds. Although many studies have been conducted on the complexation of metal ions to these components in non-wine-like conditions (higher pH, ethanol-free or without organic acids), the specific conditions of wine are likely to be critical in indicating the dominant binders of metals. This study was conducted to identify which wine components influence the measurement of copper and iron in their fractionated/electrochemical forms and to enable an improved understanding for the use of such measurements in following metal activity in wine.

The wine components provided by the AWRI that were assessed by CSU included those extracted from wine (red wine tannin, white wine protein, white wine polysaccharide, red wine polyphenol, white wine polyphenol that had been isolated and characterised at the AWRI), and commercially available monomeric compounds, including phenolic compounds and sulfur-containing compounds such as H_2S. The wine components to be assessed were added at typical wine-like concentrations: control (no further addition), white wine protein (50 mg/L), white wine polysaccharide (200 mg/L), white wine polyphenol (200 mg/L), red wine polyphenol (2000 mg/L), red wine tannin (1000 mg/L) and H_2S (two-fold mole excess over metals ions, 2:1). Further experimental details can be found in Kontoudakis et al. (2016).

It was evident that only the addition of H_2S to the model wine system afforded a significant change in the classification of copper by the electrochemical technique (by forming non-labile CuS). The formation of Cu(I) sulfide (Kreitman et al. 2016) in the model wine system is responsible for the non-labile copper, some of which would be removed as particulate Cu(I) sulfide by the filtration step prior to electrochemical analysis of the sample (Clark et al. 2015). Part of the non-labile copper is also suspended Cu(I) sulfide particles that are not detected by the electrochemical sensor (Clark et al. 2016). For all other model macromolecules tested with copper, the predominant form of copper was labile copper, identical to the classification of copper in the model wine without any binding component added, that is, with copper present as Cu(II) tartrate and likely to be available for electrochemical reactions that may lead to changes in LMWSCs.

The results demonstrated that it is likely that the majority of copper in wine is in the form of Cu(I) sulfide (Cu_2S), and the presence of the macromolecules and other matrix compounds tested are unlikely to change the electrochemical state of the copper, regardless of the wine being white or red.

The impact of the quantity and timing of copper and SO_2 additions when producing wine to ensure the optimum sensorial outcome and eliminate the development of negative LMWSCs both in tank and in bottle

This study explored the interaction of copper and SO_2 in the evolution of reduced aromas as the use of SO_2 is an essential part of winemaking. The dosage and timing of SO_2 addition are similarly key activities under winemaking control and may impact H_2S formation. Sulfur dioxide naturally occurs in wines at very low concentrations (10–30 mg/L, Jackson 2008); however, the main contributor to the final concentration in commercial wines is added SO_2 at concentrations in the range of 50–200 mg/L. Sulfur dioxide exists in free and bound forms, with the majority of free SO_2 present as bisulfite ions (HSO_3^-) at normal wine pH (all of the different species of sulfur dioxide in equilibrium found in wine, which includes the molecular sulfur dioxide, bisulfite, and sulfite, will be referred to generically as 'SO_2' throughout the text). Sulfur dioxide plays a critical role in the prevention of microbial spoilage, and is important as an antioxidant, through reaction with hydrogen peroxide (H_2O_2) generated from oxygen via a series of redox steps, as well as with quinones to regenerate polyphenols (Danilewicz, 2011). It is also known that SO_2 influences the loss of thiol compounds post-bottling (Waterhouse and Laurie 2006). It has also been proposed that SO_2 may act as a source of LMWSCs. Rankine suggested that the reduction of SO_2 may occur through the interaction of metal ions such as manganese and zinc with tartaric and malic acids during fermentation (Rankine 2004). No evidence for the post-bottling formation of H_2S via the metal-catalysed reduction of SO_4^{2-} or SO_3^{2-}during low oxygen conditions has yet been shown, even though this pathway has been proposed by Ribéreau-Gayon et al. (2006) and Lopez et al. (2007). Another possible pathway through which SO_2 can be involved in the modulation of 'reductive' LMWSCs post-bottling is through reactions with wine compounds, such as quinones. Nikolantonaki et al. (2012) described the reaction and kinetics between certain o-quinones and nucleophilic thiols and has shown that the addition of SO_2 directly influenced the rates and the yields of all of the o-quinone sulfur adducts.

The separate effects that copper and SO_2 additions may have on H_2S formation in wines post-bottling have been discussed above, however, copper and SO_2 are usually both present in wine and may have synergistic effects with wine matrix compounds. Given that the final concentrations of copper and SO_2 in finished wines, as well as timing of the addition of copper and SO_2, are under a winemaker's control it is important to understand the synergistic interactions between them and the effects that these compounds may have on H_2S formation. The synergistic effects of the combined treatment of copper and SO_2 on H_2S formation in a white wine (Verdelho) and a red wine (Shiraz) were investigated. Some of the factors modulating the formation of H_2S involving copper and SO_2 interactions during wine storage under low oxygen conditions were also studied.

