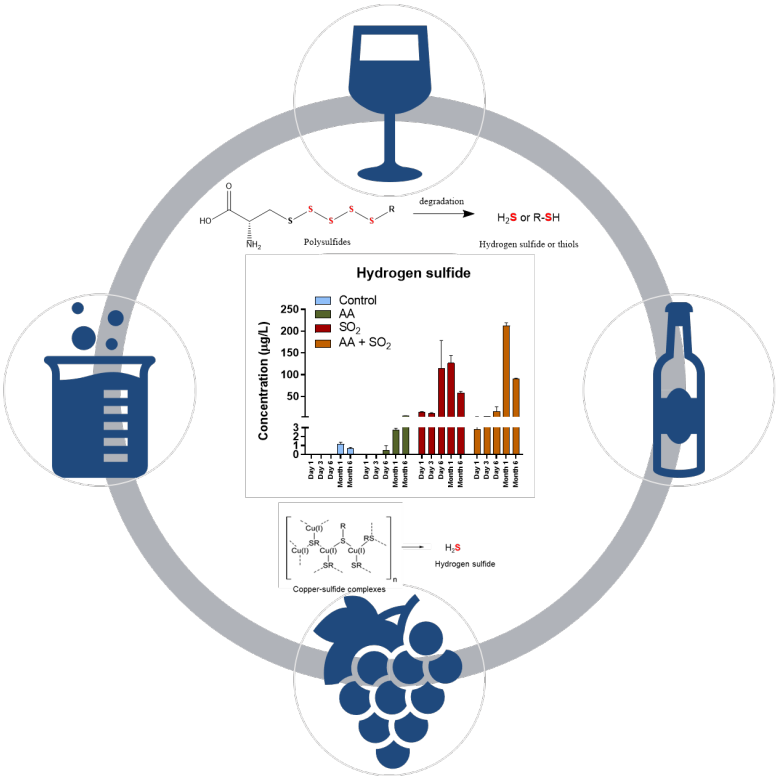
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Formation and fate of sulfur compounds associated with negative attributes in wines



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1. Abstract

Volatile sulfur compounds (VSCs) are important contributors to wine style, as they impart both varietal aromas and aromas associated with premium character. They are also associated with 'reductive' aromas, which account for approximately 30% of all wine faults and have significant economic implications. Many aspects of the formation and fate of VSCs were investigated in this project, including chemical and biochemical formation pathways, new analytical methods and the development of novel smart surfaces. Each aspect contributed to improved understanding of how VSCs are produced, how to modulate their formation through winemaking practices, and ultimately, how to remediate unwanted VSCs.

2. Executive summary

Volatile sulfur compounds are a group of compounds that contribute significantly to wine aroma. These compounds are associated with important varietal character and stylistic expression through their contribution to 'passionfruit', 'tropical', 'blackcurrant' and 'struck flint' aromas. The latter is associated with premium aroma character present in high-quality white and sparkling wines. VSCs are also associated with negative 'reductive' aromas, such as 'rotten egg', 'rubber', 'sewage' and 'canned corn'. The management of 'reductive' off-aromas is one of the main concerns for the wine sector, as these aroma faults make up to one-third of all faults in wine and result in economic losses (Goode et al. 2008).

This project aimed to develop an in-depth understanding of the role of compounds suspected to be the main precursors of sensorially important VSCs, with a focus on negative sensory characters deriving from hydrogen sulfide (H_2S), methanethiol (MeSH), methylthioacetate (MeSAc), and the generally positive 'tropical' sensory characteristics of 3-sulfanylhexan-1-ol (3-SH or 3-MH) and 3-sulfanylhexyl acetate (3-SHA or 3-MHA). The project was also focused addressing the knowledge gap surrounding phenylmethanethiol (PMT), also known as benzyl mercaptan, a compound that imparts 'struck flint' aroma, which is associated with premium sensory character in white and sparkling wines. The project sought to understand the metabolic and chemical pathways that lead to the formation of these sulfur 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. This knowledge can be implemented to provide practical advice to the wine sector for the development of practical strategies to control the expression of positive and negative VSCs in wine.

To be able to study these highly volatile and reactive sulfur compounds and evaluate their speciation precursors and other latent forms of VSCs, six methods were developed that allowed for the quantification of ^{34}S-labelled and ^{32}S-non labelled VSCs, their precursors and breakdown products. These methods were critical in supporting experiments elucidating the biochemical and chemical formation pathways of VSCs, as well as determining the role of copper and VSC speciation in the perception of 'reductive' aromas.

A significant finding of this project was establishing the importance of polysulfides as latent sources of 'reductive' aromas in wine post-bottling. The project demonstrated their stability and susceptibility to decomposition and liberation of H_2S when exposed to sulfur dioxide or ascorbic acid, two reducing agents that are commonly used in the winemaking process. This work addressed the knowledge gap of identifying latent sources of VSCs that are responsible for the post-bottling formation of VSCs. Follow-up studies investigated conditions that would promote the formation of polysulfides or promote the formation of irreversible polyphenolic adducts with VSCs. These experiments aimed to elucidate the mechanisms that drive the success of aerative remediation techniques, such as splashy racking and macro-oxygenation. These techniques have previously been considered to pose a risk of producing latent sources of VSCs, such as disulfide or polysulfide species. Experiments in this project showed that stable oxidation reaction products with polyphenolic compounds were preferentially produced after macro-oxygenation and that 'reductive' aromas were permanently removed from the experimental

wines. This work identified the mechanism that drives the long-term effectiveness of macro-oxygenation in removing unwanted VSCs that had been observed in previous AWRI studies.

This project also identified genetic pathways that favour the formation of VSCs. Genes that play a pivotal role in H_2S, 3-SH and 4-methyl-4-sulfanylpentan-2-one (4-MSP or 4-MMP) expression, as well as pathways that modulate MeSH, MeSAc and methional formation, were identified. The expression of tropical aroma in red wine was also investigated, and strains were identified that were high producers of the desirable tropical sulfhydryl compound 3-SH, as well as low producers of the undesirable VSCs, MeSH, MeSAc and methional. This work provided evidence for yeast strain selection to modulate the formation of VSCs and provided information to winemakers to aid their selection of yeast strains to control the expression of positive or negative flavour profiles of their wine.

Commercial yeast manufacturers offer a wide range of yeast strains, but little is known about their ability to modulate the formation of PMT. This compound is associated with 'struck flint' character, which is a sought-after aroma character for certain white wine styles. This project addressed the large knowledge gap regarding yeast selection and winemaking conditions that promote the formation of PMT, as well as factors that affect the preservation of PMT once it has been produced. This work identified yeast strains that are high PMT producers, identified the dependence of PMT formation on the presence and timing of H_2S production, the important role of benzaldehyde as a precursor to PMT, and demonstrated the critical role of nitrogen levels in the fermentation media on PMT formation by yeast. The studies also demonstrated that oxygen and elevated concentrations of copper and iron negatively affected PMT formation by decreasing concentrations of its precursor, H_2S. Factors that influence the preservation of PMT, such as residual metal concentrations and elevated SO_2 levels, were also identified.

To understand the prevalence of PMT in Australian wine and establish consumer preference for wines that contain 'struck flint' aroma, a targeted survey of PMT was conducted in a range of commercially available white wines. This enabled the natural variability of PMT in wine to be determined, as well as the relationship between increasing concentrations of PMT and the transition from 'flint' aroma to 'sulfurous'/'plastic' aroma at higher concentrations. An important finding of this trial was that both PMT and 2-furanmethanethiol (2-FMT) were associated with 'struck flint' aroma. A follow-up odorant addition study showed that 2-FMT exerted a 20 times larger effect on 'struck flint' aroma than PMT in Chardonnay wine.

Winemakers use various remediation strategies to manage 'reductive' aroma formation; however, the relative effectiveness of these techniques has not previously been evaluated. An experiment was designed to evaluate the effectiveness of five commonly used 'reductive' aroma remediation strategies to provide the wine sector with clear guidelines and practical advice on which remediation strategy would be best suited for their requirements. This work demonstrated that macro-oxygenation during fermentation was the most effective strategy over a period of 12 months for ameliorating 'reductive' character when compared to diammonium phosphate (DAP) addition, copper fining and lees addition. Macro-oxygenation also produced wines with increased 'red fruit' aroma and flavour, compared to the control. The use of tannin products in removing the appearance of 'reductive' faults was also evaluated, following anecdotal evidence that oenological tannin products can 'freshen' up 'reductive' wine. Experiments were performed to explore the effectiveness of this treatment and the mechanism by which it removes VSCs, with results showing that H_2S, MeSH, and ethanethiol (EtSH) were effectively removed from the wine through the formation of oxidation reaction products with tannins.

A novel smart technology was developed which was successful in removing up to 45% of free H_2S. The smart surface was also more effective in removing free MeSH compared to copper fining. This technology has the potential to overcome the limitations of copper addition currently used to remove unpleasant 'reductive' aromas.

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External collaborators and contributions

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4. Background

The occurrence of VSCs is 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 the availability and speciation of transition metal ions such as copper. As a defence against the formation of undesirable VSCs, especially H_2S, winemakers typically rely on monitoring must nitrogen, additions of DAP and copper, and choice of closure and its oxygen transfer rate. However, despite these commonly used management practices, post-bottling release of VSCs and sulfur-related off-flavours can still be observed in some wines.

In previous AWRI research, a number of obvious sulfur-containing precursors (thioacetates, *S*-methyl methionine, cysteine, dimethyl sulfoxide, glutathione (GSH), methionine, dimethyldisulfide (DMDS) and methionol) were assessed for their role in releasing VSCs and results indicated that many of these do not play a significant role in post-bottling VSC release. This suggested that other precursors are present which are yet to be identified. Sufficient observational experiments have been established across a range of wines to show that the accumulation patterns of VSCs are relatively consistent post-bottling, but that they differ significantly between red and white wine. It remains to be established whether the accumulation patterns observed are driven only by release from precursors or, more likely, a combination of release and subsequent loss of VSCs by incorporation into other chemical forms (e.g. binding into quinones, or the formation of compounds such as disulfides or polysulfides). In addition to the *de novo* formation of VSCs, their accumulation might also reflect their stability and reactions causing their 'loss' in wine. Thus, there remains a knowledge gap relating to novel precursors and their degradation mechanisms, addressing of which was an aim of this project.

This project also aimed to characterise the role of yeast in forming precursors for VSCs and the biochemical pathways that affect VSC formation. The yeast biochemical pathways associated with release of H_2S from cysteine have been established. However, recent work indicated that sulfate, for example, is a key sulfur source incorporated into EtSH, ethylthioacetate (EtSAc) and diethyl disulfide, but, interestingly, not into MeSH, MeSAc or dimethylsulfide (DMS). Research by international teams has also indicated that some vineyard interventions such as foliar spraying have an impact on VSC formation, as does the addition of compounds like GSH to must. Hence different classes of VSCs are likely being derived from different pathways at different points of production, and therefore understanding VSC accumulation from fermentation through to bottle-aged wine requires a multidisciplinary approach.

The overall objectives for this project were to:

- Develop improved methods of analysis for known and new VSCs, their precursors and breakdown products.
- Develop an in-depth understanding of the main precursors to sensorially important VSCs, with a focus on H_2S, MeSH, thioacetates, disulfides, polysulfides and PMT, and elucidate their release mechanisms.
- Develop an in-depth understanding of the role of microbiological factors (biochemical pathways, microbial ecology, nutrition etc.) that lead to the formation of VSCs and loss after formation.
- Develop practical strategies to decrease the occurrence or impact of negatively perceived VSCs through developing in-depth understanding of the chemical, biochemical and environmental factors that influence the occurrence of VSCs.
- Develop practical strategies for the removal of negatively perceived VSCs.
- Develop practical advice and extension materials for winemakers.

5. Project aims

Compounds such as H_2S, MeSH, EtSH, thioacetates, and DMS are responsible for a range of negative sensory attributes in wine, whereas PMT and the tropical sulfhydryls, 3-SH, 3-SHA, and 4-MSP, are responsible for a range of positive sensory attributes in wine. Their control in a winery environment and post-bottling is important for maintaining and increasing the quality and value of wine. The aim of this project was to generate new knowledge that will inform winemakers' decisions on how and when to control, and to decrease, the concentrations of VSCs in their wines. Project deliverables and associated activities are detailed in Tables 1 to 5 below.

Table 1. 2017-18 Deliverables and activities

Deliverables	Activities	Target date
Improved methods of analysis for known and new VSCs, and their precursors and breakdown products (2018).	• Finalise method development and optimisation of the LCMS method for derivatisation of free sulfhydryls and sulfhydryls for disulfide and polysulfide forms. • Initiate method development for a GCMS-based method for measurement of VSCs.	• December 2017 • June 2018
Knowledge of precursors to VSCs and their release mechanisms (2019).	• Finalise and publish existing 'VSC precursors' research characterising the roles of known sulfur compounds (thioacetates, disulfides etc.) as sources of post-bottling release of VSCs. • At appropriate timepoints continue characterisation of chemical and sensory effects of vintage 2017 winemaking trial which focussed on practical remediation strategies for during-ferment VSC production. • Initiate discovery and characterisation of polysulfides in wines, toward understanding their role as sources of post-bottling release of VSCs. • Initiate synthesis or isolation of individual or mixtures of polysulfides. • Design and execute a vintage 2018 winemaking trial to assess factors influencing the formation of putative precursors to VSC formation post-bottling (e.g. thioacetates, polysulfides).	• December 2017 • June 2018 • June 2018 • June 2018 • June 2018
Improved understanding of the role of microbiological factors (biochemical pathways, microbial ecology, nutrition etc.) in VSC formation (2020).	• Assess formation of MeSH using CS-lyase gene-modified yeast (e.g. STR3 or IRC7 gene-modified), using methionine as a precursor. • Assess natural genetic variability of yeast strains in relation to CS-lyase gene(s) and MeSAc formation.	• June 2018 • June 2018

Table 2. 2018-19 Deliverables and activities

Deliverables	Activities	Target date
Improved methods of analysis for known and new VSCs, and their precursors and breakdown products	1. Optimise a GCMS method for VSC analysis to complement the current GC-SCD method. 2. Expand the GC-SCD method to differentiate between free and total volatile sulfur compounds present in wines. 3. Initiate comparison study between GC-SCD, LCMS, and GCMS methods to understand the strengths, weaknesses and interoperability of methods.	June 2019
Knowledge of precursors to VSCs and their release mechanisms	1. Finalise and prepare a manuscript summarising the existing polysulfide research investigating sulfhydryl release mechanisms in model wine systems. 2. At appropriate timepoints continue characterisation of vintage 2018 experiments focused on polysulfide discovery in laboratory-scale and winery-scale trials. 3. Study the morphology of *in situ* generated CuS nanoparticles to gain a better understanding of their putative role as a latent source of H_2S in wines post-bottling. 4. Review data from vintage 2018 winemaking trials in red wine, and design and execute a vintage 2019 winemaking trial in white wine to assess factors influencing the formation of putative precursors to VSCs (e.g. polysulfides, CuS), and their release mechanisms. 5. Initiate the development of novel platforms for the selective removal of 'reductive' aroma compounds and their precursors. 6. Summarise results to date on 'reductive' aromas for presentation at the AWITC in 2019.	June 2019
Improved understanding of the role of microbiological factors (biochemical pathways, microbial ecology, nutrition etc.) in VSC formation	1. Continue to assess formation of MeSH and MeSAc using at least three CS-lyase gene-modified yeast (e.g. STR3 or IRC7 gene-modified), and methionine as a precursor. 2. Assess the evolution of the concentration of VSCs in 2017 Grenache bottled wines, fermented with six wine strains with different abilities to produce these compounds. 3. Prepare peer-reviewed manuscript covering the role of the yeast enzyme IRC7 in the formation of both negative and positive VSCs.	June 2019

Table 3. 2019-20 Deliverables and activities

Deliverables	Activities	Target date
Improved methods of analysis for known and new VSCs, and their precursors and breakdown products.	1. Develop an untargeted GCMS method for the analysis of unknown sulfur compounds. 2. Prepare a manuscript describing the comparison study between SCD, LCMS and GCMS. 3. Evaluate the potential of electron spin resonance spectroscopy (ESR) to support research on the formation and fate of volatile sulfur compounds in wine.	June 2020
Knowledge of precursors to VSCs and their release mechanisms	1. Analyse the vintage 2019 juices and wines to assess the formation and release mechanisms of H_2S and other sulfhydryls in white wines. 2. Design and execute an experiment investigating the chemical reactions during oxidative winemaking that influence the formation of irreversible sulfhydryl-complexes (sulfur traps) or reversible sulfhydryl-complexes (latent precursors). 3. Design and execute a laboratory-scale experiment to investigate the formation pathways of PMT in model systems. 4. Evaluate novel fining agents such as colloidal nanoparticles (NPs) (e.g. gold, silver) and/or functionalised NPs for selective H_2S detection. Validate the practicality of novel fining agents by H_2S detection in model and wine samples.	June 2020
Improved understanding of the role of microbiological pathways (biochemical pathways, microbial ecology, nutrition,..) in VSC formation	1. Assess the role of the yeast alcohol acetyltransferases *ATF1* and *ATF2* in the formation of MeSAc from MeSH. Assess the production of VSCs using at least two modified yeast strains (e.g. deletion and overexpression of one or both alcohol acetyltransferases) and using methionine as precursor. 2. Investigate the links between the Ehrlich and demethiolation pathways in yeast, which compete for the precursor methionine, using some of the previously isolated strains from Project 3.2.1. Assess the production of VSCs and methionol/methional using at least four of these strains in a synthetic media.	June 2020