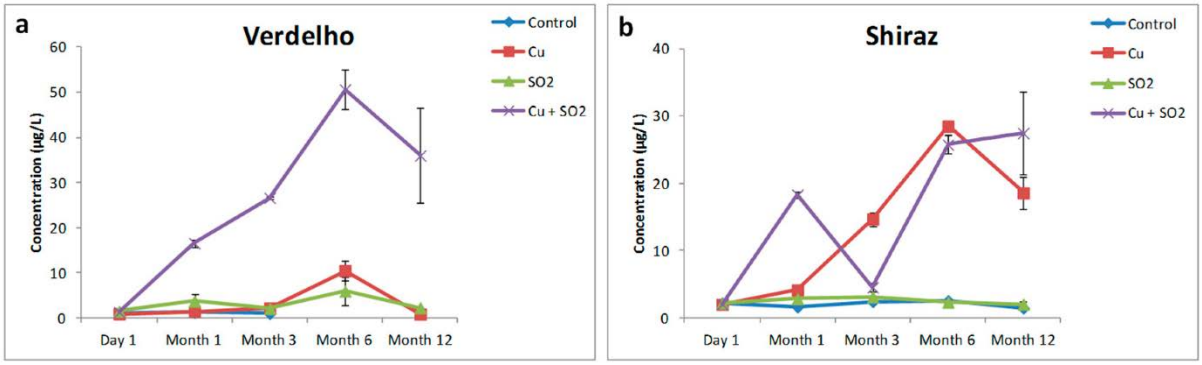
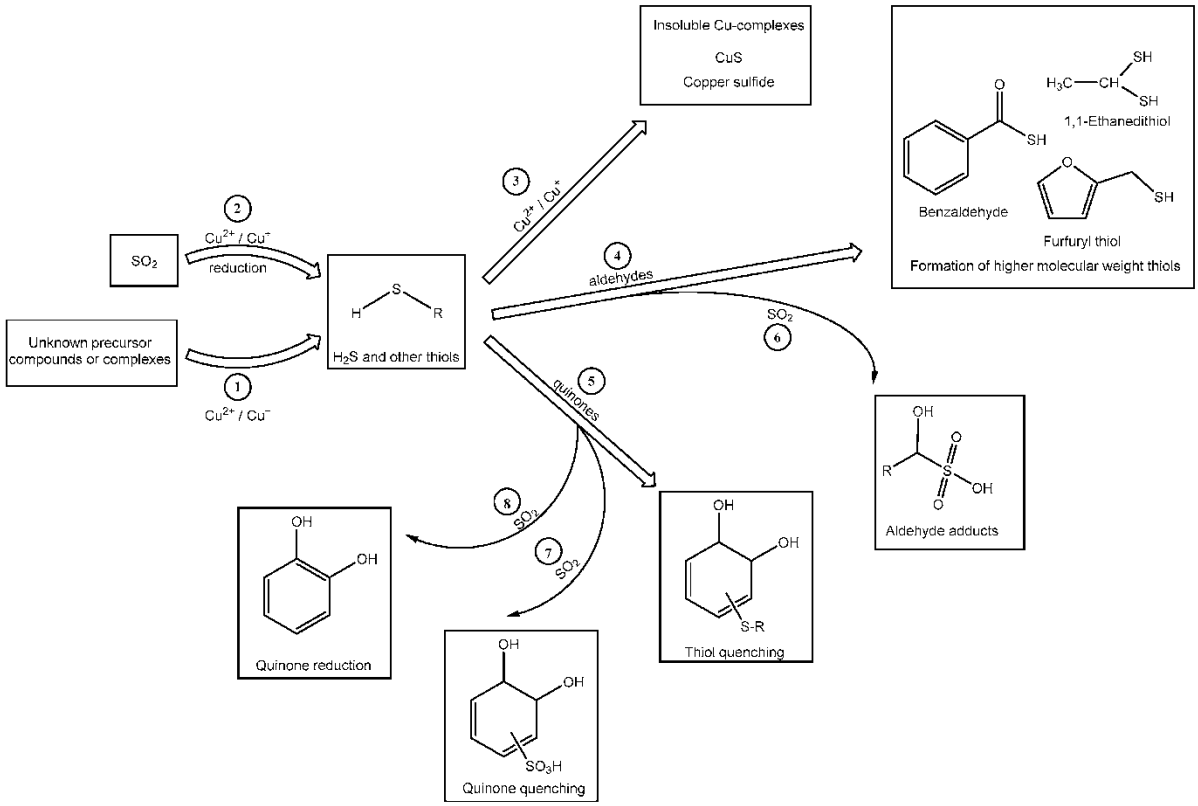


Figure 8. The effects of Cu^{2+}, SO_2, and Cu^{2+} + SO_2 on the formation of H_2S in (a) Verdelho and (b) Shiraz wine samples that were stored under nitrogen (N_2) and measured over the course of 12 months.

The combined copper and SO_2 treatment in the Verdelho wine samples resulted in significant and large increases in H_2S concentrations (Bekker et al. 2016b). For the Verdelho samples more H_2S was measured in samples treated with copper + SO_2 than by treating samples with copper alone (Figure 8a). Using multi-way ANOVA it was possible to separate the effects of copper treatment from the combined copper and SO_2 effects, with the increase in H_2S concentration significantly associated with copper + SO_2 treatment in the Verdelho samples ($p < 0.001$). The concentration of H_2S produced in Verdelho samples treated with copper + SO_2 was 36.01 (±18.1) µg/L compared to the 0.879 (±0.028) µg/L of H_2S measured in the control samples after 12 months of storage under low oxygen conditions, which is a significant increase in H_2S concentration.

Significantly increased H_2S concentrations were also measured in the Shiraz samples treated with copper + SO_2 (Figure 8b). By making use of multi-way ANOVA it was clear that these increases were only associated with a significant effect of copper ($p < 0.001$) and not associated with the combined copper and SO_2 treatments (Figure 8b). If the evolution of H_2S in Shiraz samples is considered over the course of the 12 months of storage in a low oxygen environment, the trends of H_2S formation for Cu^{2+}-treated samples and samples treated with both copper and SO_2 are remarkably different (Figure 8b). Shiraz samples treated with only copper displayed a steady increase in H_2S concentrations from Month 1 to Month 6, followed by a decrease to Month 12, whereas samples treated with copper and SO_2 displayed an initial increase from Day 0 to Month 1, followed by a significant decrease in H_2S concentrations at Month 3, which was then followed by increased H_2S concentrations from Month 6 through to Month 12. The exact mechanism for these different H_2S evolution trends are not clear; however, this demonstrates the complex underlying interactions of different wine compounds with one another and how the addition of SO_2 to a wine already containing copper could have significant implications for H_2S evolution. Previous studies have also shown the non-linear increase in thiols such as H_2S and MeSH evolution over time, which suggests that compounds such as H_2S and MeSH may be dynamically bound and released by other wine compounds after bottling and during storage.

Although the exact reaction pathway for the formation of H_2S associated with the combination of copper and SO_2 is not yet known, there are a few possible mechanisms (Scheme 1). When a higher ratio of free SO_2 to H_2S is available, it can form adducts with wine quinones or reduce them back to their corresponding phenols, and in doing so prevent the quenching of the newly formed thiols. Competing reactions between H_2S and SO_2 could also result in a smaller percentage of H_2S lost through, for example, reactions with acetaldehyde. This could indirectly lead to a relative increase in thiol concentration in the treatments with a high concentration of carbonyl compounds.



Scheme 1. Possible mechanisms for the formation of H_2S and other low molecular weight thiol compounds and the interaction between copper, SO_2, and copper + SO_2 with wine compounds such as aldehydes and o-quinones, during H_2S formation (from Bekker et al. 2016).

In summary, these investigations have shown that the presence of wine additives, such as copper and SO_2, play a fundamental role in the modulation of H_2S profiles in both red and white wine post-bottling.

Understanding differences among wine yeast strains in their ability to release volatile sulfur compounds (LMWSCs)

During fermentation, wine yeast not only produce ethanol and CO_2, but also a range of volatile compounds such as esters, higher alcohols or volatile sulfur compounds (LMWSCs). Different strains produce varying amounts of these flavour compounds, which means that wine yeast can be used as a tool by winemakers to modulate wine style. To assess this variability among different wine yeast strains, a series of triplicated small-scale fermentations were carried out in a synthetic grape juice (SGJ), and quantitative data on the production of LMWSCs was obtained for 100 yeast strains (Figure 9).

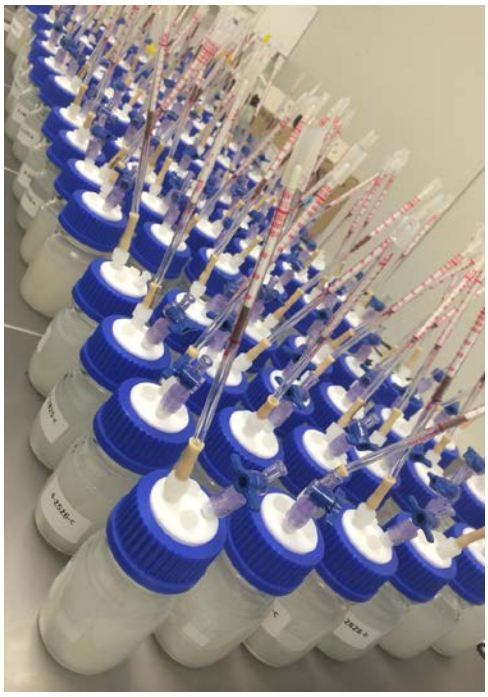


Figure 9. Picture showing the small-scale ferments (90 mL) in synthetic grape juice (SGJ).

SGJ was supplemented with grape-like concentrations of the cysteinylated and glutathionylated precursors of the volatile thiols 3-mercaptohexan-1-ol (3MH) and 4-mercapto-4-methylpentan-2-one (4MMP), which are responsible for the 'tropical fruit' characters of Sauvignon Blanc wines (Figure 10).

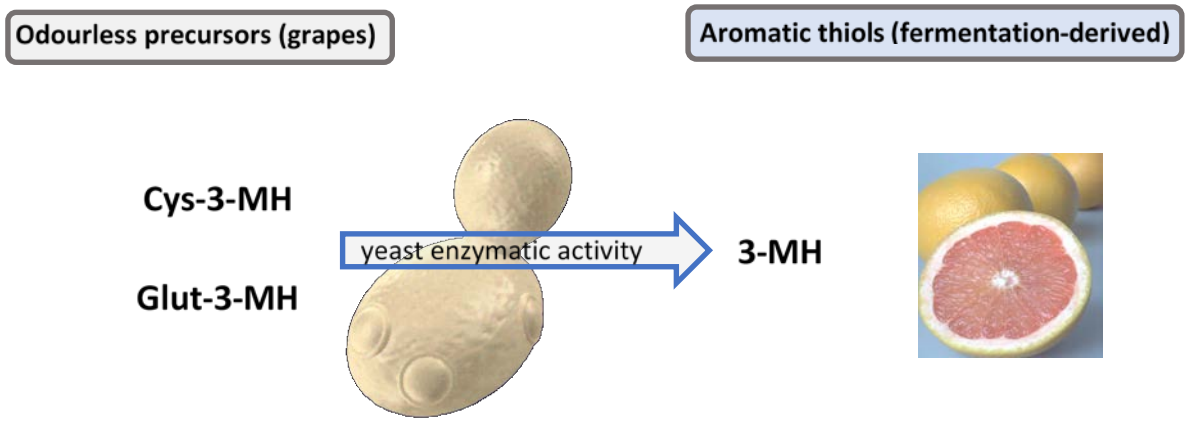


Figure 10. The odourless precursors of 3MH, cysteine-3-MH (cys-3MH) and glutathione-3-MH (glut-3-MH) are found in grapes. During alcoholic fermentation, the yeast converts glut-3MH in a series of enzymatic steps into cys-3MH. Then cys-3MH is cleaved by different yeast enzymes to release the thiol 3-MH, with aroma descriptors of 'grapefruit' and 'passionfruit'.