Table 4. 2020-21 Deliverables and activities

Deliverables	Activities	Target date
Practical strategies to decrease the occurrence or impact of negatively perceived VSCs: chemical and biochemical factors influencing the occurrence of VSCs	1. Conduct a targeted survey of PMT in a range of commercially available white, red, and sparkling wines, using chemical and sensory analysis, to establish natural variability of PMT as well as determining the relationship between increasing concentrations of PMT and the transition from 'flint' aroma to 'sulfurous/plastic' aroma as PMT approaches higher concentrations. 2. Design and implement an experiment investigating the chemical formation pathway of PMT. Set up ampoule experiments to investigate the chemical formation of PMT from the reaction of benzaldehyde with H_2S, while also monitoring benzoic acid and benzyl alcohol formation. 3. Design and implement an experiment investigating the biochemical formation pathway of PMT. Screen at least 10 *Saccharomyces* isolates for their ability to produce PMT in laboratory-scale ferments, both in a chemically defined media (CDM) and in a grape must. Evaluate the influence of nitrogen compounds (amino acids and ammonium) on PMT concentrations in laboratory-scale ferments in a CDM. Assess yeasts from three different genetic backgrounds. 4. Design and implement a laboratory-scale fermentation experiment investigating the biochemical formation pathways of MeSH and MeSAc using ^{34}S-labelled methionine in CDM. Use at least three yeast strains with differences in sulfur metabolism to study the formation of VSCs derived from ^{34}S-methionine.	June 2021
Practical strategies to decrease the occurrence or impact of negatively perceived VSCs: removal of VSCs.	1. Evaluate smart surfaces which incorporate immobilised nanoparticles as an alternative to copper fining for their effectiveness in removing 'reductive' aroma compounds in different varieties of red and white wines. Evaluate the selectivity of the smart surfaces in removing 'negative' and 'positive' sulfhydryls and benchmark this against copper fining. 2. Review results and identify options/essential steps for upscaling and progressing towards commercialisation.	
Practical advice and extension materials for winemakers	1. Collate the data and summarise results from the 2019 experiments investigating irreversible sulfhydryl-complexes (sulfur traps, polyphenolic complexes) and reversible sulfhydryl-complexes (latent precursors, i.e. polysulfides). 2. Prepare two papers for peer-reviewed journals. 3. Prepare a webinar on the latest findings on sulfur research. 4. Update the 'reductive' aroma roadshow talk.	June 2021

Table 5. 2021-22 Deliverables and activities

Deliverables	Activities	Target date

Practical strategies to decrease the occurrence or impact of negatively perceived VSCs: chemical and biochemical factors influencing the occurrence of VSCs	1. Conduct sensory analysis and consumer testing of effects of PMT additions to Chardonnay wine. 2. Investigate factors that influence PMT formation during wine ageing in laboratory-scale experiments. French and American oak products will be evaluated for their ability to introduce benzoic compounds (benzaldehyde, benzoic acid, benzyl alcohol) into a model system, and the subsequent formation of PMT from these benzoic compounds when in the presence of H_2S. The effect of oxygen and SO_2 on PMT formation and preservation of PMT will also be studied. 3. Explore opportunities to collaborate with industry partners to gain knowledge on winemaking practices that promote PMT formation in Chardonnay wine. 4. Investigate the influence of yeast genetic background and nutrient status (DAP additions) on PMT production in laboratory-scale ferments of a low- yeast assimilable nitrogen (YAN) Chardonnay juice. 5. Conduct a pilot-scale winemaking trial in Chardonnay to assess the influence of different yeast strains and nutrient status (DAP additions) on PMT production. 6. Study the effect of yeast phenylpyruvate decarboxylase *ARO10* on benzyl alcohol/benzaldehyde production through deletion and overexpression experiments. 7. Further investigate the biochemical formation pathway of MeSAc from MeSH and methionine by overexpression/deletion of at least three yeast genes.	June 2022
Practical strategies to decrease the occurrence or impact of negatively perceived VSCs: removal of VSCs	1. Evaluate the use of smart surfaces in terms of surface exposure time, surface geometry, and regeneration potential to aid the maximum removal of unwanted sulfhydryl compounds from wine. Benchmark the effectiveness of the smart surface in removing H_2S, MeSH, and EtSH against copper fining (currently the most commonly used remediation tool by the wine industry). 2. Map out a route for commercialisation for products developed under this project. 3. Determine how copper speciation (total Cu, labile/non-labile Cu) modulates 'free' and 'total' H_2S and MeSH, and how it relates to 'reductive' character and the sensory profile of white wine as part of a collaborative study with NWGIC (NSW). Perform a survey of 50 white wines with half of the wines selected for 'reductive' character. The wines will be analysed for free and total H_2S by both the AWRI and NWGIC. Copper fractions, free and total SO_2 and metals and ascorbic acid will be measured by NWGIC, and the AWRI will conduct the sensory analysis of the wines. 4. Support collaborators through the provision of samples and analysis, where appropriate.	June 2022
Practical advice and extension materials for winemakers.	1. Prepare peer-reviewed and/or industry journal manuscripts with practical advice for winemakers on PMT modulation in wine. 2. Prepare extension material for the 18th AWITC on	June 2022

	how to increase sulfur compounds associated with 'premium' character and how to decrease sulfur compounds associated with 'negative' aromas. 3. Support Projects 2.1.1 (Extension) and 2.1.2 (Communication and awareness-raising activities) through identifying topics and preparing materials for awareness-raising and practice change activities.	

6. Methods

6.1 Analysis of wine chemical composition

To study the formation and fate of key sulfur compounds in wine, a range of methods was used to determine their speciation in wine, their precursors and other latent forms of VSCs. These included specialised methods that could differentiate between ^{32}S and ^{34}S labelled VSCs, as well as high-throughput methods using 96-well plates to allow for the analysis of large sets of samples. These methods are described below.

6.1.1 Analysis of volatile sulfur compounds

- Volatile sulfur compounds were analysed according to Siebert et al. (2010) and Cordente et al. (2022) using static headspace gas chromatography sulfur chemiluminescence detection (HS-GC-SCD) on an Agilent 7890B GC, coupled to an Agilent 8355 SCD and equipped with a Gerstel MPS2 XL (Lasersan).
- The gas-chromatography - mass spectrometry (GC-MS) method used for the detection of ^{32}S- and ^{34}S-VSCs was based on the method by Nguyen et. al (2012), with slight modification. An Agilent 7890A GC equipped with cryogenic cooling function was connected to an Agilent 5975 mass spectrometer (MS). The oven temperature was initially held at 5°C for 5 mins, followed by a ramp of 5°C/min until 150°C. This was held for 5 mins and then ramped at 20°C/min until 250°C and finally held for 5 mins. The total run time was 49 mins. The transfer line was held at 250°C. Single ion monitoring was performed on the MS with a source temperature of 230°C and a quad temperature 150°C. Headspace solid phase-micro extraction (HS-SPME) was used for sample introduction using a DVB/CAR-PDMS (50/30 mm x 2 cm) fibre with an extraction time of 30 mins at 45°C. Samples were stirred during extraction. For sample preparation 1 mL of wine was added to 9 mL of brine. To limit the risk of loss of VSCs, all sample preparation was performed in an anaerobic environment using degassed solutions. Samples were stored at 4°C prior to analysis.
- To quantify 'metal-bound', 'free' and 'total' fractions of VSCs present in wine, the HS-GC-SCD method and/or the GC-MS method described above were used; however, samples were prepared according to Franco-Luesma and Ferreira (2014) and Ontañón et al. (2019) with slight modification. All sample preparation was conducted in an anaerobic environment. The samples used to determine the 'metal-bound' VSC fraction were prepared by conducting a 1:10 dilution of sample using a 35% cold and degassed brine solution. The samples used to determine the 'free' VSC fraction were prepared as described in Siebert et al. (2010); however, no salt or stir bar were added to the vials, the samples were incubated at 30°C with agitation for 15 mins, and injected into the GC inlet using a syringe with temperature set at 40°C. The 'total' fraction was calculated by adding the 'free' and 'metal-bound' fractions.
- Polyfunctional sulfhydryls (including PMT, 2-FMT and varietal sulfhydryls 3-SH, 3-SHA and 4-MSP) were initially quantified using liquid chromatography with tandem mass spectrometry (LC-MS/MS) as per Capone et al. (2015). After modifications to improve sample throughput, polyfunctional sulfhydryls were quantified as described in Cordente et al. (2022). Screening ferments with higher concentrations of polyfunctional sulfhydryls was carried out by 96-well plate-SPE-LC-MS/MS (unpublished method).

- Unknown VSCs related to 'struck flint' aroma in white wine were investigated using liquid chromatography high-resolution mass spectrometry (LC-HRMS), based on Vichi et al. (2015).

6.1.2 Analysis of non-volatile sulfur compounds

- A robust high performance liquid chromatography (HPLC) method for quantification of reduced L-glutathione (GSH) and oxidised glutathione (GSSG) was developed based on ultra-violet (UV) detection. Derivatisation of GSH using N-ethylmaleimide (NEM) to produce N-ethylsuccinimido-S-glutathione was used for analyte stability, thus avoiding GSH oxidation while awaiting analysis and preventing under-estimation of GSH and over-estimation of GSSG (New and Chan 2008). The enhanced stability allowed for the storage of samples prior to analysis at 4°C and the simple equipment required to quantify the analytes increased the accessibility of the method to wine analysis laboratories, analysis centres and research laboratories. The method is described in Bekker et al. (2022).
- Dicysteinyl and diglutathionyl polysulfides were initially analysed using the method described in Bekker et al. (2018). Method development was undertaken to modify the method used for GSH and GSSG analysis (Bekker et al. 2022) to allow for the quantification of a larger range of polysulfides using LCMS. Modification of the original HPLC method included a 1:2 dilution step and multiple reaction monitoring (MRM) of analyte ions using MS, which allowed for the highly specific and sensitive quantification of analytes within complex mixtures. The target analytes quantified with this method were expanded to include cysteinyl- and glutathionyl-polysulfides and allowed for the quantification of ^{34}S-labelled isotopes of these analytes.

6.1.3 Analysis of precursors of phenylmethanethiol

GC-MS analysis of benzaldehyde, a potential precursor of PMT, as well as related compounds (benzyl alcohol, benzyl acetate and benzoic acid) was developed. The methodology developed is described in Espinase Nandorfy et al. (2022).

6.1.4 Analysis of metals

The metal ions were measured by Affinity Labs using an inductively coupled plasma mass spectrometer (ICPMS) (Nexion 350D PerkinElmer, Boston, MA, USA) as described in Bekker et al. (2020).

6.2 Sensory analysis

The detailed sensory studies conducted for the remediation trial (Section 7.5.1) and for the trial investigating consumer preference for 'flint' aroma in white wine (Section 7.4.1.7) used a generic quantitative sensory descriptive analysis method as described in Heymann et al. (2014), Day et al. (2021) and Bekker et al. (2020). All panellists were part of the external AWRI trained descriptive analysis panel and had extensive experience in wine sensory descriptive analysis.

Evaluating the intensity of 'reductive' aromas in 49 white wines was deemed too large for classical descriptive analysis (Section 7.5.4). As such this investigation was undertaken using AWRI trained descriptive analysis assessors, following a similar laboratory protocol to routine smoke taint evaluations. Before all sensory studies, a blind preliminary sensory evaluation was conducted by a panel of expert technical assessors to ensure experimental samples were free of dominant off-flavours or winemaking artefacts not under investigation.

6.3 Development of novel remediation tools

The following methods were used for the development and analysis of novel smart surfaces consisting of deposited plasma polymers (Mierczynska-Vasilev et al. 2022).

6.3.1 Plasma polymerisation

Allylamine (AA) (reagent grade, 98%) and 2-methyl-2-oxazoline (POx) (98%) were obtained from Sigma-Aldrich (Australia) and used as supplied. Microscope glass slides and 100-mesh stainless steel sheets were used as a substrate for plasma deposition. Plasma polymerisation was carried out in a custom-built reactor equipped with a 13.56 MHz plasma generator (Vasilev et al. 2010). Allylamine was deposited at a precursor pressure of 0.13 mbar, and 2-methyl-2-oxazoline at 0.08 mbar. The power used for deposition of both monomers was 40 W and 50 W, respectively. In both cases, the plasma deposition time was two minutes. Before deposition, all surfaces were cleaned by applying air plasma for two minutes at 50 W.

6.3.2 Synthesis of gold nanoparticles

Gold nanoparticles were synthesised by reducing hydrogen tetrachloroaurate ($HAuCl_4$) with trisodium citrate. A 50 mL solution of 0.01 % HAuCl4 was brought to boiling temperature with vigorous stirring. Under vigorous stirring, 1% solution of trisodium citrate (TSC) in water was added. To achieve particle sizes of 38 and 68 nm in diameter, 0.5 mL and 0.3 mL of TSC solution were added, respectively. After adding trisodium citrate, the colour of the solution changed from light yellow to wine red within minutes. The solution was kept for a further 20 minutes at boiling temperature and then cooled to room temperature (Turkevich et al. 1951).

6.3.3 X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) spectra were obtained using a Kratos Axis Ultra XPS spectrometer (Kratos Analytical Ltd, UK) with a monochromatic Al source and operated at 15 keV and 15 mA to get a survey spectrum from 0 eV to 1,100 eV for all surface coatings. To compensate for surface charge effects, all the binding energies were referenced to the C1s neutral carbon peak at 285 eV. CasaXPS software was used for processing and curve fitting.

6.3.4 Thickness measurements

The thickness of the deposited plasma polymers was determined using a variable angle ellipsometer (VASE, J. A. Woolam Co. USA). The experimental data were analysed by WVASE32 (J. A. Woolam) software. The optical properties of the silicon wafer and the native oxide layer were taken from the software. A refractive index of 1.55 (Coad et al. 2012) was assumed for all plasma polymer layers.

6.3.5 Fourier transform infrared spectroscopy

IRTracer-100 Fourier transform infrared spectrometer (Shimadzu) equipped with liquid nitrogen-cooled mercury cadmium telluride detector was used for all measurements. Measurements were performed using the Quest Single Reflection Attenuated Total Reflectance (ATR) Accessory (Specac), fitted with a diamond ATR crystal. In all cases, 128 scans at a resolution of 4 cm^{-1} were taken to obtain a satisfactory signal-to-noise ratio. The ATR effect and atmospheric contributions from carbon dioxide and water vapour were corrected by background performed on an empty ATR device.

6.3.6 Scanning electron spectroscopy

Scanning electron spectroscopy (SEM) was employed to determine the morphology and density of gold nanoparticles immobilised on a glass or mesh surface. An FEI Quanta 450 FEG-ESEM equipped with an EDAX Apollo X Energy Dispersive X-Ray (EDX) spectrometer was used for analysis. SEM images were analysed using Image J software. To calculate the number of nanoparticles per mm^2, % surface coverage and interparticle distance three samples were

prepared per nanoparticle size. These samples were analysed by taking three images per sample.

6.4 Improved understanding of the role of microbiological factors in volatile sulfur compound formation

6.4.1. Laboratory-scale fermentations

Laboratory-scale fermentations were performed in triplicate in different fermentation media, including an unfiltered Pinot Noir must, a filtered Chardonnay must, and a chemically defined must (CDM). Fermentations were conducted at 17°C in 100 or 250 mL Schott bottles fitted with stir bars. The lids of the Schott bottles were fitted with gas detector tubes to measure the release of H_2S during fermentation. Fermentation progress was followed by CO_2 weight loss, measured every 24 hours. All yeast strains were obtained from the AWRI Wine Microorganism Culture Collection.

6.4.2. Pilot-scale winemaking of Grenache

Small-lot wines were produced by WIC Winemaking Services, according to a standardised red winemaking protocol. Grenache grapes (2017 vintage) from a high-value McLaren Vale vineyard were hand-picked. Crushed and destemmed grapes were portioned into 50 kg lots and inoculated with the different strains in triplicate and fermented on skins at 20°C. Wines were pressed and seeded for malolactic fermentation. Wines were bottled in screw-cap-sealed bottles (375 mL).

7. Results and discussion

7.1 Improved methods of analysis for known and new volatile sulfur compounds, and their precursors, and breakdown products

7.1.1 Improved methods for analysing volatile sulfur compounds

The method developed by Siebert et al. (2010) has been successfully used for routine analysis of VSCs for the last 12 years. This method allows for the quantification of ten VSCs in wine. The recent advancement in the understanding of the speciation of VSCs in wine, which includes 'free', 'metal-bound' and 'total' VSC species (Franco-Luesma and Ferreira 2014, Ontañón et al. 2019) initiated the development of a larger sweep of methods to allow for the in-house quantification of the 'free', 'metal-bound' and 'total' fractions of VSCs using both GC-SCD and GC-MS.

The new and improved methods for VSC analysis were used in a sensory-based project that was undertaken in collaboration with Charles Sturt University. In this project, the factors that drive the perception of 'reductive' faults were investigated by determining VSC speciation and copper speciation in a range of wines rated as 'reduced' and comparing it to wines with no 'reductive' aroma faults. The results of this trial are discussed in Section 7.5.4. These methods were also used for the projects described in Sections 7.5.3 and for collaborative projects with Stellenbosch University, South Africa.