A large range of concentrations among the yeast was observed for the negative LMWSCs H_2S, methyl thioacetate (MeSAc), and ethyl thioacetate (EtSAc). In addition, a 20-fold and 35-fold difference in their ability to release the 'tropical' thiols 3-MH and 4-MMP, respectively, was observed among yeast strains. Interestingly, about 70% of the strains released low amounts of both 3-MH and 4-MMP; about 20% were classified as moderate releasers, and only 10% produced high amounts of these thiols. No trends were observed between commercial wine strains and natural isolates in their ability to release 4-MMP and 3-MH; however, strains recommended by yeast manufacturers for white winemaking tended to produce higher levels of both thiols than strains recommended for red wine. Finally, a strong positive correlation was observed in the formation of 3-MH and 4-MMP by the different wine yeast, suggesting that their formation might share common genetic determinants.

These studies were then reproduced in a red must, and analysis indicated a strong correlation between LMWSC results from the model study in SGJ and fermentations with real grape juice. Unfortunately, no data on 'tropical' thiol release was obtained on these red ferments.

To further explore whether the information obtained from fermenting SGJ could be used to predict the ability of a particular wine strain to release 'tropical' thiols in real grape juice, a subset of 26 of the 100 strains was used to ferment a small volume of a Chardonnay juice (200 mL). Again, a great variability in LMWSC and 'tropical' thiol formation was observed, and wine yeast strains were classified into different groups according to their sulfur production profile (Figure 11).

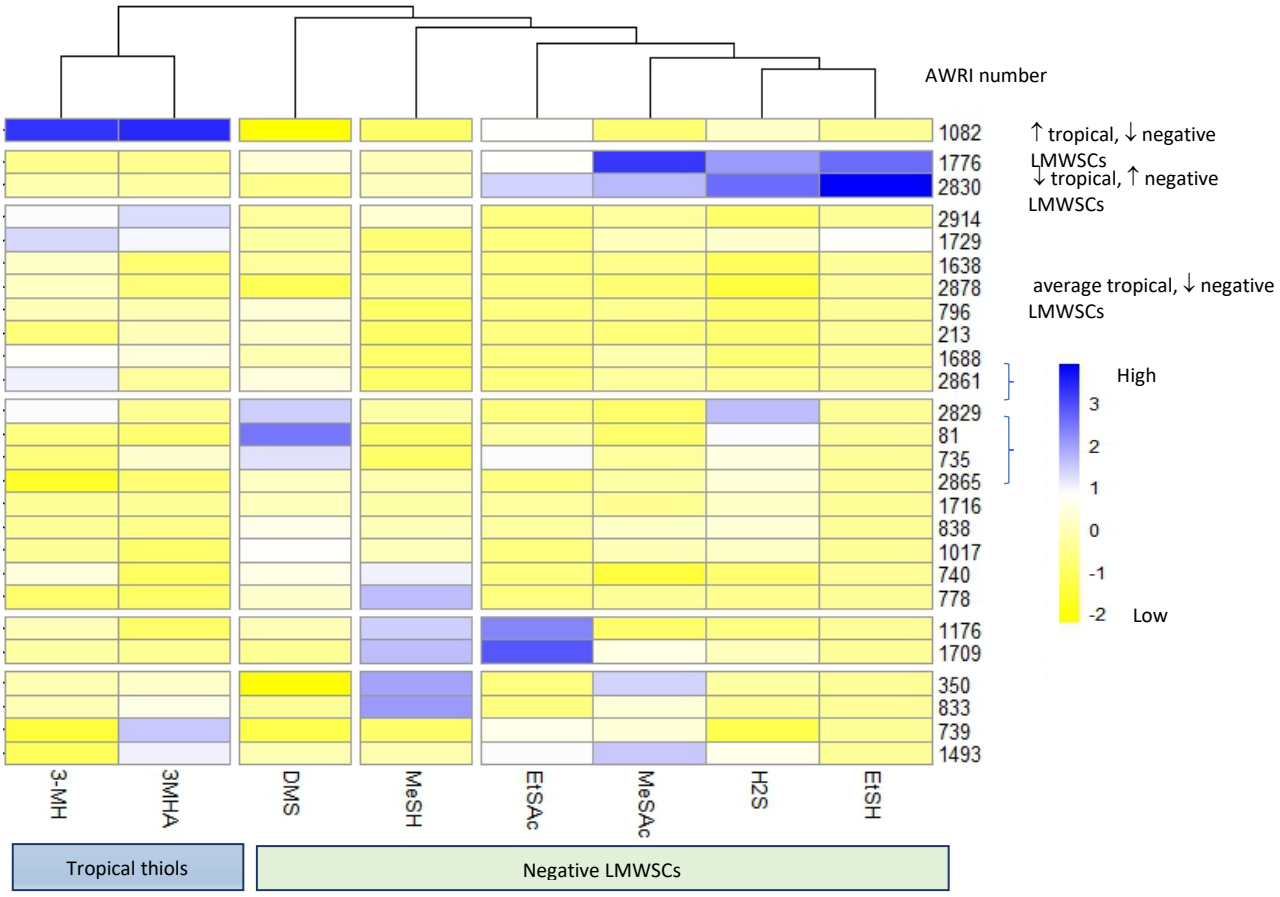


Figure 11. Heatmap showing the levels of different sulfur compounds ('tropical' thiols: 3-MH and 3-MHA, and negative LMWSCs: DMS, MeSH, EtSAc, MeSAc, H_2S and EtSH) after fermentation of a Chardonnay juice (200 mL) using 26 wine strains. Blue and yellow colours depict high and low levels of these compounds, respectively. The AWRI number identifier of each of the wine yeast strain is also shown.

A strong correlation was found between the concentration of 3-MH produced in the Chardonnay wine and those previously found in the SGJ (Figure 12). These results confirmed that model ferments using SGJ were good predictors of a strain's capacity to release 'tropical' thiols under winemaking conditions. A strong positive correlation was also observed between the levels of H_2S, ethanethiol (EtSH) and MeSAc in the Chardonnay wines, suggesting that the formation of these negative LMWSCs is to some extent dependant on yeast enzymatic activity.

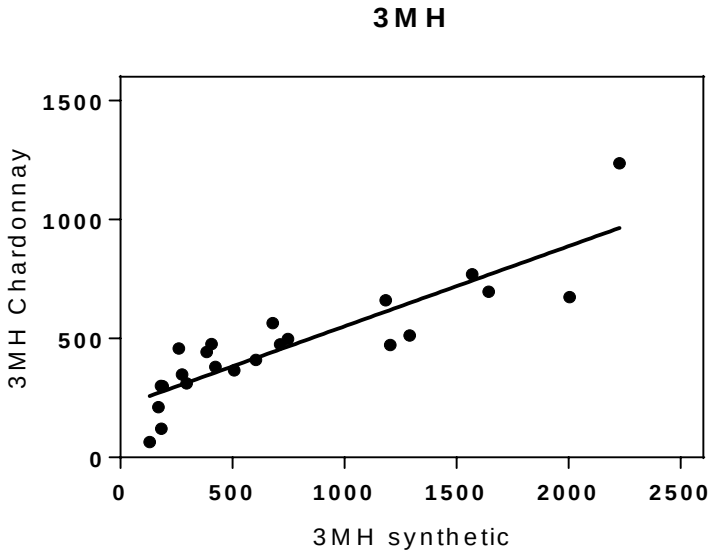


Figure 12. Relationship between 3-MH concentrations (ng/L) in fermentations of a synthetic grape juice and a Chardonnay juice with 26 different wine strains. The goodness of the fit between both data sets is expressed as $R^2 = 0.765$ ($P<0.0001$).

Based on these results, a high and a low-LMWSC producer were selected for a pilot-scale ferment in Grenache grapes (50kg) for the 2017 vintage. Preliminary chemical analysis of the wines confirmed the same trends observed in these small-scale ferments. These two strains could be used to investigate the genetic determinants involved in the formation of different LMWSCs by yeast.

Formation of 'tropical' thiols 3-MH and 4-MMP from grape odourless precursors: glutathionylated precursors of 3-MH and 4-MMP.

The 'tropical fruit' characters of Sauvignon Blanc wines are attributed to the presence of the aromatic thiols 3-MH, 3-MHA and 4-MMP. These thiols are not detectable in grape juice to any significant extent but are released by yeast during alcoholic fermentation. While the processes involved in the release of 3-MH and 4-MMP from their cysteinylated precursors have been studied extensively, degradation pathways for glutathione-*S*-conjugates (GSH-3-MH and GSH-4-MMP) have not. A candidate-gene approach was taken, focusing on genes known to play a role in glutathione and glutathione-*S*-conjugate turnover in *Saccharomyces cerevisiae*. Several of these genes were deleted in a laboratory strain background, and the ability of the single deletants to release 3-MH and 4-MMP was assessed in SGJ spiked with their respective glutathionylated precursors (Cordente et al. 2015). Results confirmed several yeast genes as playing a critical role in thiol release: Opt1p was found as the major transporter responsible for uptake of GSH-3-MH and GSH-4-MMP inside the yeast cell for their cleavage, and vacuolar Ecm38p as a key determinant of 3-MH release from GSH-3-MH. These results were in agreement with a previous genome-wide screening of the release of H_2S from the amino acid cysteine (Winter et al. 2014), in which the vacuolar function was found to be critical for H_2S formation.

Formation of 'tropical' thiols 3-MH and 4-MMP from grape odourless precursors: cysteinylated precursors of 3-MH and 4-MMP.

As described previously, fermentations conducted using 100 yeast strains in both a synthetic grape juice and a red must, as part of the AWRI's yeast research, were analysed for LMWSCs. The analytical results, combined with genomic data on these yeast strains, revealed that the potential of a particular strain to release the 'tropical' thiols 3-MH and 4-MMP from cysteine-bound precursors correlated with certain alleles (versions) of the gene *IRC7*. The majority (70%) of yeast strains analysed were classified as low thiol releasers, and it was established that the cause for this phenotype was the presence of inactivating mutations in the yeast *IRC7* gene. Therefore, DNA sequence of *IRC7* can be used as a molecular marker to determine the likely potential of any given strain to release volatile thiols. Identification of this marker opens the door for the breeding of wine strains with enhanced thiol-producing capabilities, enabling the release of untapped 'tropical' thiol aroma in different white wine varieties, such as Chardonnay.

Formation of H_2S and other negative LMWSCs from cysteine

To identify genes important to H_2S release from the amino acid cysteine, a series of strains were constructed expressing different candidate enzymes at high levels. Six of these yeast genetic determinants were found to regulate the formation of H_2S from cysteine during fermentation of a SGJ. In particular, overexpression of *CYS3* and *STR3* led to significant increases in the levels of H_2S, while overexpression of *CYS4* had the opposite effect. Finally, overexpression of *MET17* only led to an increase in the levels of MeSAc, suggesting that this gene might play an important role in the formation of this negative LMWSC. Interestingly, among all these genes, *IRC7* was found to play the most critical role in the formation of H_2S and other negative LMWSCs, as observed previously with the 'tropical' thiols 3-MH and 4-MMP.