7.1.2 Improved methods for analysing polyfunctional sulfhydryl compounds

The throughput for the quantitative analysis of the polyfunctional sulfhydryls (3-SH, 3-SHA, 4-MSP, PMT and 2-FMT) was substantially increased with modifications to sample preparation and improvements to the LC-MS/MS methodology (five-fold faster run time), as reported in Cordente et al. (2022). Furthermore, screening large numbers of samples with higher

concentrations of polyfunctional sulfhydryls was enabled by developing a method using 96-well-plate solid phase extraction (SPE) with a positive pressure manifold for the sample preparation. In the well-plate method, ebselen reagent for derivatisation of the sulfhydryl compounds rather than the usual 4,4'-dithiodipyradine (DTDP) as ebselen forms more stable derivatised compounds.

To investigate whether Chardonnay wines with 'struck flint' character contained other unknown sulfhydryl compound(s) also associated with 'struck flint' aroma, a method was developed also using ebselen reagent to derivatise any sulfhydryl compounds in the targeted wines, followed by liquid-liquid extraction for the sample preparation and then LC-high-resolution accurate mass spectrometry with an ID-X™ instrument. One hundred and thirty-four sulfhydryls were found in the wines using Compound Discoverer 3.3 for feature identification, followed by PLS-R (Unscrambler 11) and a correlation matrix (Python 3) to determine significance. Consequently, two compounds of interest were tentatively identified.

To monitor the potential precursors, other related compounds, and the breakdown products of PMT, a GC-MS method was developed to quantify benzaldehyde, benzyl alcohol, benzyl acetate and benzoic acid. This method was used for experiments investigating the formation, evolution and preservation of PMT in model studies, fermentations and commercial wines.

7.1.3 Method for quantifying ^{34}S-glutathione, oxidised glutathione, and cysteinyl- and glutathionyl polysulfides.

It is known that GSH plays a role in modulating post-bottling release of H_2S (Bekker et al. 2018a, 2022, Ugliano et al. 2011). Additionally, GSH acts as a precursor to glutathionyl-polysulfides, compounds that have the potential to act as latent sources for H_2S post-bottling (Bekker et al. 2018b). To quantify reduced L-glutathione (GSH), oxidised glutathione (GSSG) and glutathionyl polysulfides, as well as ^{34}S-labelled polysulfide isotopes, a rapid and high throughput HPLC-UV method was developed.

This method was also used to quantify and used to monitor yeast GSH metabolism during alcoholic fermentation with two yeast strains (AWRI1688, AWRI2861) in collaboration with Project 3.1.6 'Winemaking interventions to modulate glutathione status'. The results demonstrated strong evidence that high GSH addition rates (100 and 250 mg/L) increased post-ferment H_2S concentration in low-nitrogen fermentations (155 mg/L); however, the effect of GSH on VSC formation was yeast strain-dependent. This method was published in Bekker et al. 2022 (submitted July 2022) and the method was used in the work described in Section 7.5.4.

7.2 Knowledge of precursors to volatile sulfur compounds and their release mechanisms

7.2.1 Discovering novel precursors to hydrogen sulfide

Hydrogen sulfide is one of the main compounds associated with unwanted 'reductive' aromas in wine. Its odour is often described as 'rotten egg' and 'sewage', and it can significantly affect wine aroma and quality when present above its aroma threshold of 1.1 to 1.6 µg/L (Siebert et al. 2010). It is known that H_2S is often produced in large quantities during fermentation as a product of yeast metabolism (Dzialo et al. 2017); however, it only becomes problematic when residual concentrations of H_2S remain in wine post-fermentation.

'Reductive' aromas often reappear post-bottling, which indicates that there are latent H_2S sources present in bottled wine that release H_2S during bottle ageing. This can be ascribed to the reversible equilibria between free, metal-complexed and latent sources of H_2S (Vela et al. 2018). In fact, the main techniques used for H_2S removal are oxidative handling and/or copper fining; however, these treatments may only temporarily remove H_2S, as it often reappears post-bottling. It has been proposed that the copper-based remediation treatments applied in

oxidative environments may produce polysulfide species which could act as latent sources of H_2S.

A collaborative study with world-renowned researchers from Plumpton College (UK), E. & J. Gallo Winery (USA) and the University of Adelaide was initiated to investigate the role of dicysteinyl polysulfides as putative precursors to H_2S during post-bottling storage. Model wine experiments enabled the identification of four dicysteinyl polysulfides when H_2S was oxidised in the presence of cysteine, copper and iron. The stability of the polysulfides was shown to be related to the sulfur chain length, with decreased stability as the chain length increased. Importantly, H_2S was released from the polysulfides over the course of six months, and the largest amount of H_2S (up to 212 µg/L) was produced when the model wines containing the polysulfides were treated with commonly used reducing agents, such as ascorbic acid and sulfur dioxide (SO_2) (Figure 1).

The results of this trial played a significant role in establishing the importance of polysulfides as latent sources of 'reductive' aromas in wine and have initiated a large body of international research that is further investigating the prevalence of polysulfides in wine. This study emphasised the importance of taking care with copper fining, as copper addition promotes the formation of polysulfides which act as latent sources of H_2S post-bottling. This work was published in Bekker et al. (2018b) and presented locally and internationally at conferences focused on wine science. The results of this trial were also communicated to the Australian wine industry through webinars and as part of the AWRI roadshow seminar series.

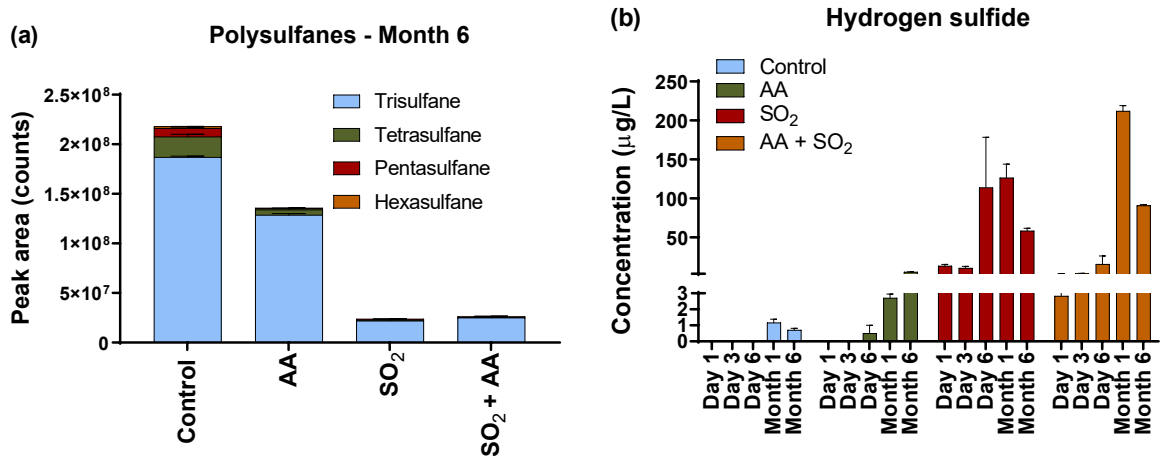


Figure 1. (a) The stability of polysulfides after six months of storage (control) and as affected by commonly used reducing agents such as ascorbic acid (AA) and sulfur dioxide (SO_2), and (b) the subsequent liberation of hydrogen sulfide (H_2S) over the course of six months (control samples) as well as the accelerated liberation of H_2S as facilitated by the addition of AA and SO_2.

7.2.2 Does macro-oxygenation favour the formation of latent sources of volatile sulfur compounds or produce irreversible 'sulfur traps'?

The two main methods used by winemakers to remove VSCs from wine are aerative racking/splashing techniques during fermentation and copper fining post-fermentation or pre-bottling. These treatments may be effective in the removal of 'reductive' aromas in the short term, but in some instances H_2S can reappear post-bottling when 'reductive' conditions are re-established in the wine. It was previously thought that the insoluble copper sulfide complexes produced as a result of copper fining could be readily removed from wine by filtration and racking; however, it has been demonstrated that the copper sulfide complexes exist as nanoparticles that remain dispersed in the wine (Kreitman et al. 2016). Additionally, the mechanisms by which aerative treatments are thought to remove VSCs include the straightforward volatilisation or the formation of less detrimental oxidation products, which

can also occur with copper fining. The substances that subsequently release VSCs may potentially be disulfides and diorganopolysulfides, although the mechanisms and conditions for such release are not clearly established. Sulfhydryl compounds may also add to quinones produced when polyphenols are oxidised, but these adducts are presumed to be stable and unable to release VSCs again.

To investigate the chemical mechanism through which aerative remediation strategies successfully remove H_2S, an experiment was designed to investigate the formation of oxidation reaction products that produce irreversible complexes with H_2S (quinone reactions) as well as the formation of latent sources of H_2S after a wine with 'reduced' character has been treated with either copper fining or macro-oxygenation. Ferments were conducted in chemically defined grape juice media with added elemental sulfur (50 mg/L or 100 mg/L), cysteine (20 mg/L), GSH (50 mg/L), and 4-methylcatechol (180 mg/L). The ferments were treated with either aerative sparging (macro-oxygenation) when 20% of the total sugars were consumed, or with copper fining when the ferments reached 1°Bé. The formation of H_2S, polysulfides as well as polyphenolic-sulfhydryl adducts was studied using GC-SCD, GC-MS and HPLC-MS/MS.

The results of this study showed that macro-oxygenation induced the formation of both oxidation reaction products ('sulfur traps') as well as polysulfides ('latent sources). A 4-methylcatechol sulfhydryl adduct (4MC-S-4MC) was identified in oxygen-treated samples and the accumulation of this diarylsulfide correlated with the decrease of 4-methylcatechol in oxygen-treated samples. The diarylsulfides produced were stable for 12-months (Figure 2). A diglutathionyl polysulfide (GS-S-SG) was also identified in oxygen-treated samples. The concentration of GSH decreased in oxygen-treated samples, which correlated with an increase in GS-S-SG. Only a small yield of GS-S-SG was produced as a result of macro-oxygenation and this compound was unstable and decomposed after bottling. Copper fining treatment deployed just before bottling did not increase polysulfide or diarylsulfide formation in this experiment (Figure 2).

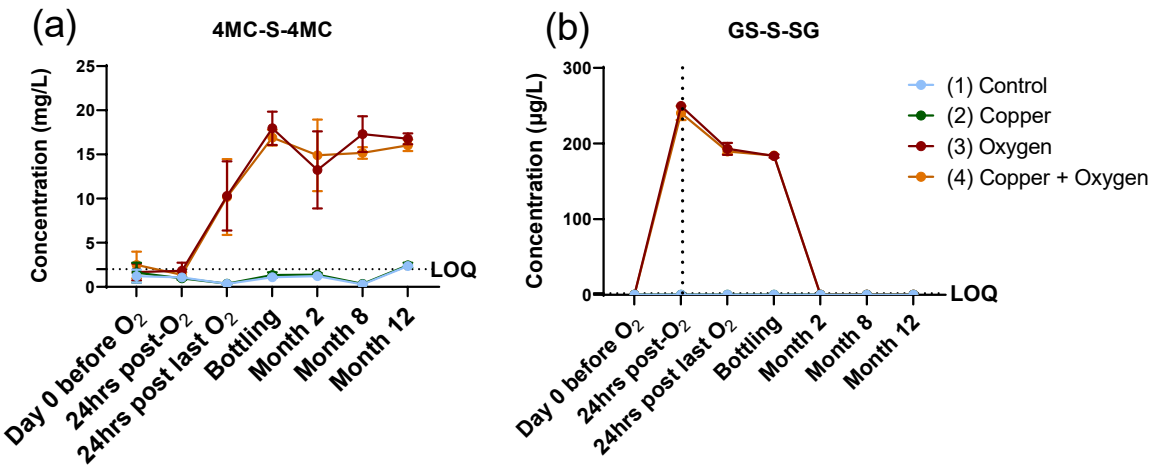


Figure 2. Formation of (a) oxidation reaction products (4-methyl catechol sulfhydryl adduct) that act as 'sulfur traps' and (b) latent sources of hydrogen sulfide (H_2S) (glutathionyl polysulfides) as a result of macro-oxygenation remediation treatments.

The results of this study provided insights into the mechanism underpinning the successful removal of H_2S when aerative winemaking treatments are employed. These results demonstrated that the formation of stable oxidative reaction products with quinones was favoured and only small quantities of symmetrical and asymmetrical di- and polysulfide species were produced. As such, macro-oxygenation did not increase the risk of developing 'reductive' aromas in wine post-bottling since 'sulfur traps' were preferentially produced. This work was presented at the 2021 International Chemical Congress of Pacific Basin Societies Virtual Conference and shared with the wine sector in presentations showcasing the latest research of Project 3.4.2 in the AWRI roadshow seminar series.

7.3 Improved understanding of the role of microbial factors in volatile sulfur compounds formation

7.3.1 The amino acid methionine is the precursor to some volatile sulfur compounds associated with negative aroma descriptors

Saccharomyces cerevisiae performs a wide range of industrial fermentations that functionally depend upon its ability to convert sugars to ethanol and CO_2 efficiently. While performing this primary function, *S. cerevisiae* also produces a range of secondary metabolites, such as esters, acids, higher alcohols and VSCs, which contribute substantially to the flavour and aroma of wine. Some of these important metabolites are derived from yeast amino acid metabolism. For example, the desirable 'rose' aroma compound 2-phenylethanol (2-PE) is derived from phenylalanine, while the sulfur-containing amino acid methionine is believed to be the precursor of the undesirable VSCs MeSH ('sewage and rubber' aromas), methional ('cooked potato' aroma) and MeSAc ('sulfurous and cheesy' aromas).

It is well known that different wine yeast strains produce varying amounts of VSCs, such as H_2S and the 'tropical' sulfhydryls, 3-SH and 4-MSP, which means that careful yeast selection can be a simple and effective way to modulate the concentration of these compounds and optimise wine style while minimising the formation of off-flavours. While the formation of both H_2S and 'tropical' sulfhydryls by yeast has been extensively studied, and for example, low-H_2S producing strains have been generated, little is known about the biochemical pathway that leads to the formation of odoriferous VSCs from methionine, and few strategies exist to decrease their formation during fermentation.

Initially, the role of methionine as a potential source of VSCs was studied. The addition of high concentrations of methionine to a CDM resulted in a substantial increase in the levels of MeSH, MeSAc and DMDS produced by the yeast AWRI1631 (Figure 3). On the other hand, the formation of H_2S seemed to be unaffected by methionine supplementation, while the formation of EtSAc by yeast was dramatically decreased in these conditions (Figure 3).

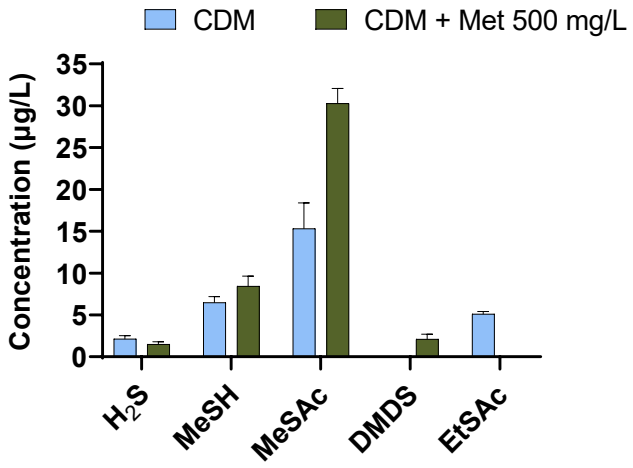


Figure 3. Concentrations of hydrogen sulfide (H_2S), methanethiol (MeSH), methylthioacetate (MeSAc), dimethyldisulfide (DMDS) and ethylthioacetate (EtSAc) by the yeast strain AWRI1631 after fermentation of 100 mL of a chemically defined must (CDM). The media was also supplemented with 500 mg/L of methionine (green bars).

7.3.2 How the choice of yeast starter culture modulates the formation of volatile sulfur compounds derived from methionine

The formation of MeSAc and MeSH is also dependent on the genetic makeup of the yeast used for fermentation, as shown previously in laboratory-scale fermentation experiments in CDM.

This was later confirmed in a pilot-scale winemaking trial in Grenache (50 kg scale) with six different wine strains. One of these strains assessed, AWRI1776, produced high concentrations of MeSAc (Figure 4), and a formal sensory evaluation showed that wines made with this strain were rated high in 'drain' ('reductive') and 'tinned vegetable' aroma attributes. On the other hand, strains AWRI1833 and AWRI2914 that produced relatively low levels of odoriferous VSCs and high levels of 'tropical' sulfhydryls, were rated very low in negative 'reductive' aromas and high in 'red fruit' and 'cherry/confectionary' aromas. These results showed that the choice of starter culture to carry out fermentation could be used as a strategy to decrease the levels of these undesirable compounds and minimise off-flavours in wine.