To further study the role of *IRC7,* three versions of this gene with different LMWSC-releasing abilities were assessed in a SGJ at three different concentrations of cysteine. A strong positive correlation was observed between the amount of H_2S released during fermentation and the initial concentration of cysteine in the media, for each of the three *IRC7* alleles (Figure 13). In addition, the amount of H_2S released by yeast also strongly correlated with the levels of EtSH, EtSAc, MeSAc, and carbon disulfide (CS_2). These results confirm the important role that yeast (and in particular the *IRC7* gene) play in the formation of H_2S and other negative LMWSCs from organic sulfur compounds such as cysteine, and possibly glutathione (GSH).

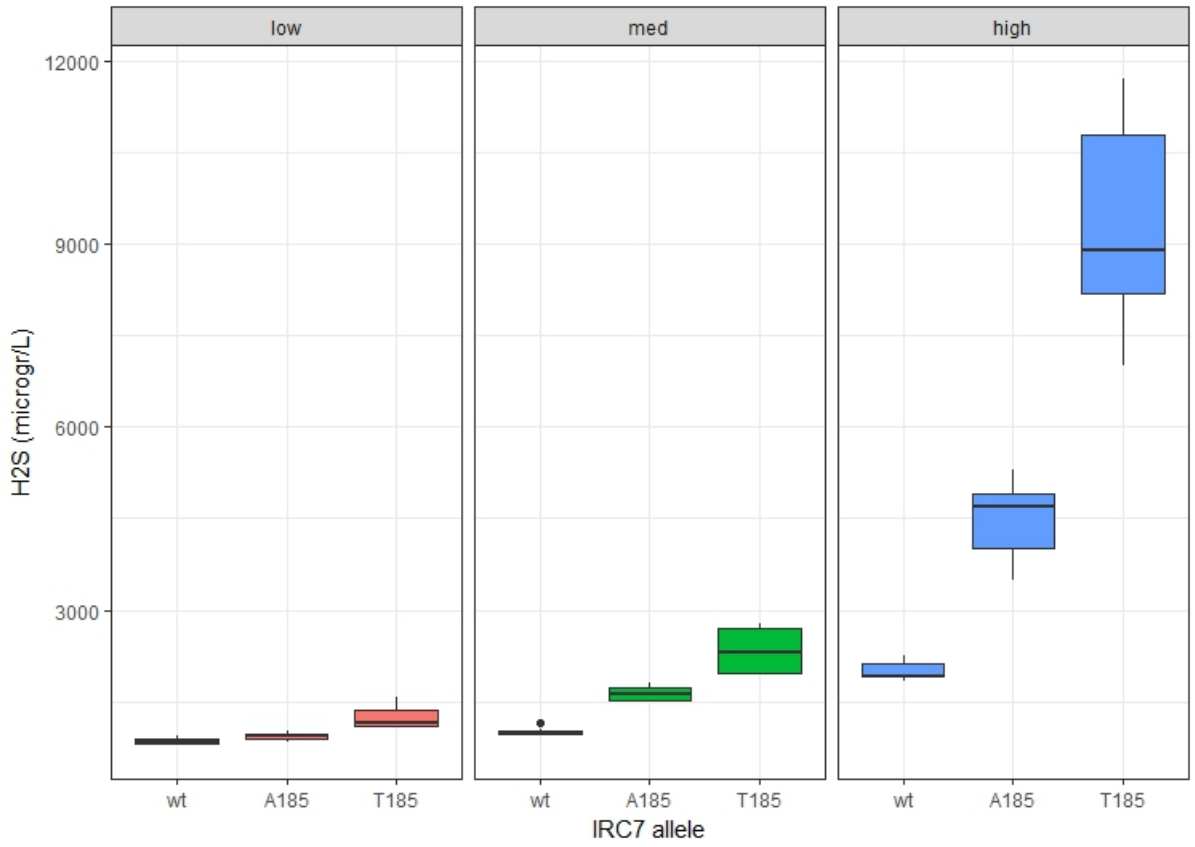


Figure 13. Production of H_2S during fermentation of a SGJ supplemented with different concentrations of cysteine (low, med and high). Three yeast strains expressing different versions (alleles) of the gene *IRC7* were used in this fermentation experiment (wt, A185, and T185)

Effect of copper on yeast performance and biochemistry

The effect of copper on yeast performance and biochemistry has been a focus of research in Project 3.2.2 – 'Fit for purpose yeast' and the final report for that project should be referred to for more details of this work.

Outcome and conclusion

The objectives of this project have been achieved to a high standard. New methods were developed to compliment the current technologies and expand the AWRI's ability to quantify a wider range of LMWSC and precursor compounds. Precursor compounds associated with increased LMWSC concentrations in wines post-bottling were thoroughly investigated and the risk that these compounds pose to 'reductive aroma' formation were determined. Factors that affect the formation and fate of LWMSCs, such as wine pH, residual metal concentrations and SO_2 treatment were also thoroughly investigated and the effects of these compounds on H_2S and MeSH formation in wine post-bottling were determined. Differences among wine yeast in their ability to release both positive and negative LMWSC were also determined.

Practical implications for the Australian grape and wine industry include:

- The impacts of residual copper, aluminium, iron, manganese, and zinc on 'reductive aroma' formation in wines post-bottling have clearly been defined. Winemakers can assess the risks and rewards of using a metal such as copper as a fining agent during wine production.
- Management strategies for using copper as a fining agent and removing it to reduce the risk of 'reductive aromas' have been proposed.
- Precursor compounds responsible for H_2S, MeSH, and EtSH formation in finished wines have been assessed and winemakers can measure the concentrations of these specific precursors in their wines to determine their risk for 'reductive aroma' formation in wines post-bottling.
- Several yeast markers have been identified that modulate the formation of important LMWSCs, such as the 'tropical' thiols 3-MH and 4-MMP and H2S, from their precursors. This information will help winemakers to make an informed yeast strain choice to suit their wine style, and potentially reduce the formation of negative LMWSCs in a winery environment.

Recommendations

Based on results from this project, the sulfur-containing amino acids Cys, Met and GSH are not be considered to pose a direct or significant risk as precursors to H_2S or MeSH formation through direct desulfurisation in wines post-bottling. It should be noted that their interactions as antioxidants or in other roles may have an effect on LMWSCs. However, the concentration of the sulfur-containing amino acid SMM is related to DMS formation, as previously shown by Segurel et al. (2005), and researchers and producers interested in understanding the sensory characters associated with DMS could consider monitoring SMM.

The presence of thioacetates and disulfides does appear to represent a direct risk as precursors to their associated thiols such as MeSH and EtSH. As such, it is recommended that thioacetates and disulfides are monitored to assess potential risk of post-bottling LMWSC release. In addition, it is recommended that further research into the role of yeast in modulating thioacetate production is performed, as well as additional research to better characterise disulfides and polysulfanes as potentially significant precursors.

This project has shown that metals can act singly or in combination to greatly influence evolution of undesirable 'reduced' aromas. Winemakers that wish to lower the risk of such characters should therefore take steps to minimise metal concentrations in wine. Oxygen concentration in wine also significantly interacts with metals in the evolution of LMWSCs. As such, it is recommended that winemakers ensure they know the 'total packaged oxygen' (TPO) concentration of their products and its likely progression post-bottling. 'Macro-oxygenation' during fermentation can also be considered as a tool for decreasing the residual metal concentrations in wines. An awareness of the impact of additives (for example bentonite) on metal concentrations in wine should also be developed by winemakers.

Copper has been noted for its role in accelerating post-bottling formation of LMWSCs. As such, winemakers should ensure they understand the risk profile of copper addition processes and should be aware of the timing options for copper additions. This research indicates that the ideal time for such additions is late in fermentation in the presence of fermentation solids. The risk from the presence of copper in juice (potentially from vineyard sprays etc.) on certain copper-sensitive yeast strains should also be considered prior to fermentation. The differential behaviour of LMWSC evolution in reds compared to whites in relation to copper should be noted. The predominant form of copper remaining in wine is usually copper sulfide and research into the mechanisms of copper sulfide reactions during wine aging is recommended.

It is recommended that winemakers consider the effects of pH and SO_2 timing in relation to their modulation of LMWSC development as it relates to copper and precursors such as thioacetates.

Several markers have been identified that can be used to predict a yeast's potential to release 'tropical' volatile thiols and H_2S from their precursors. Little is known about the role of wine yeast in the production of other important LMWSCs such as MeSH or thioacetates. Therefore, it is recommended that research be directed toward the characterisation of yeast markers responsible for the metabolism of sulfur compounds and formation of relevant precursors and negative volatile compounds. Identification of these markers will help winemakers to choose the best strain to modulate their wine style, and will open the door for the breeding of novel wine strains with reduced production of negative LMWSCs.

References cited

Clark, A.C., Kontoudakis, N., Barril, C., Schmidtke, L., Scollary, G.R. 2016. Measurement of labile copper in wine by medium exchange stripping potentiometry utilising screen printed carbon electrodes. *Talanta*. 154: 431-437.

Clark, A. C., Grant-Preece, P., Cleghorn, N., Scollary, G. R. 2015. Copper(II) addition to white wines containing hydrogen sulfide: residual copper concentration and activity. *Aust. J. Grape Wine R*es. 21(1): 30-39.

Bekker, M.Z., Mierczynska-Vasilev A., Smith P.A., Wilkes, E.N. 2016a. The effects of pH and copper on the formation of volatile sulfur compounds in Chardonnay and Shiraz wines post-bottling. *Food Chem*. 207: 148-56.

Bekker, M.Z., Smith, M.E., Smith, P.A., Wilkes, E.N. 2016b. Formation of hydrogen sulfide in wine: Interactions between copper and sulfur dioxide. *Molecules* 21(9): 1214.

Bekker, M. Z., Day, M. P., Holt, H. E., Wilkes, E., Smith, P. A. 2016c. Effects of oxygen exposure during fermentation on volatile sulfur compounds in Shiraz wine and a comparison of strategies for remediation of reductive character. *Aust. J. Grape Wine R*es. 22(1): 24-35.

Bekker, M.Z., Smith, P.A., Wilkes, E.N., Johnson, D. 2016d Wine pH, copper and 'reductive' aromas in wines. *Wine Vitic. J.* 31(6): 36–38.

Bekker, M. Z., Wilkes, E.N., Smith, P. A. 2017. Evaluation of putative precursors of key 'reductive' compounds in wines post-bottling. *Food Chem.* (accepted).

Cantoni, G. L. 1960. Onium Compounds and Their Biological Significance. Florkin, M., Mason, H.S. (eds.) *Comparative Biochemistry. A Comprehensive Treatise* New York: Academic Press Inc.: 215.