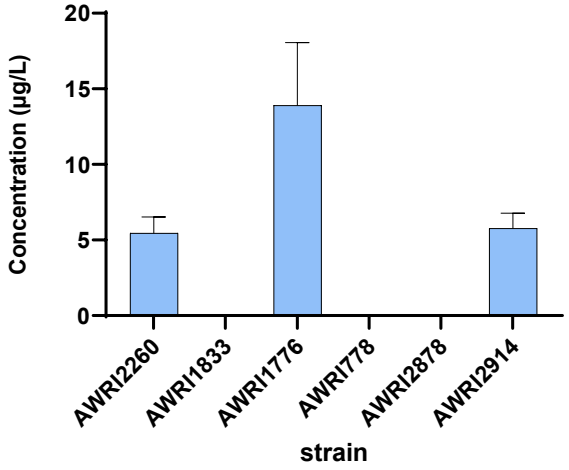


Figure 4. Formation of methylthioacetate (MeSAc) in Grenache wines (2017 vintage) fermented with six different yeast strains

7.3.3 Biochemical pathways that lead to the formation of volatile sulfur compounds derived from methionine

The potential biochemical pathway(s) that lead to the formation of VSCs derived from the sulfur-containing amino acid methionine were also studied. In yeast, two competing pathways are involved in methionine degradation: the Ehrlich and the demethiolation pathways. In the Ehrlich pathway, methionine is transaminated and decarboxylated to methional, and subsequently reduced to the higher alcohol methionol ('cooked potato' aroma). Methionine is also the precursor of MeSH, the production of which can occur enzymatically via demethiolation or non-enzymatically. Once MeSH is formed, it is believed that this compound can be modified by unknown enzymatic activity to form MeSAc.

The yeast enzyme(s) responsible for the demethiolation of methionine to yield MeSH were not previously identified before this study. Several candidates were initially considered: *STR3, CYS3, CYS4, MET17, IRC7, YHR112c* and *TUM1*. The enzymes encoded by these genes are known to catalyse similar reactions, such as the β-elimination of the sulfur-containing amino acid cysteine to yield H_2S, or the cleavage of cysteine-bound conjugates of the 'tropical' sulfhydryls, 3-SH and 4-MSP. To prove whether any of these seven enzymes were also involved in the degradation of methionine, the corresponding genes were individually deleted (inactivated) or overexpressed (more copies of the enzyme were being produced) in the yeast strain AWRI1631.

Of the seven genes assessed, only *TUM1* showed some involvement in the release of MeSH from methionine as its overexpression resulted in a significant increase in the levels of MeSH when compared to the control strain (17.3 µg/L vs 5.2 µg/L, respectively) (Figure 5). *TUM1*

inactivation did not result in a decrease in the levels of MeSH produced by yeast, indicating that there might be other enzymatic activities also responsible for MeSH release.

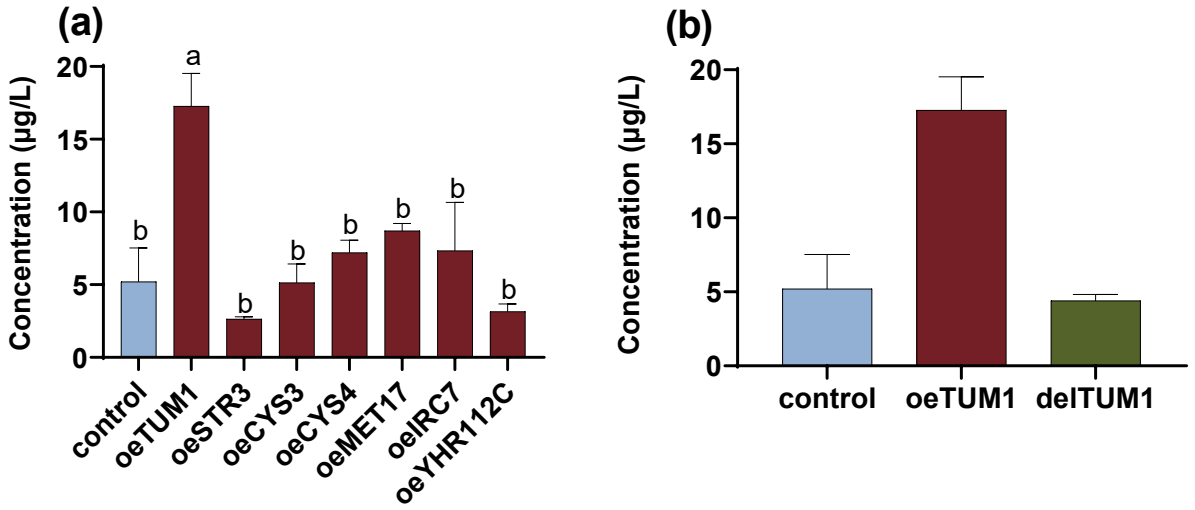


Figure 5. Formation of methanethiol (MeSH) from methionine after fermentation of 100 mL of a chemically defined must (CDM) supplemented with methionine (500 mg/L). Several yeast genes were overexpressed (a) (oe, depicted in red); or deleted (del, depicted in green) (b) in the control wine yeast strain AWRI1631 (blue).

When considering the formation of MeSAc from MeSH, it is believed that this reaction might require an enzyme with a very specific activity, known as 'sulfhydryl S-acetyltransferase' activity (Scheme 1). The most similar candidate in the yeast genome was the alcohol O-acetyltransferase ATF1, which is responsible for acetate ester production during fermentation (Scheme 1).

A) sulfhydryl *S*-acetyltransferase activity (unknown)

CH_3-**S**H (MeSH) + acetyl-CoA = CH_3-CO-**S**-CH_3 (MeSAc)

B) alcohol *O*-acetyltransferase activity (encoded by *ATF1*)

CH_3-CH_2-**O**H (ethanol) + acetyl-CoA = CH_3-CO-**O**-CH_2-CH_3 (ethyl acetate)

Scheme 1. Enzymatic activities required for the formation of thioacetates (A) and acetate esters (B) by yeast

The potential role of *ATF1* in MeSAc formation was assessed through its deletion or overexpression in the model yeast AWRI1631. These modified strains, alongside with the control strain, were used to ferment a small volume of CDM supplemented with different concentrations of MeSH. Independent of the initial MeSH present in the fermentation media, the overexpression of *ATF1* resulted in a slight but consistent decrease in MeSAc and a slight increase in MeSH in the final wines (Figure 6). While these results strongly suggest that *ATF1* is not involved directly in the thioesterification of MeSH to produce MeSAc, *ATF1* might still play an indirect role in this reaction. Both acetate esters and MeSAc share acetyl-CoA as the common precursor for their formation (Scheme 1), and it is well known that overexpression of *ATF1* leads to an overproduction of acetate esters, which might deplete yeast from the acetyl-CoA necessary for MeSAc formation. This could explain the slight decrease in MeSAc formation when

ATF1 is overexpressed. Alternatively, *ATF1* has also been shown to have a 'thioesterase' activity, which might be responsible for the cleavage of MeSAc back to MeSH.

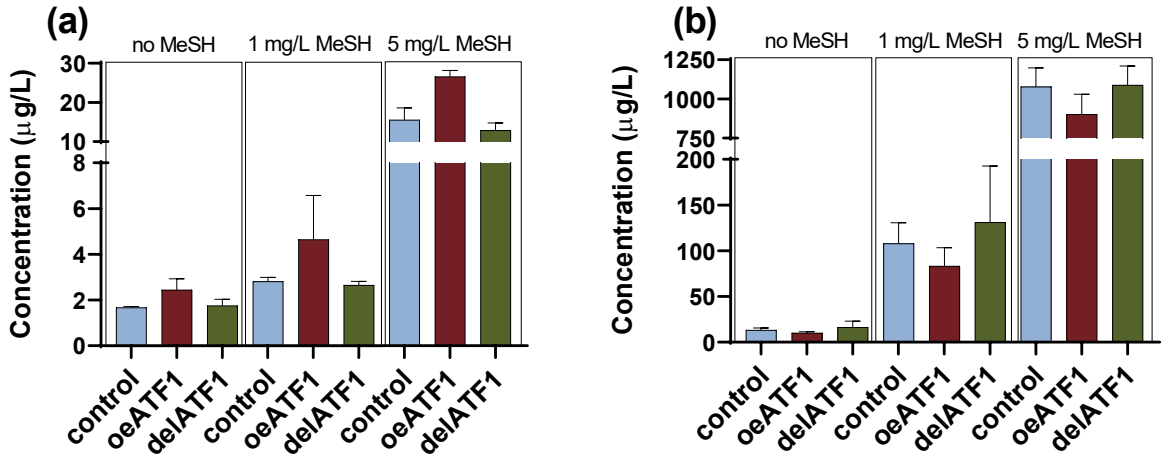


Figure 6. Formation of (a) methanethiol (MeSH) and (b) methylthioacetate after fermentation of 100 mL of a chemically defined must. The media was supplemented with either 1 or 5 mg/L of MeSH. The control strain is shown in blue, *ATF1* overexpression (oeATF1) is depicted in red, while its deletion (delATF1) is depicted in green.

7.3.4 Links between sulfur and nitrogen metabolism in yeast and its effect on volatile sulfur compound formation

Higher alcohols are alcoholic compounds with more than two carbon atoms. These alcohols are derived from yeast amino acid metabolism via the Ehrlich pathway. This pathway is responsible for the formation of the 'rose' aroma compound, 2-PE from the aromatic amino acid phenylalanine, while the degradation of methionine results in the formation of the higher alcohol methionol, with methional as the precursor. Both methionol and methional are associated with 'cooked potato' aromas in wine. While the metabolism of aromatic amino acids via the Ehrlich pathway has been extensively studied, little is known about the branch of the pathway involved in methionine catabolism.

From Project 3.2.1 (Microbial diversity) a series of strains with an activated Ehrlich pathway that results in the overproduction of 2-PE was isolated. Sensory analysis of pilot-scale Chardonnay wines produced with three of these strains revealed that these wines not only exhibited a strong 'rose/floral' aroma but were surprisingly rated really low in the undesirable 'cooked potato/vegetable' aroma descriptor. Chemical analysis of the wines confirmed that two of these 'rose' strains produced significantly lower levels of methional and MeSH when compared to the control, which might explain the low ratings for the 'cooked potato/vegetable' attribute in wine (Figure 7). This study suggests that activation of the Ehrlich pathway in yeast not only results in an increase in positive aromas ('rose/floral') in wine, but also leads to lower production of some negative VSCs derived from methionine. Although these strains were originally developed with the objective of enhancing 'rose/floral' aromas in white wines, they could also be used as a tool for winemakers to decrease the formation of off-flavours. These results have been published by Cordente et al. (2021) and one of the strains has been recently commercialised by the yeast manufacturer AB Biotek as AWRI Rosa.

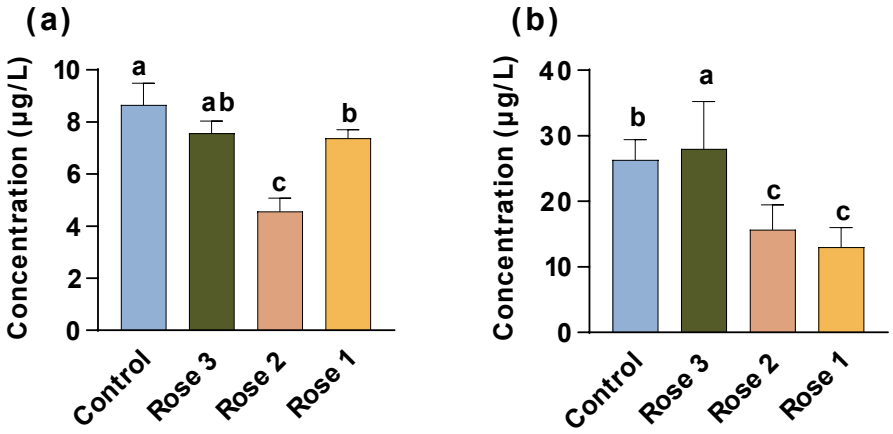


Figure 7. Concentrations of (a) methanethiol and (b) methional (µg/L) in pilot-scale Chardonnay wines made with the control (AWRI796) and three 'rose' strains. The results are expressed as the mean and standard deviation of three independent replicates.

7.4 Chemical and biochemical factors that influence the occurrence of volatile sulfur compounds

7.4.1 Practical strategies for managing 'flint' aroma in wine

Commercial yeast manufacturers offer a wide range of yeast strains, but little is known about their ability to modulate the formation of PMT. This compound is associated with 'struck flint' character in wine, which may be perceived as a positive character for certain wine styles. To address this knowledge gap, factors affecting PMT formation during fermentation and its preservation once produced, were investigated.

7.4.1.1 Modulation of phenylmethanethiol formation by yeast

To improve understanding of the role of yeast in PMT production, the ability of a group of 11 *Saccharomyces* strains to produce PMT was initially screened in several different fermentation media: CDM, a filtered Chardonnay must and an unfiltered Pinot Noir must.

Some variability was found between yeast in their ability to produce the 'flinty' compound PMT during fermentation (Figure 8). A five-fold difference between the lowest and the highest PMT-producing strain was found in the CDM ferments, while this figure was slightly larger in the Chardonnay ferments, around seven-fold. The lowest PMT concentrations and the lowest variability between strains (three-fold) were observed in the Pinot Noir ferments. Interestingly, strain AWRI3442, which produced the highest concentrations of H_2S of all the strains assessed in Pinot Noir, was also found to produce the highest levels of PMT.

From these initial experiments, a subset of these strains was selected for further studies as they produced consistently high amounts of PMT in at least two of the three fermentation media assessed.

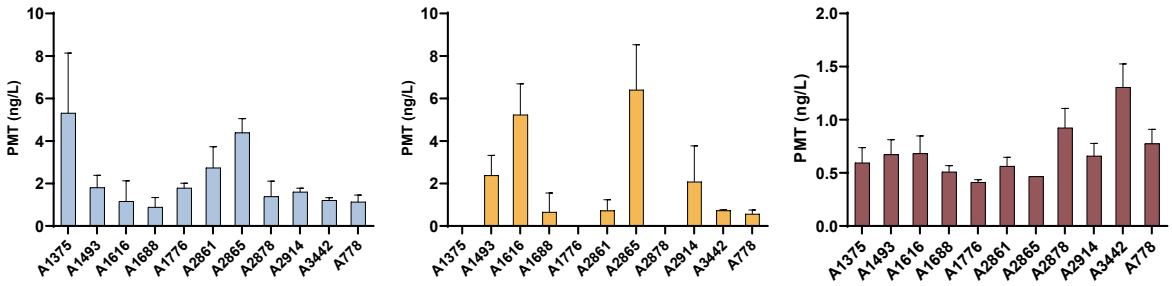


Figure 8. Production of PMT (ng/L) by 11 wine yeast strains after laboratory-scale fermentation (100 to 200 mL) of different fermentation matrices: chemically defined media (blue), Chardonnay (yellow) or Pinot Noir (red).

7.4.1.2 Precursors involved in phenylmethanethiol formation: role of hydrogen sulfide and benzaldehyde

Little is known about the pathways that lead to the formation of PMT during fermentation by yeast. The fact that relatively high concentrations of this 'flinty' compound are observed in CDM (~ 6 ng/L), indicates that the wine yeast can synthesise PMT *de novo* from simple precursors.

Previous research on the 'tropical' sulfhydryl, 3-SH, and on 2-furanmethanethiol (2-FMT) ('roasted coffee' aroma) has shown that two precursors are needed for the formation of sulfhydryl compounds in wine (Tominaga et al. 2003). The first precursor (sulfur donor) is H_2S, which is actively produced by the wine yeast during alcoholic fermentation. The second precursor is an aldehyde, and depending on the sulfhydryl, the nature and the origin of this aldehyde might be different. For 2-FMT the aldehyde is known as furfural, which is mainly extracted from wood when wines are fermented and/or aged in oak barrels. Benzaldehyde is considered to be the precursor to PMT formation, and while oak barrels are believed to be the main source of this compound, wine yeasts are also able to produce some small concentrations of benzaldehyde during fermentation. These low concentrations might be enough for some PMT to be produced, as this 'flinty compound' can be found in unoaked wines.

To confirm whether H_2S and benzaldehyde act as precursors to PMT formation, a series of laboratory-scale fermentations was conducted in CDM with a low concentration of yeast assimilable nitrogen (YAN), to encourage H_2S formation by yeast. Four strains with different H_2S-producing abilities were used for this purpose. The amount of PMT produced depended on the strain used, and positively correlated with the amount of H_2S produced by yeast during fermentation (Figure 9). These results agree with the previous studies by Tominaga et al. (2003) that H_2S is an important precursor necessary for PMT formation.

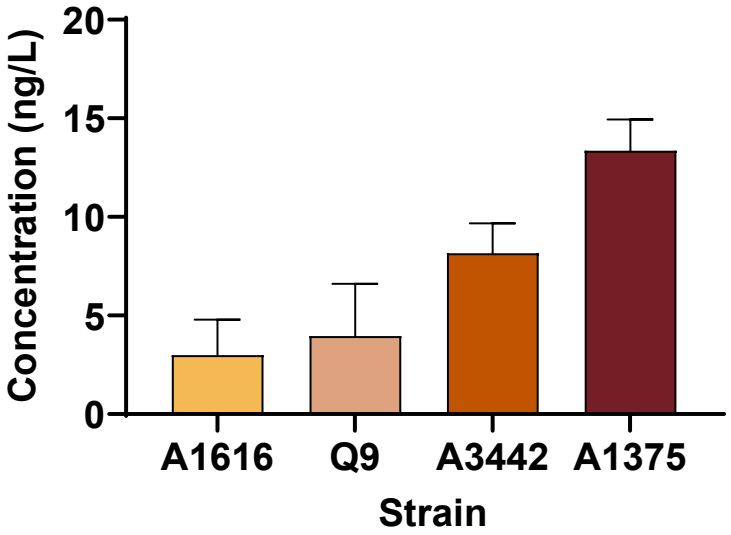


Figure 9. Formation of phenylmethanethiol by four strains with different hydrogen sulfide (H_2S) releasing abilities after fermentation of CDM (100 mL). Strains are shown in ascending order in their ability to produce H_2S. The yeast assimilable nitrogen (YAN) content was set to 150 ppm.

The role of benzaldehyde as a precursor for PMT was also assessed. For this purpose, the CDM medium was spiked with different concentrations of benzaldehyde. The low H_2S producing strain AWRI1616 did not respond to benzaldehyde additions, and the concentration of PMT did not change regardless of whether benzaldehyde was added to the ferments or not (Figure 10). Conversely, for the three other moderate and high H_2S producing strains that were assessed (Q9, AWRI3442 and AWRI1375), the production of PMT was enhanced by increasing concentrations of benzaldehyde in the fermentation media, particularly with the largest benzaldehyde addition (2 mg/L).

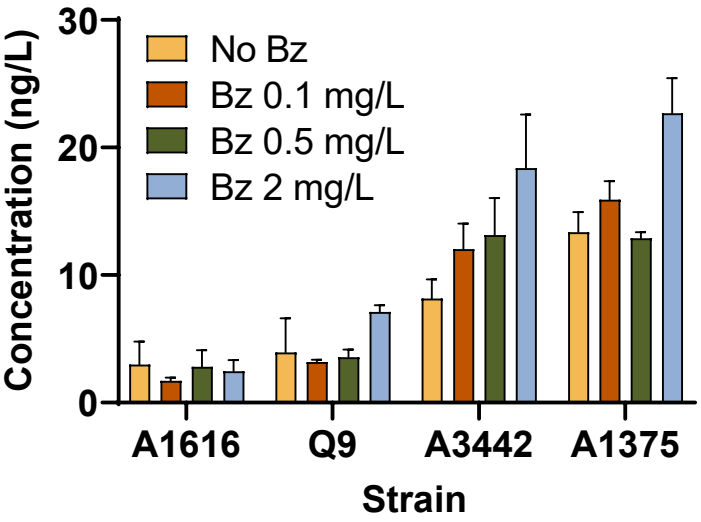


Figure 10. Formation of phenylmethanethiol (PMT) by four yeast strains in chemically defined media (CDM) (100 mL). Strains are shown in ascending order in their ability to produce hydrogen sulfide (H_2S). The yeast assimilable nitrogen content was set to 150 ppm, and different concentrations of benzaldehyde (Bz) were spiked in the ferments.

7.4.1.3 Biochemical pathways leading to formation of the precursor benzaldehyde: role of ARO10

The biochemical pathways leading to the formation of benzaldehyde by wine yeast are poorly understood. It is believed that the branch of the Ehrlich pathway responsible for the degradation of the aromatic amino acid phenylalanine, which leads to the formation of the 'rose' aroma compound 2-PE, also plays a major role in benzaldehyde formation. In particular, the yeast phenylpyruvate decarboxylase *ARO10* has been suggested to catalyse one of the most important steps that leads to the formation of benzaldehyde.

The role of *ARO10* in the modulation of the formation of benzaldehyde was investigated in CDM through its deletion and overexpression in the model yeast strain AWRI1631. Even though the overexpression of *ARO10* resulted in a 10-fold increase in the levels of benzaldehyde when compared to the control strain, this did not translate into higher PMT levels in the final wine (Figure 11). The deletion of *ARO10* resulted in a small decrease in the levels of both benzaldehyde and PMT when compared to the control strain. These results indicate that the *ARO10* gene might play a crucial role in the formation of the precursor benzaldehyde.

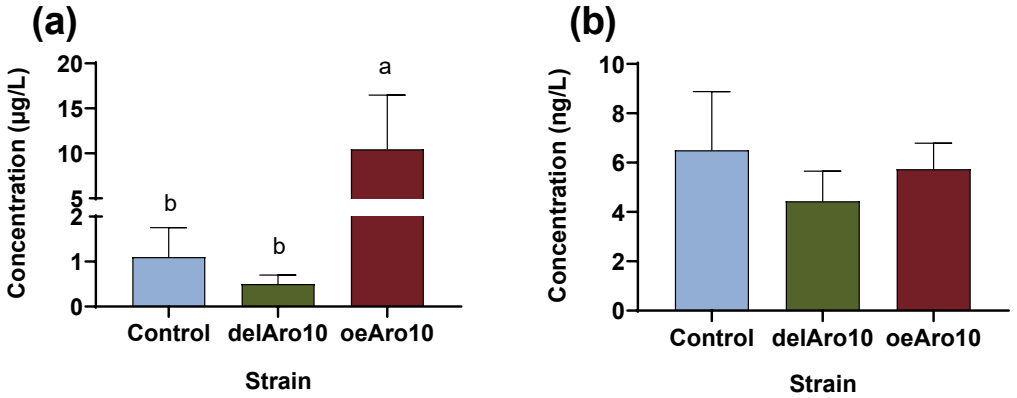


Figure 11. Production of (a) benzaldehyde and (b) phenylmethanethiol after fermentation of 100 mL of a chemically defined must by the yeast strain AWRI1631 (control, blue), in which the *ARO10* gene was deleted (delAro10, green) or overexpressed (oeAro10, red).

7.4.1.4 Role of nitrogen content in grape must on phenylmethanethiol formation

It is well known that the nitrogen content of grape must plays an important role in the formation of VSCs by wine yeast during fermentation. For example, low nitrogen (or low YAN) juices are associated with enhanced H_2S formation by yeast and 'reductive' characters.

To investigate whether PMT formation is also affected by the nitrogen content of the fermentation media, laboratory-scale fermentations were carried out in both CDM and Chardonnay musts, to which increasing concentrations of nitrogen in the form of inorganic ammonia were added prior to fermentation. In CDM the addition of nitrogen resulted in a dramatic increase in the levels of PMT produced by three of the four yeast strains assessed (Figure 12a). Similar results were also obtained with a filtered Chardonnay must (Figure 12b), which confirmed the important role of the nitrogen content of the must in PMT formation by yeast.

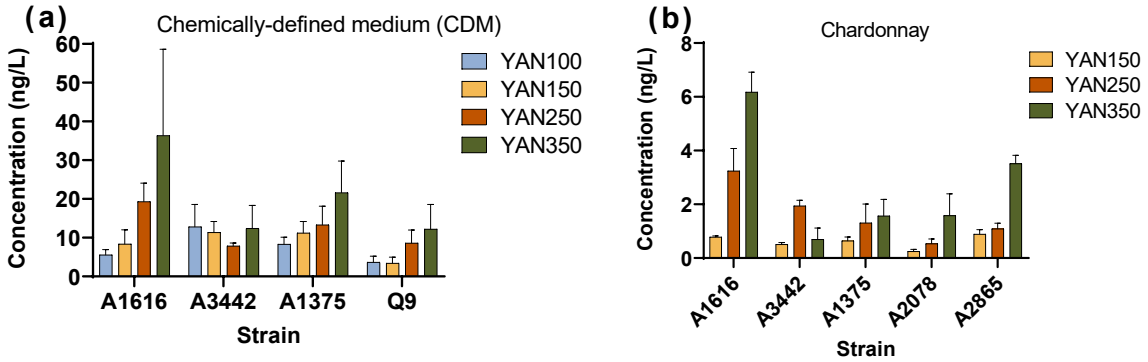


Figure 12. Production of phenylmethanethiol by different yeast strains after fermentation of 100 mL of CDM (a) or Chardonnay must (b) with different concentrations of yeast assimilable nitrogen (YAN). The original Chardonnay must, with a YAN concentration of 150 mg/L, was supplemented with inorganic nitrogen to achieve a final concentration of either 250 or 350 mg/L.

7.4.1.5 Factors that promote and inhibit the formation and preservation of phenylmethanethiol (PMT)

'Struck flint' aroma in wine, associated with the volatile sulfur compound, PMT, is often sought-after in premium Chardonnay and sparkling wine. Although there is an increasing demand for premium wines with this enhanced stylistic aroma, there is little information available on practical winemaking techniques and strategies that can be used to increase or control its intensity. There is also no information available on the effects of winemaking techniques and commonly used winemaking additives on the preservation of PMT in ageing wine.

In this study, model systems were used to evaluate the effects of oxygen exposure, copper (0.5 mg/L) and iron (4 mg/L) on the formation of PMT from its likely precursor compounds, benzaldehyde (5 µM) and H_2S (5 µM). It was found that samples treated with oxygen as well as samples treated with either copper or iron, contained significantly less H_2S than samples protected from oxygen and without metal treatment. The decrease in H_2S concentrations, a key precursor to PMT, resulted in significantly less PMT being produced compared to the control samples that were completely protected from oxygen and contained no metal ions (Figure 13).

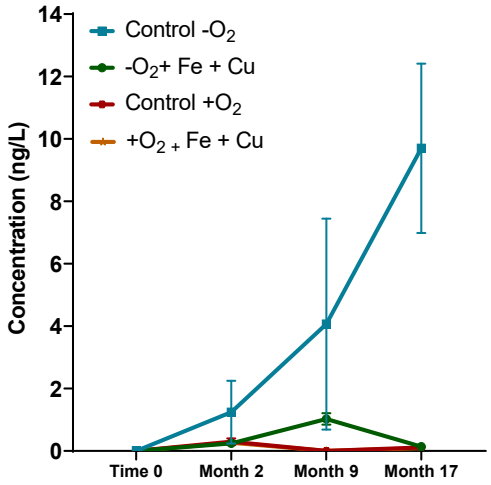


Figure 13. Evaluating the effects of oxygen (O_2) exposure and iron (Fe) and copper (Cu) on the formation of phenylmethanethiol from its precursor compounds, benzaldehyde, and hydrogen sulfide

Once PMT has been produced in wine, either as a product of the yeast metabolic pathway, or as a result of the chemical reaction of benzaldehyde and H_2S during the wine ageing process, it is important to preserve this compound in ageing wine. However, the natural evolution of PMT in wine post-bottling had not yet been studied, and neither had factors that may impact its

preservation in ageing wine. Considering that PMT is a VSC that contains a terminal sulfhydryl group, it has the potential to undergo oxidation reactions, bind to metal ions, or form adducts with other reactive wine compounds, such as H_2S and SO_2, all factors that may negatively affect its preservation post-bottling. Oxidation of PMT to produce larger molecular mass compounds due to exposure to oxygen is another factor that may impede the preservation of 'flint' aroma.

To address this knowledge gap, experiments were designed to:

1) evaluate the natural evolution of PMT in ten Chardonnay wines over the course of six months
2) evaluate the effects of copper (0.5 mg/L), iron (4 mg/L) and oxygen exposure, as well as elevated residual concentrations of H_2S (10 and 40 µg/L) and SO_2 (20 and 40 mg/L) on the preservation of PMT in model systems.

The concentration of PMT was found to naturally decrease in the ten wines evaluated over the course of six months, but the rate of decrease was unique to each wine (Figure 14). This suggests that there are many factors that may influence the rate of PMT degradation in wine.

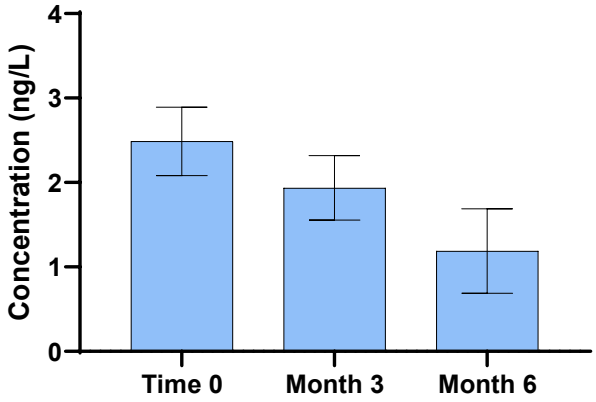


Figure 14. The average concentration of phenylmethanethiol measured in 10 wines over the course of six months.

To evaluate the factors that influence PMT concentrations during bottle ageing, the effects of common winemaking additives and compounds that PMT may be exposed to during ageing were evaluated in model systems. The conditions evaluated were exposure to elevated residual concentrations of copper (0.5 mg/L) and iron (4.0 mg/L), elevated residual concentrations of H_2S (50 µg/L) and elevated residual concentrations of SO_2 (20 mg/L). PMT decreased in all samples over the course of six months (Figure 15), which is in agreement with the study that evaluated the evolution of PMT in real wine (Figure 14). Exposure to elevated residual metal concentrations had the most detrimental effect on PMT preservation, with a decrease of approx. 88% measured after six months in samples exposed to elevated residual copper and iron concentrations compared to control samples with no added metals (Figure 15). The factor with the second most detrimental impact on PMT preservation was exposure to elevated H_2S concentrations, with a 76% decrease measured after six months in samples exposed to high levels of H_2S compared to control samples (Figure 15). Interestingly, exposure to O_2 had no significant effect on PMT preservation, suggesting that PMT is not as susceptible to oxidation as other VSCs. Exposure to elevated SO_2 concentrations also decreased PMT concentrations; however, the changes in PMT concentration compared to the control samples were relatively small (Figure 15).

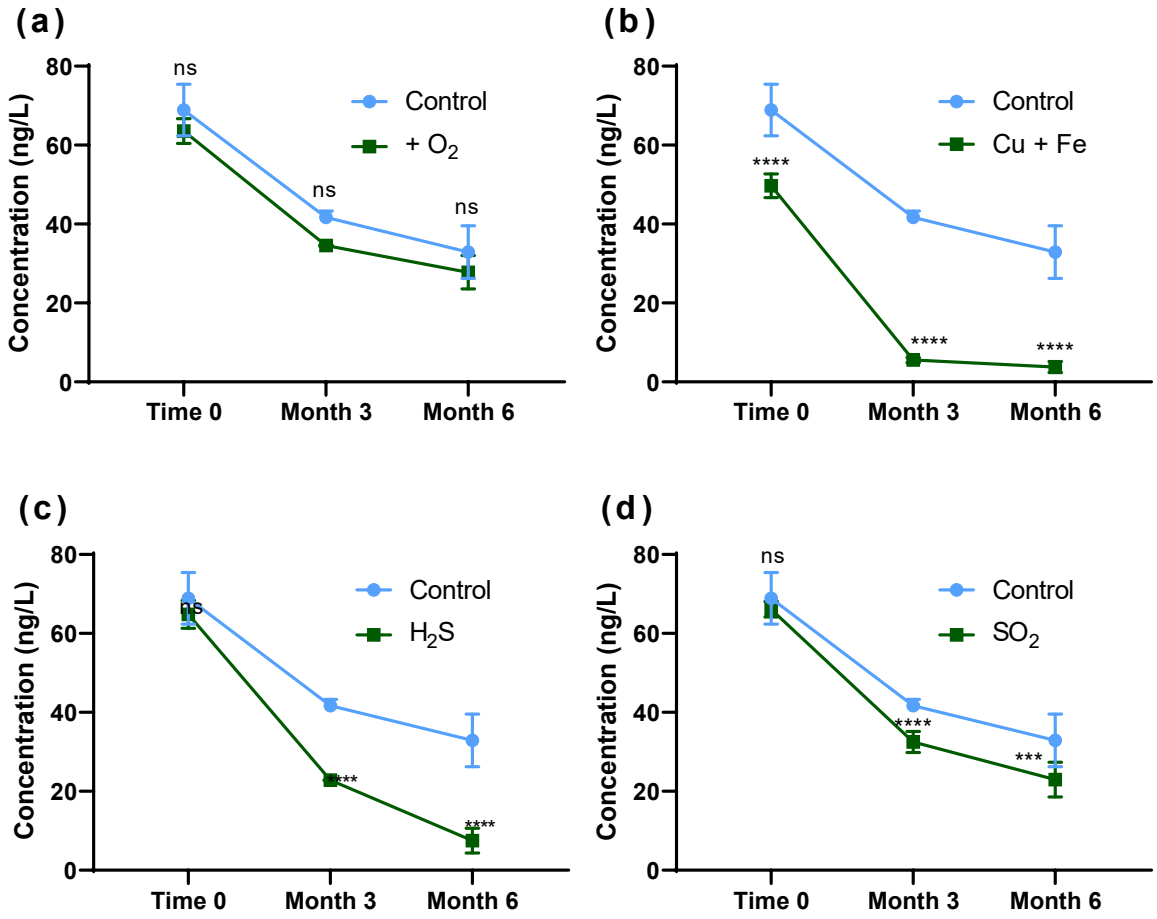


Figure 15. The effects of (a) oxygen (O_2) exposure (b) residual copper (Cu) and iron (Fe), (c) elevated hydrogen sulfide (H_2S) and (d) elevated sulfur dioxide (SO_2) concentrations on the preservation of phenylmethanethiol (PMT) in model wine.

This study demonstrated that anaerobic conditions promoted the formation of PMT from its precursor compounds, benzaldehyde and H_2S, but once produced, PMT was not as susceptible to loss due to oxidation as other smaller molecular mass VSCs. Exposure to metal ions, such as copper and iron, had significantly detrimental effects on both the formation and preservation of PMT. This implies that care should be taken when treating wines with copper as it may both inhibit formation of PMT and have detrimental effects on its preservation.

7.4.1.6 Pilot-scale trial in Chardonnay to assess the role of yeast strain and different additions on phenylmethanethiol formation

To confirm some of the previous results obtained under controlled conditions in the laboratory, a pilot-scale winemaking trial in Chardonnay (2022 vintage) is underway. The purpose of the trial was to assess the influence of different yeast strains and nutrient status (nitrogen additions) on PMT production. For this pilot-scale winemaking trial, high-quality hand-picked Chardonnay grapes were used from the McLaren Vale region (DogRidge). Nitrogen additions, in the form of the widely used winemaking additive DAP, were carried out in 30-litre lots of the Chardonnay must to achieve yeast assimilable nitrogen (YAN) concentrations of 150, 250 and 350 mg/L. Each of these nitrogen treatments was fermented with two different yeast strains. The resulting wines have just been bottled.