Cordente, A.G., Capone, D.L., Curtin, C.D. 2015 Unravelling glutathione conjugate catabolism in *Saccharomyces cerevisiae*: the role of glutathione/dipeptide transporters and vacuolar function in the release of volatile sulphur compounds 3-mercaptohexan-1-ol and 4-mercapto-4-methylpentan-2-one. *Appl. Microbiol. Biotechnol*. 99: 9709-9722.

Cordente, T., Schmidt, S. Curtin. C. 2017. Understanding differences among wine yeast strains in their ability to release 'tropical' thiols. *AWRI Technical Rev.* (228): 6-10.

Danilewicz, J. C. 2011. Mechanism of Autoxidation of Polyphenols and Participation of Sulfite in Wine: Key Role of Iron. *Am. J. Enol. Vitic.* 62(3): 319-328.

Kreitman, G.Y., Danilewicz, J.C., Jeffrey, D.W., Elias, R.J. 2016. Reaction mechanisms of metals with hydrogen sulfide and thiols in model wine. Part 1: Copper-catalysed oxidation*. J. Agric. Food Chem.* 64: 4095-4104.

Jackson, R. S. 2008. *Wine Science Principles and Applications* (3rd ed.): Elsevier Inc.

Kontoudakis, N., Smith, M., Guo, A., Smith, P.A., Scollary, G.R., Wilkes, E.N., Clark, A. 2016. The impact of wine components on fractionation of Cu and Fe in model wine systems: Macromolecules, phenolic and sulfur compounds. *Food Res. Int.* in press.

Lopez, R., Lapena, A. C., Cacho, J., Ferreira, V. 2007. Quantitative determination of wine highly volatile sulfur compounds by using automated headspace solid-phase microextraction and gas chromatography-pulsed flame photometric detection. Critical study and optimization of a new procedure. *J. Chromatogr. A.* 1143(1-2): 8-15.

Nikolantonaki, M., Jourdes, M., Shinoda, K., Teissedre, P. L., Quideau, S., Darriet, P. 2012. Identification of adducts between an odoriferous volatile thiol and oxidized grape phenolic compounds: kinetic study of adduct formation under chemical and enzymatic oxidation conditions. *J. Agric. Food Chem.* 60(10): 2647-2656.

Ovalles, J.F., Brunetto, M d R., Gallignani, M. 2005. A new method for the analysis of amikacin using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatization and high-performance liquid chromatography with UV-detection. *J. Pharma Biomed. Anal.* 39: 294-298.

Rankine, B. C. 2004. *Making good wine*. Sydney, Australia: Pan Macmillan Australia Pty Ltd.

Reschke, S., Tran, T., Bekker, M., Wilkes, E., Johnson, D. 2015. Using copper more effectively in winemaking. *Wine Vitic. J.* 30(5): 35-37.

Ribereau-Gayon, P., Glories, Y., Maujean, A., Dubourdieu, D. 2006. *Handbook of Enology Volume 2. The Chemistry of Wine Stabilization and Treatments* (2nd ed.). Chichester, England: John Wiley & Sons Ltd.

Segurel, M.A., Razungles, A.J., Riou, C., Trigueiro, M.G.L., Baumes, R.L. 2005. Ability of possible DMS precursors to release DMS during wine aging and in the conditions of heat-alkaline treatment. *J. Agric. Food Chem.* 53: 2637−2645.

Seiwert, B. Karst, U. 2007. Simultaneous LC/MS/MS determination of thiols and disulfides in urine samples based on differential labeling with ferrocene-based maleimides. *Anal. Chem.* 79: 7131-7138.

Siebert, T. E., Solomon, M. R., Pollnitz, A. P., Jeffery, D. W. 2010. Selective determination of volatile sulfur compounds in wine by gas chromatography with sulfur chemiluminescence detection. *J. Agric. Food Chem.* 58(17): 9454–9462.

Smith, M.E., Bekker, M.Z., Smith, P.A., Wilkes, E.N. 2015. Sources of volatile sulfur compounds in wine. *Aust. J. Grape Wine Res.* 21: 705–712.

Thiel, G., Geisler, G., Blechschmidt, I., Danzer, K. 2004. Determination of trace elements in wines and classification according to their provenance. *Anal. Bioanal. Chem.* 378: 1630-1636.

Ugliano, M., Kwasniewski, M. T., Travis, B., Francis, I. L., Waters, E. J., Herderich, M., Pretorius, I. S. (2009). Post-bottling management of oxygen to reduce off-flavour formation and optimise wine style. Wine Industry Journal, 24(5), 24-28.

Ugliano, M., Kwiatkowski, M., Vidal, S., Capone, D., Siebert, T., Dieval, J.-B., Waters, E. J. 2011. Evolution of 3-mercaptohexanol, hydrogen sulfide, and methyl mercaptan during bottle storage of Sauvignon blanc wines. Effect of glutathione, copper, oxygen exposure, and closure-derived oxygen. *J. Agric. Food Chem.* 59(6): 2564–2572.

Viviers, M.Z., Smith, M., Wilkes, E., Smith, P.A. 2013. Effects of Five Metals on the Evolution of Hydrogen Sulfide, Methanethiol and Dimethyl Sulfide during Anaerobic Storage of Chardonnay and Shiraz Wine. *J. Agric. Food Chem.* 61: 12385-12396.

Waterhouse, A. L., Laurie, V. F. 2006. Oxidation of wine phenolics: a critical evaluation and hypotheses. *Am. J. Enol. Vitic.* 53(3), 306-313.

Winter, G., Cordente, A.G., Curtin, C. 2014. Formation of hydrogen sulphide from cysteine in *Saccharomyces cerevisiae* BY4742: Genome wide screen reveals a central role of the vacuole. *PLoS ONE* 9: e113869.