To further explore practical winemaking strategies that can be implemented to modulate the formation and preservation of PMT during vinification, a second pilot-scale winemaking trial in Chardonnay (2022 vintage) was designed. In this experiment, the effect of different additions on PMT formation during the ageing process was evaluated. These treatments include the addition of the fermentation lees back to the base wine combined with regular lees stirring, the adding of

either American or French oak chips, or adding additional SO_2 prior to bottling to try to preserve PMT in the bottled wine.

7.4.1.7 What is the consumer perception of 'flint' in wine?

The first targeted survey of PMT in a range of commercially available white wines was conducted, using chemical and sensory analysis to establish the natural variability of PMT. Phenylmethanethiol was found in all wines as well as a similarly potent sulfur compound, 2-furylmethanethiol (2-FMT). Both PMT and 2-FMT were linked with 'struck flint' aroma but with higher levels of 2-FMT, the aroma character of the wines tended toward 'burnt rubber'. A subset of the survey wines was characterised by sensory descriptive analysis and comprehensive quantitative chemical analysis. Descriptive sensory analysis showed the subset of wines had varied intensity ratings of 'struck flint' aroma. The sensory attribute ratings were related to volatile composition by PLS-R. 'Struck flint' aroma was positively associated with both PMT and 2-FMT.

Six of the wines covering a wide range of 'struck flint' aroma intensities were then chosen for consumer testing. Overall, the most liked wine was moderately high in 'peach' and moderate in 'struck flint' character. From the cluster analysis, two-thirds of consumers preferred 'fruity' wines high in 'peach' aroma, 'sweetness' and 'viscous mouthfeel' and low in 'flint/struck match' aroma, but one-third liked wines with the 'struck flint' and 'toasty' characters that were low in 'hotness' and 'bitterness'.

A follow-up odorant addition study was conducted to test the relative contribution of compounds PMT and 2-FMT that were associated with 'flint' aroma in the study of commercial wines. These potent sulfhydryls were added to a fruity Chardonnay using a full factorial design approach at five concentrations representing the range found in previously surveyed wines. Statistical analysis revealed strong evidence that both compounds contribute to 'flint' aroma rating, and surprisingly, 2-FMT appeared to exert an effect 20 times larger than PMT in the Chardonnay wine despite their identical detection threshold values reported in the literature (Figure 16). These compounds strongly suppressed the intensity of fruit aroma attributes.

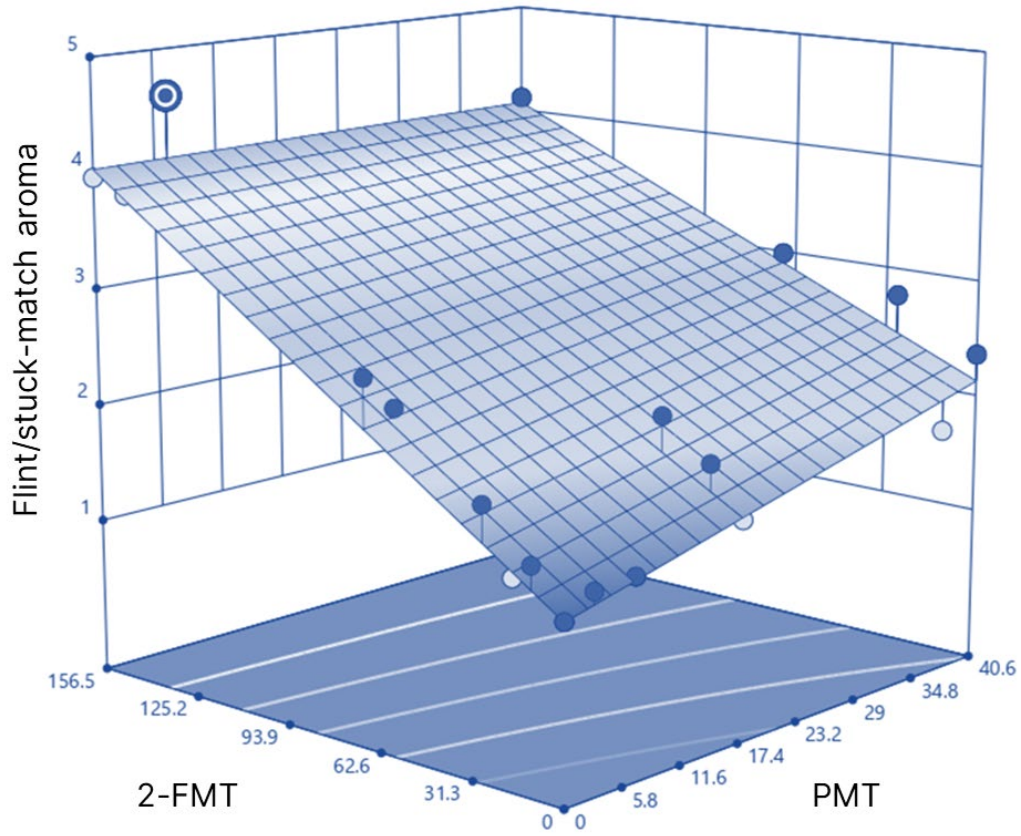


Figure 16. The response surface methodology (RSM) analysis of variance (ANOVA) graph representing the contribution to 'flint'/'struck-match' aroma as imparted by phenylmethanethiol (PMT) and 2-furylmethanethiol (2-FMT)

This work investigating the formation, preservation and consumer preference for wines containing 'flint' aroma was presented in Workshop #17 and in posters at the 18th AWITC in June 2022, at the In Vino Scientia Analytica conference, Neustadt, Germany in July 2022, and at the 2020 Tasmanian Winemaker Symposium. A paper describing the sensory evaluations has been submitted to the *Australian Journal of Grape and Wine Research* AWITC special edition (Espinase Nandorfy et al. 2022).

7.4.2 Practical strategies for increasing tropical sulfhydryl aroma

The genetic pathways that favour the formation of the 'tropical' sulfhydryls, 3-SH and 4-MSP, were also investigated. It was found that the choice of yeast starter culture plays a pivotal role in the formation of both 4-MSP and 3-SH in laboratory-scale fermentations in CDM. In addition, the yeast gene *IRC7* was identified as a molecular marker that could be used to predict a yeast's potential to release 'tropical' sulfhydryls. A peer-reviewed manuscript describing these results has been published (Cordente et al. 2019).

The 'tropical' sulfhydryls 3-SH and 4-MSP are important contributors to the flavour of white wines, particularly certain styles of Sauvignon Blanc and Chardonnay, but little is known about their formation and impact in red wine aroma and flavour. Tropical sulfhydryls can also be found in different red wines, although in lower concentrations than in white wines (Siebert et al. 2019). The ability of six wine yeast strains to release 'tropical' sulfhydryls in pilot-scale Grenache wines was also investigated. A commercial wine strain, AWRI2914, commonly used for expressing varietal sulfhydryls in Sauvignon Blanc wines, produced twice as much 3-SH as the other five strains assessed (Figure 17). This strain was shown to produce Grenache wines with intense 'red fruit' and 'cherry/confectionary' aromas, and both 3-SH and its acetate ester 3-SHA were associated with the 'fruity' attributes of the Grenache wines. These results revealed

an opportunity for winemakers to shape red wine aroma and flavour by using yeast that might typically be considered for white wine production. This work was published in Cordente et al. (2018) and Cordente et al. (2022).

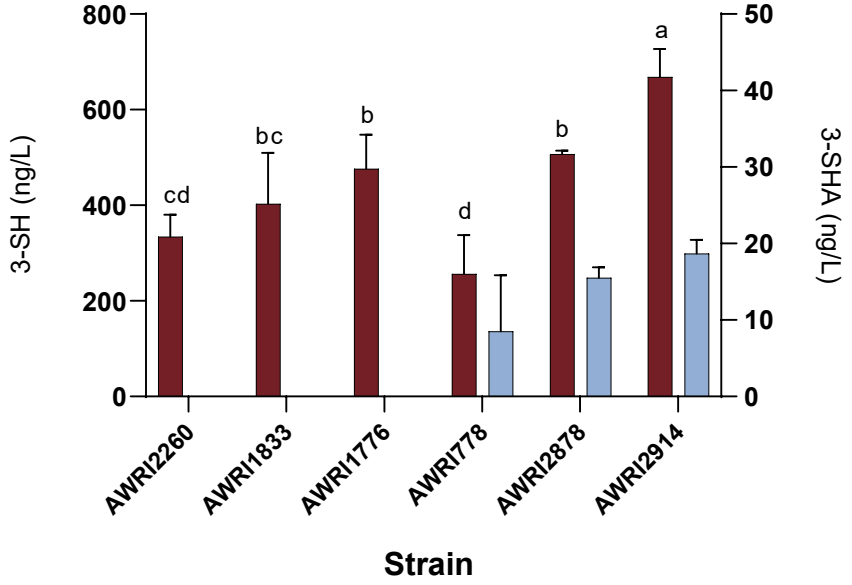


Figure 17. The concentration of 3-sulfanylhexan-1-ol (3-SH) (red) and 3-sulfanylhexyl acetate (3-SHA) (blue) in Grenache wines fermented with six yeast strains with different sulfhydryl-releasing abilities (as determined by their *IRC7* genotypes), after nine months in bottle.

7.5 Practical strategies to decrease the occurrence or impact of negatively perceived volatile sulfur compounds: removal of volatile sulfur compounds

7.5.1 Practical advice on the performance of commonly remediation strategies for removing 'reductive' aromas

Winemakers make use of various remediation strategies to manage 'reductive' aroma formation. Traditionally, copper fining is one of the most commonly used remediation strategy and it is known to be effective in the short term (i.e. weeks to months)' however, during long term storage (i.e. months to years) excess residual copper that remains in the wine post-treatments may increase the risk of liberating VSCs in wines post-bottling. Other remediation techniques, such as DAP additions, oxidative handling and racking, and fresh lees addition are also commonly employed to ameliorate unwanted VSCs.

In this study, the relative effectiveness of five commonly used 'reductive' aroma remediation strategies was compared. Five wines (in triplicate) were prepared and at the onset of 'reductive' aromas, each wine was treated with a unique remediation strategy, namely:

1) copper fining ('Copper'),
2) macro-oxygenation ('Macro-ox')
3) a combination of macro-oxygenation and copper fining ('Macro-ox + copper')
4) DAP addition ('DAP'),
5) the addition of fresh lees ('Lees').

The effectiveness of the different treatments was evaluated over the course of 12 months using chemical and sensory analysis techniques.

The results of this study showed the remediation techniques had varying levels of effectiveness in treating the 'reductive' aromas. Small changes in 'Total' VSC concentrations were associated with large differences in the sensory profiles of each wine. The wines treated with macro-oxygenation ('Macro-ox' and 'Macro-ox + copper') produced wines with enhanced 'red fruit'

aroma and flavour attributes as well as lower 'drain' and 'boiled egg' sensory attributes compared to all the other treatments (Figure 18). This showed that for this study, 'Macro-Ox' treatments were the most successful in decreasing the sensory perception of 'reductive' faults (Figure 18). Wines that were treated with 'DAP', 'Lees', or fined with 'Copper', were not effective in fully diminishing 'reductive' notes; instead, these treatments were each associated with increased notes described as 'drain' (sewage-like), 'boiled egg' and 'rubber', respectively, when measured 12-months post-bottling (Figure 18).

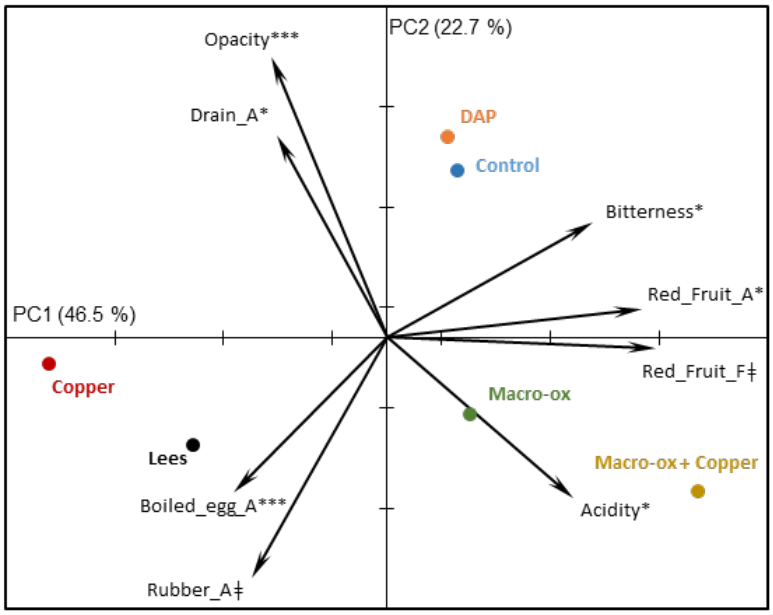


Figure 18. Mean attribute intensity scores for significant (*P<0.05; **P<0.01; ***P<0.001) and approaching significance (‡ P< 0.16) attributes for the 'reduction' remediation treatments. LSD (P=0.05) values were included for the significant attributes (P<0.05).

This work provided practical guidance to winemakers on the performance of commonly used remediation strategies employed for the removal of 'reductive' faults as well as the expected outcomes of these treatments on important red fruit wine aromas and flavours. This work was published in Bekker et al. (2020), given as a plenary presentation in a Fresh Science session at the 17th AWITC in 2019, presented at the Oeno IVAS conference in Bordeaux in 2019 and shared with the sector in numerous roadshow talks and webinars.

7.5.2 Can tannins be used to 'freshen up' 'reductive' aromas in wine?

The most commonly implemented remediation strategies were discussed and compared in the previous section. However, winemakers also implement a range of other treatments to ameliorate unwanted sulfur-related off-odours in wine. An example of such a treatment is using oenological tannins to 'freshen up' ferments. Anecdotal evidence suggests this treatment may assist in diminishing the 'reductive' character of wines, yet there is no scientific evidence supporting this observation nor an explanation of the potential mechanism of such an effect. During wine oxidation, nucleophilic addition of quinones with a variety of electrophiles, including VSCs, has been suggested as a possible mechanism for this wine aroma change. A collaborative study with Prof. Felipe Laurie (The University of Talca, Chile) was initiated where two commercial tannin products were evaluated for their ability to remove VSCs. The aims of this work were:

1) to provide winemakers with advice on whether oenological tannin treatments can be used to successfully remove VSCs
2) to unravel the underlying mechanism through which oenological tannins may diminish 'reductive' aromas in wine.

In the first experiment, oxygen-free model wine, red wine and white wine samples containing H_2S, MeSH, and EtSH (50 µg/L each) were prepared containing copper (0.3 mg/L) and iron (2.5 mg/L). The wines were then treated with two commercially available oenological tannins, Tannin V and Tannin Q (100 mg/L and 400 mg/L each, Laffort Nuriootpa SA) and the VSC concentrations were measured after 24 hours. Experiments were performed at two pH levels as it is known that pH affects the formation of quinones and subsequent adduct formation. From these experiments, it was clear that the oenological tannins indeed removed VSCs, and that H_2S was removed at the most rapid rate (Figure 19). Treating 'reductive' aromas with Tannin Q resulted in the greatest decrease in H_2S, MeSH and EtSH concentrations However, it was less clear whether changes in pH or higher concentrations of tannin affected the amelioration action of the oenological tannins.

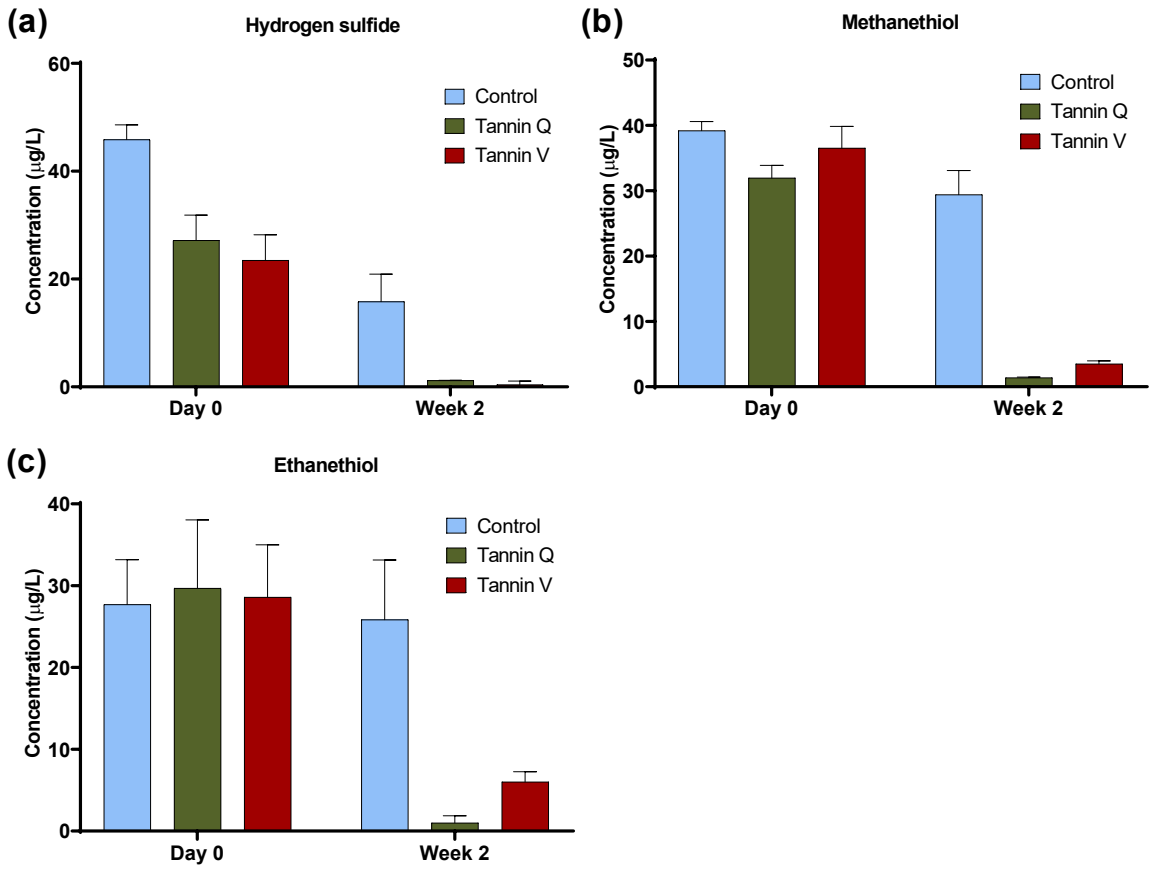


Figure 19. Decrease in (a) hydrogen sulfide, (b) methanethiol, and (c) ethanethiol in model wine after treatment with Tannin Q and Tannin V.

To unravel the mechanism by which the commercial oenological tannin products removed VSCs, an experiment was designed where Tannin Q and Tannin V (100 mg /L each) were added to model wine containing copper (0.3 mg/L) and iron (2.5 mg/L) as well as MeSH and EtSH (50 µg/L each). In this series of experiments, MeSH and EtSH concentrations decreased significantly as a result of treatment with tannin products, and the subsequent formation of vescalagin and gallic acid adducts with MeSH and EtSH were also measured (Figure 20). It was also noted that a certain percentage of disulfides were produced as catalysed by copper (Figure 20), which is in agreement with previous studies; however, a significant portion of MeSH and EtSH was removed by the tannin products.

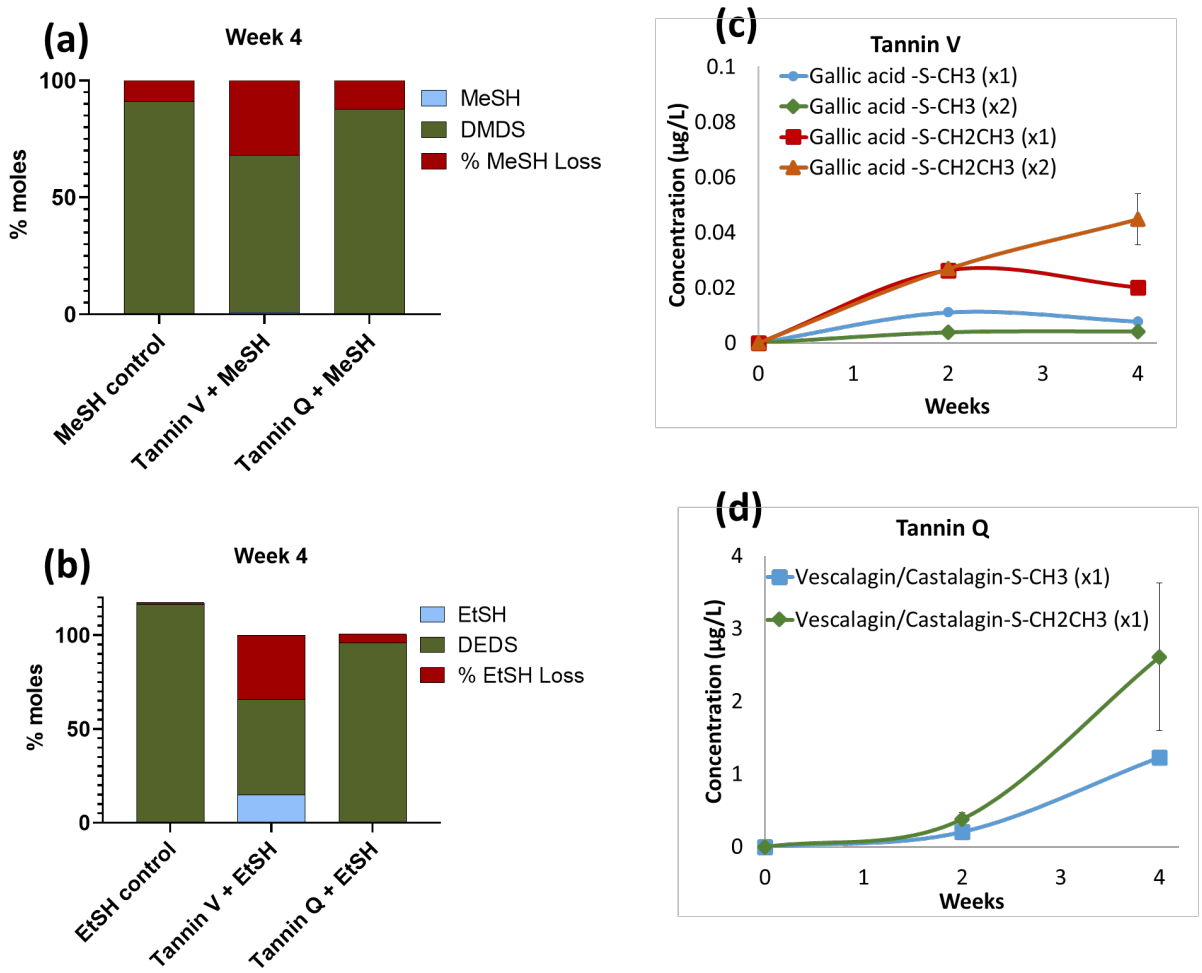


Figure 20. Effect of tannin addition on the decrease of (a) methanethiol (MeSH) and (b) ethanethiol (EtSH) measured after four weeks; as well as the subsequent formation of disulfides (dimethyldisulfide, DMDS, and diethyldisulfide, DEDS) and adducts with MeSH and EtSH in samples treated with (c) Tannin V and (d) Tannin Q.

These results demonstrated that winemakers could use commercial tannin products to successfully remediate unwanted 'reductive' aromas and that the mechanism for VSC scalping by tannins is through the formation of oxidation reaction products with the sulfur compounds.

7.5.3 Development of novel innovative tools to remediate 'reductive' faults in wine

There has been no targeted development in remediation technologies to treat 'reductive' aromas in wine for the last 20 years. The most commonly used strategy internationally is fining with copper, which increases the risk of developing latent 'reductive' aromas and is a regulated additive with low residual legal limits of 0.5 mg/L as determined by the European Union and the International Organization of Vine and Wine. To overcome the limitations of copper fining efficacy and provide a technology that does not introduce additives into wine, a novel smart technology was developed that specifically targets the removal of H_2S, MeSH and EtSH, as these compounds are the main species associated with 'reductive' aromas. The technology is based on the use of citrate-reduced gold nanoparticles immobilised on a plasma polymer-coated surface, as schematically presented in Figure 21.

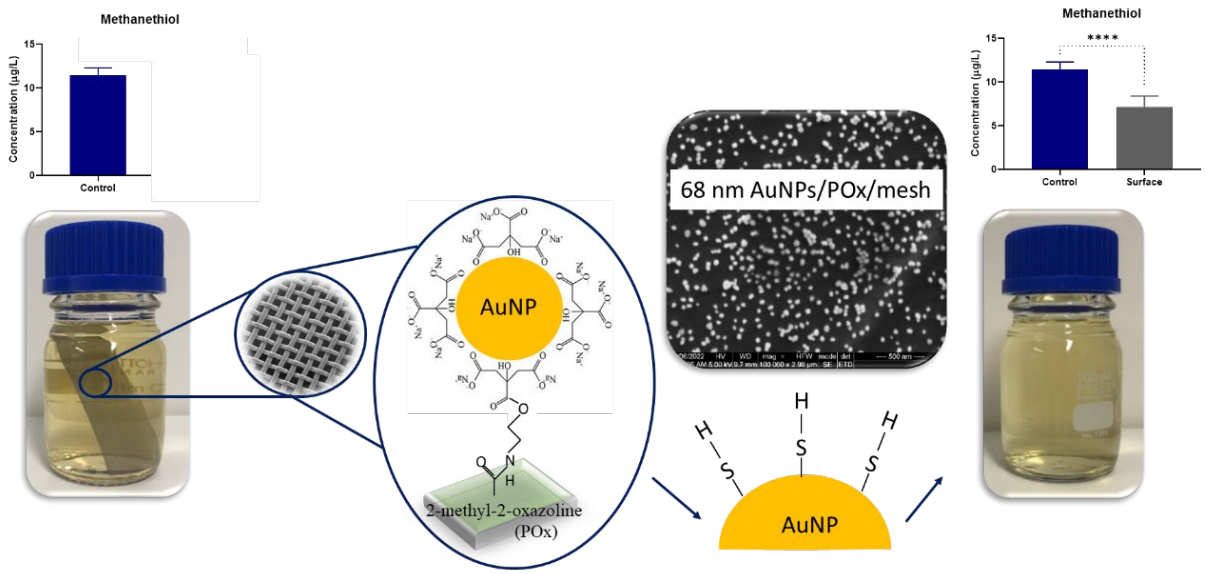


Figure 21. Schematic representation of the process of generation of smart surfaces and their use in wine

The capacity to remove H_2S, MeSH and EtSH from spiked model wine solutions was investigated using two different sizes of gold nanoparticles deposited on POx and AA underlayers. Results indicated that surfaces with 68 nm diameter gold nanoparticles immobilised on POx were the most effective. Based on these results, the POx/68 nm AuNPs platform was selected to further investigate the effect of exposure time to sulfur compounds in a model wine for effective VSC removal. Exposure times of 3, 6 and 24 hours were tested. The longest exposure time was the most effective and was used in the follow-up experiments.

To maximise the removal efficiency of undesirable sulfhydryl compounds in wine, the surface area of 'smart' surfaces was increased by immobilising gold nanoparticles on plasma-coated stainless steel mesh surfaces. The efficiency of the mesh surfaces in removing H_2S was compared with that of previously investigated glass surfaces. The results showed that the increased surface area improved H_2S removal capacity from 70% for glass to 90% for mesh surface in model wine matrices containing elevated H_2S concentrations.

Furthermore, the mesh surfaces were evaluated and compared with copper fining for their ability to remove free H_2S, MeSH and EtSH in wines containing naturally high concentrations of these VSCs (Figure 22). It was found that free H_2S, MeSH and EtSH concentrations decreased in all white wines treated with the smart surfaces, and that the treatment was as or more effective as copper fining for white wines. This suggests that the newly developed technology can be used as an alternative to copper fining.

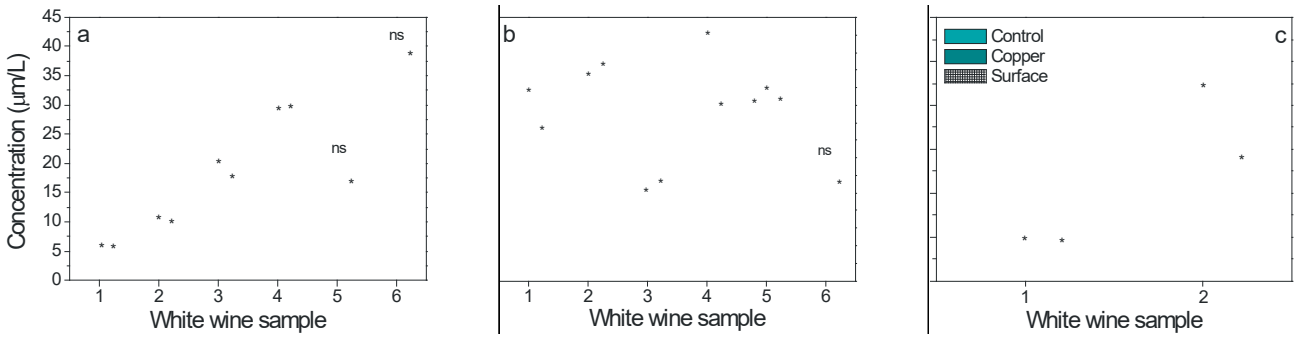


Figure 22. The concentration of a) hydrogen sulfide, b) methanethiol and c) ethanethiol in white wine samples before and after treatment with copper and smart surfaces. Data significance was assessed by Student's t-test.

In addition, the selectivity of novel smart surfaces in removing 'positive' tropical sulfhydryls was also evaluated in white wine. No significant decrease in 'positive' tropical sulfhydryl concentrations in the remediated wines was measured compared to the controls. Experiments evaluating the effects of SO_2 on H_2S removal also showed no interference of SO_2 with H_2S binding and removal as facilitated by the smart surface.

In summary, this project has developed novel nanoengineered surfaces and demonstrated that they can remove unwanted 'reductive' aromas from wine without altering its tropical fruit character. The ability of the nanoengineered surfaces to remove the 'total' fraction of unwanted sulfhydryl compounds and the 'free/non-metal bound' fraction of unwanted sulfhydryl compounds was compared in real wine. A fraction of strongly bound sulfhydryls was not able to be removed with either nanoengineered surfaces or traditional copper fining. However, 'free' sulfhydryls were readily removed with the newly developed technology. The surfaces were more effective in eliminating MeSH than copper fining, with up to four times greater amounts of MeSH removed when using the nanoengineered surface compared to copper fining. A key advantage of this technique is that it is an easily deployable and retrievable processing platform, making it a one-step process (a surface is added to the wine and then removed after a specific time). The process can be repeated if necessary.

A paper describing this work has been prepared for submission to *npj Science of Food* (Mierczynska-Vasilev et al. 2022).

7.5.4 The role of copper in the perception of 'reductive' faults

The perception of 'reductive' aroma faults is more complex than merely considering the aroma threshold values of compounds associated with unwanted sulfidic off-aromas. Even though specific sulfur compounds have been identified as the main compounds associated with certain 'reductive' aromas, the perception of these aromas is influenced by the synergistic interaction of other volatile and non-volatile compounds present in wine. The perception of VSCs is further complicated by the reactive nature of sulfhydryls and their ability to exist in reversible free and metal-complexed forms (Vela et al. 2018). It is known that copper plays an important role in determining whether VSCs such as H_2S and MeSH exist in 'free' and 'metal-complexed' forms. Furthermore, recent work by Zhang et al. (2021, 2022) has provided insight into copper speciation and the associated implications of VSC speciation in wine.

To determine how copper speciation (total copper, labile/non-labile copper) modulates 'free' and 'total' H_2S and MeSH, and how it relates to 'reductive' character and the sensory perception of white wine, a collaborative study was undertaken with Dr Andrew Clark from the Gulbali Institute (Charles Sturt University, NSW) which involved a survey of 49 white wines. Approximately half of the wines selected for the trial were selected based on having 'reductive' character. The wines were analysed for 'free' and 'total' H_2S and MeSH by both the AWRI and NWGIC using GC-SCD methods, as well as measuring H_2S using a gas detection tube (GDT) method as described in Allison et al. (2021). Other analyses were performed to evaluate the effect of factors such as pH, free-, bound- and total SO_2, ascorbic acid, GSH, and tropical sulfhydryls on the perception of 'reductive' aromas.

Assessing the differences between the 49 wines on 'reductive' aromas using ANOVA showed very strong statistical evidence ($P<0.005$) that all sensory attributes, overall 'fruit', 'boiled' and 'rotten egg', 'natural gas'/'sewage'/'durian', 'canned corn'/'asparagus', and 'rubber' aroma differed among the wine set. To aid in understanding the associations between the analytical measures (x) and the sensory attributes (y), PLS-R was conducted. Of the well modelled 'reductive' sensory attributes, 'boiled egg' 'rotten egg' and 'natural gas'/'sewage'/'durian', measurements of 'Free H_2S', 'H_2S GDT measurement', 'Free MeSH', and 'Total MeSH' were found to have highly positive regression coefficients and/or significance by the jack-knifing test.

The measures of 'Total H_2S' and 'Bound H_2S' and all copper species (Cu-I, Cu-II, and Cu-III) were not found to be significant for 'reductive' sensory attributes and had very small regression coefficients. These simple associations identified by PLS-R can be visualised by a correlogram shown in Figure 23. The 'Total Cu' and 'Cu-III' measurements correlated well with total and bound H_2S, however, these measures did not correlate with the 'reductive' sensory attributes. Conversely, 'Free H_2S', and 'Free MeSH' were well correlated with 'boiled egg', 'rotten egg' and 'natural gas'/'sewage'/'durian' aromas.

This study provided valuable insights into how different VSC measurements related to 'reductive' aromas. Measuring 'Total H_2S' may provide insights into the major portion of this VSC in wine; however, this fraction of the VSCs does not correlate with the perception of 'reductive' faults. This study demonstrated that quantifying 'Free H_2S', 'Total MeSH' and 'Free MeSH' provides the most accurate indicator of whether a wine may be perceived as 'reductive' by consumers. Another important finding of this trial was that 'Total copper' indeed correlates with 'Total H_2S'; however, this does not correlate with 'Free H_2S' or the sensory perception of 'reductive' faults.

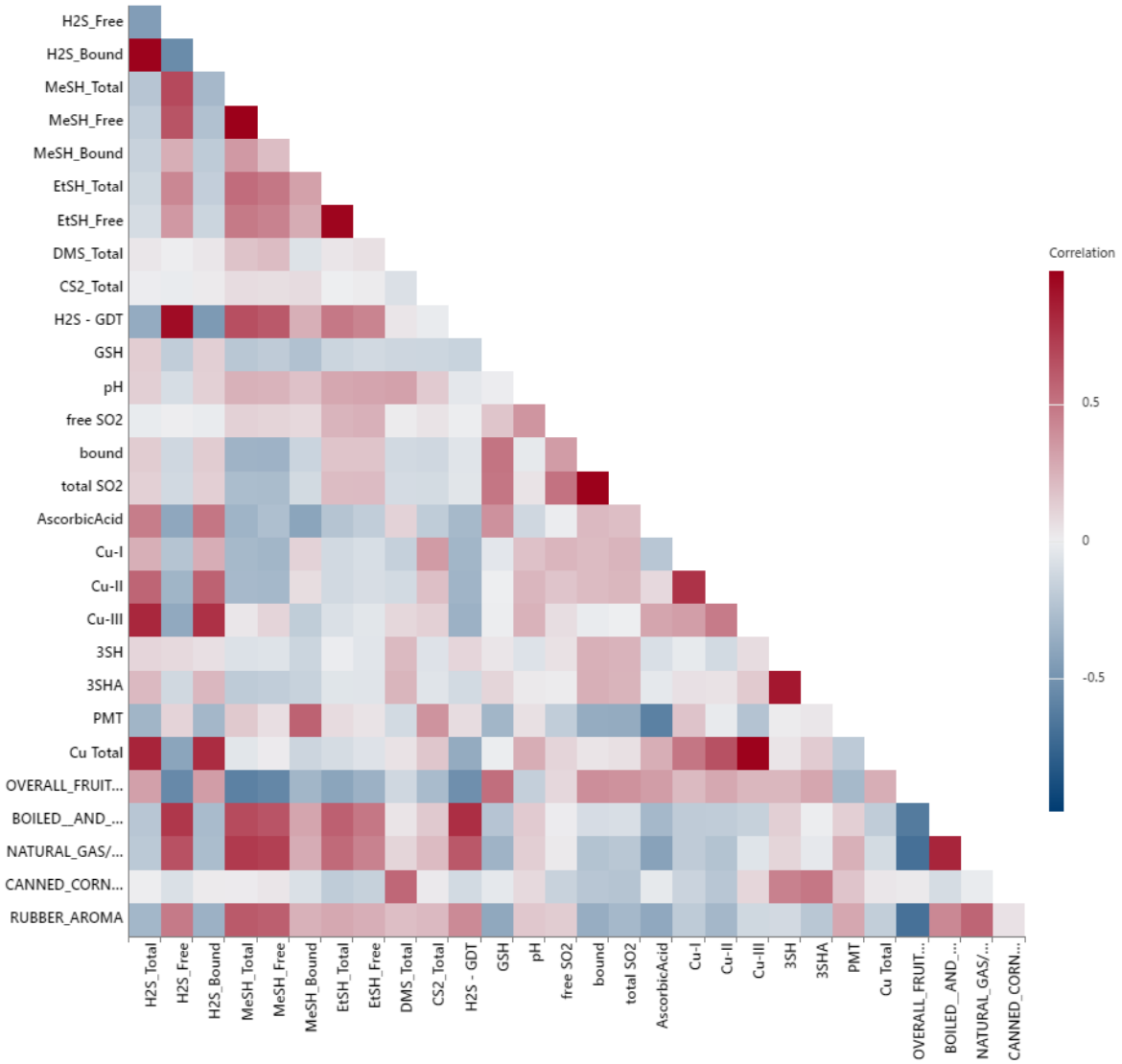


Figure 23. Correlogram of mean analytical measurements and sensory attributes associated with 'reductive' aromas. The colour gradient describes the strength of the correlation between the analytical measurements and sensory attributes, with strong positive correlations shown in dark red and strong negative correlations shown in dark blue.

8. Outcome and conclusion

8.1 Improved methods of analysis for known and new VSCs, and their precursors and breakdown products

To be able to study 'reductive' aroma compounds, their speciation in wine, their precursors and other latent forms of VSCs, six new methods were either developed as original methods or adapted and improved from published methods.

A method for the quantification of GSH, GSSG and glutathionyl-based polysulfides was developed utilising HPLC and LCMS capabilities. It is known that these compounds may act as precursors to H_2S, which makes this method an important tool for evaluating risk and elucidating the formation pathways of VSCs and their important precursor compounds. This method has been submitted for publication (Bekker et al. 2022).

Two GC-MS methods, based on the methods published by Ontañón et al. (2019), Nguyen et al. (2012), and Franco-Luesma and Ferreira (2014) were developed to quantify up to ten ^{34}S-labelled and unlabelled ^{32}S-VSCs. These methods can differentiate between 'Total', 'Metal-bound', and 'Free' sulfhydryls. These methods have been used in this project to elucidate the chemical formation pathways of polysulfides, for sensory-based experiments for national and international collaborative projects. The methods have been validated by Affinity Labs to be made available to the Australian wine sector for routine analysis in the future. These methods have also been used by Affinity Labs for project work for external clients from the Australian wine sector.

The analysis of polyfunctional sulfhydryls, which includes the method for analysing PMT ('flint' aroma compound) is of significant importance for the majority of projects at the AWRI, especially for experiments that produce wine that will undergo sensory evaluation. These methods have been substantially improved to allow for high-throughput analysis and improved stability by using a novel derivatising agent. These methods have been critical for the success of the experiments of this project, have increased the number of samples analysed for other internal projects and for external clients, and have been used for collaborative projects with national and international collaborators to elucidate the role of copper/VSC speciation on the sensory perception of 'reductive' faults well as for experiments with national and international collaborators to elucidate the chemical formation pathways of VSCs (E. & J. Gallo, USA, Plumpton College, UK, University of Adelaide, Australia), as well as for sensory-based experiments to determine the effect of free- and metal-bound 'reductive' and tropical sulfhydryl fractions on the sensory characteristics of wine (Stellenbosch University, South Africa; Charles Sturt University, New South Wales).

Finally, a novel method was developed to monitor putative benzoic precursors and related benzoic breakdown products of PMT. This method was used to elucidate the chemical and biochemical formation pathways of PMT.

8.2 Knowledge of precursors to volatile sulfur compounds and their release mechanisms

Building on the work from previous years (2011 – 2016) that evaluated the contribution of the main precursors to VSCs in modulating 'reductive' aroma formation in wine, it was clear that the prevalence of certain compounds, such as H_2S, was not fully explained by the precursors proposed in the literature. In collaboration with world-renowned researchers from Plumpton College (UK), E. & J. Gallo Winery (USA) and the University of Adelaide, a study was initiated that investigated the ability of a novel class of sulfur compounds, namely polysulfides, to release H_2S in wine during bottle ageing. This study demonstrated that polysulfides can indeed act as latent sources of H_2S; the stability of dicysteinyl-polysulfides was established over a six-month period; and commonly used wine additives that facilitate the breakdown and subsequent release of H_2S were identified. This work was published in Bekker et al. (2018), and in 2019 this was the paper most requested from the AWRI library by the Australian wine industry. This body of work played a significant role in establishing the importance of polysulfides as latent sources of

'reductive' aromas in wine and has initiated a large body of international research that is further investigating the prevalence of polysulfides in wine.

The formation of VSC precursors was further explored by evaluating commonly used winemaking strategies that may promote the formation of unwanted sulfidic off-aromas. Aerative winemaking practices, which include aerative racking and splashing as well as macro-oxygenation, have the potential to promote the formation of symmetrical and asymmetrical disulfides and polysulfides, which may act as latent sources of VSCs. To evaluate whether latent sources of VSCs may be produced as an unwanted consequence of these aerative winemaking practices, an experiment was designed that monitored the formation of polysulfides as well as the formation of irreversible 'sulfur traps' that bind and permanently remove unwanted VSCs from wine. This study demonstrated that macro-oxygenation only produced a small amount of polysulfides, and mainly promoted the formation of 'sulfur traps' in the form of oxidative reaction products that remain stable for up to 12-months post-bottling. This work elucidated the chemical mechanism and provided evidence for the long-term success of macro-oxygenation techniques that has been observed in previous trials (Bekker et al. 2016, 2020, Day et al. 2021). This work provides evidence that winemakers can implement oxidative remediation strategies with confidence without increasing the risk of developing latent sources of VSCs that may have detrimental effects on wine aroma post-bottling. This work was presented at the 2021 PacifiChem Conference and shared in AWRI roadshow talks (2019, 2020, 2021, 2022), and presented in two AWITC workshops (17th AWITC Workshop #15, 18th AWITC Workshop #17).

8.3 Improved understanding of the role of microbiological factors (biochemical pathways, microbial ecology, nutrition etc.) in volatile sulfur compound formation

This project has provided major insights into the nature of the different biochemical pathways responsible for the formation of different VSCs. The gene *IRC7* was found to play a pivotal role in the formation of 3-SH and 4-MSP, the latter two compounds associated with 'tropical fruit' aroma. This work identified *IRC7* as a molecular marker that could be used to predict a yeast's potential to release tropical sulfhydryls. Furthermore, the biochemical pathways responsible for the formation of the odoriferous VSCs, MeSH and MeSAc from the amino acid methionine were also studied, and this work showed that there is a natural genetic variability of yeast strains in relation to their ability to release these negative VSCs. This information can guide winemakers to choose the right strain to avoid the formation of these off-flavours in wine.

The diversity in the ability of wine yeast strains to release tropical sulfhydryls in the red variety Grenache was also demonstrated. A commercial wine yeast strain, commonly used for expressing tropical sulfhydryls in Sauvignon Blanc wines, was shown to produce Grenache wines with intense 'red fruit' aromas, highlighting that yeast strain selection is an easy and effective way to drive red wine style.

The results of these trials have provided winemakers with clear evidence on which yeast strains can be selected to increase sought-after 'red fruit' aromas and decrease unwanted 'reductive' aromas. They have been published in peer-reviewed journals (Cordente et al. 2019, 2022), in *AWRI Technical Review* articles (Cordente et al. 2017, 2018), presented at national and international conferences, presented in two AWITC workshops (17th AWITC Workshop #15, 18th AWITC Workshop #17).

8.4 Chemical and biochemical factors influencing the occurrence of VSCs

Before starting this project there was virtually no information in the scientific literature regarding the factors driving the formation of the 'flinty' compound PMT during fermentation, or factors that affect the preservation of this compound in finished wine. A series of experiments demonstrated that managing a range of practical winemaking strategies, such as yeast selection and juice nitrogen status, has significant implications on the final concentration of PMT in wine. This project demonstrated in different fermentation media that the amount of PMT produced during fermentation depended on the starter culture used, and under some

experimental conditions, the amount of PMT in wine positively correlated with the amount of H_2S produced by the wine yeast. The nutritional level of the fermentation media was also shown to significantly affect PMT formation by yeast, as nitrogen additions in the form of inorganic nitrogen promoted the formation of PMT by yeast.

Practical winemaking practices and additives that may affect the formation of PMT from its precursor compounds, benzaldehyde and H_2S, were also investigated. It was demonstrated that protecting wine from oxygen facilitated the reaction between benzaldehyde and H_2S and resulted in the production of a substantial amount of PMT. Factors that have detrimental effects on this formation reaction were also identified, and experiments showed that the presence of copper and iron significantly inhibited the formation reaction of PMT. These experiments showed that wines should be protected from oxygen and any exposure to metals such as copper or iron should be limited if winemakers aim to produce the sought-after 'flint' aroma in their wine.

The winemaking conditions and additives that may promote or inhibit the preservation of PMT during ageing and post-bottling were also investigated. Oxygen exposure did not affect the preservation of PMT, but high residual concentrations of metal ions such as copper and iron as well as SO_2 and H_2S negatively affected the preservation of PMT in model systems. Vintage experiments are currently underway to confirm these results in winery-scale trials.

As a result of this research there is now a better understanding of the role of the microbiological, nutritional and winemaking conditions that modulate PMT formation. These results provide winemakers with guidance on how to choose a particular yeast strain to modulate the formation of PMT in wine, how to manage winemaking conditions to promote the formation of PMT, and which factors to avoid to facilitate the preservation of PMT post-bottling. All these factors can be employed as simple and effective methods to influence white wine style.

To better understand the prevalence of 'flint' aroma in Australian white wine and evaluate when this aroma character goes from 'flint' (accepted and sought after) to plastic/reduced/burnt rubber/smoky (unaccepted), a target survey of predominantly Australian Chardonnay wines was undertaken. This survey highlighted that both PMT and 2-FMT are linked to 'struck flint' aroma, but when higher levels of 2-FMT are present, the character might tend toward 'sulfurous/burnt'. From the comprehensive sensory and chemistry study, 'struck flint' aroma was modestly related to PMT, as previously reported, and 2-FMT was found to be strongly implicated. This is the first study that has demonstrated the role of 2-FMT in the perception of 'flint' aroma. The consumer testing study highlighted specific consumer segments that either preferred wine with 'fruity/peachy' character, or preferred wines with 'flinty' character.

The results of these experiments were presented at local and international conferences, presented at the 18th AWITC conference as posters and in Workshop #17. A section of this work was presented in the Fresh Science Session B at that same conference. A manuscript describing the sensory evaluations has been submitted to the *Australian Journal of Grape and Wine Research*, AWITC special edition (Espinase Nandorfy et al. 2022).

8.5 Practical strategies to decrease the occurrence or impact of negatively perceived volatile sulfur compounds: removal of volatile sulfur compounds

For this part of the project, the commonly used remediation strategies were evaluated for their ability to ameliorate 'reductive' aromas. This is the first trial of this nature that provided winemakers with the ability to compare and evaluate the effectiveness of remediation strategies when applied to the same base wine. From this experiment it was clear that macro-oxygenation was the most successful remediation strategy in terms of removing 'reductive' aromas and increasing 'red fruit' aroma and flavour in the Shiraz wine tested. This work was published in a peer-reviewed journal (Bekker et al. 2020), presented in national and international conferences,

presented in AWRI roadshow seminars and webinars (2017 – 2022), and presented for invited local and international seminars. The wines produced in this trial were presented to the sector at the 17th AWITC Workshop #15 as well as during the AWRI roadshow seminar series (2019). The paper describing this work was recognised by the *Australian Journal of Grape and Wine Research* as one of the top 10 most downloaded papers among work published between January 2019 to December 2020.

Alternative remediation strategies were also evaluated. There was some anecdotal evidence about using commercial tannin products to remediate 'reductive' faults; however, there was no evidence in the scientific literature to support these claims. This project investigated the chemical mechanism that facilitates the removal of unwanted sulfur compounds associated with 'reductive' faults through binding with the polyphenolic functional groups of ellagic tannins. Results showed a decrease of VSC concentrations and a subsequent increase in tannin-VSC adducts, providing evidence for the successful application of this remediation strategy, as well as elucidating the mechanism driving the removal of VSCs when using commercial ellagic tannin products to improve 'freshness' of 'reductive' wines. This work was presented at the 18th AWITC as a poster.

There has been no targeted development in remediation technologies to treat 'reductive' aromas in wine for the last 20 years. The main strategy employed by the Australian wine sector, as well as internationally, is using copper fining to ameliorate unwanted sulfidic off-aromas. Copper fining has been associated with an increased risk of developing latent 'reductive' aromas, as well as increasing the rate of wine oxidation and decreasing sought-after tropical sulfhydryl aromas. Furthermore, the use of copper is regulated with low residual legal limits of 0.5 mg/L. To address the limitations in the effectiveness of copper fining and provide a technology that does not introduce additives into wine, a novel smart technology was developed specifically targeting removal of H_2S, MeSH and EtSH, as these compounds are the main species associated with 'reductive' aromas. The surfaces were successful in removing the 'free' fractions of H_2S, MeSH, and EtSH from model and real wines, and performed as well or better than copper fining in the wines evaluated in this study. This new technology has future potential for commercialisation based on its effectiveness in removing 'reductive' sulfhydryls. It has the potential to overcome the limitations of copper addition currently used to remove unpleasant 'reductive' aromas. Potentially, this technology could be applied to filtration apparatus, remediation application devices, novel aerators and decanters, wine packaging materials and wine closures, to translate the research outputs into industry outcomes. A major advantage of using plasma polymer coated surfaces in wine processing is that the functionalised surfaces are considered processing aids rather than wine additives.

8.6 Practical advice and extension materials for winemakers

The scientific and technical achievements of Project 3.4.2 were of the highest standard, with novel scientific findings published in international peer-reviewed journals and the results presented at national and international conferences and workshops, often by invitation. Throughout the five-year investment period, Project 3.4.2 prioritised the dissemination of the novel findings of the research. This was done using easily accessible formats, which were made available to the sector in a practical manner with clear guidelines on how to apply the research in their winemaking. As described in the Final Report for Project 2.2.1 – Helpdesk, one-third of all the sensory investigations performed by the AWRI helpdesk were related to 'reductive' winemaking faults, predominantly off-aromas associated with H_2S and MeSH. The investigations revealed that some producers still lack understanding of the impacts that yeast choice, for example selecting a high-nitrogen demand yeast vs a moderate nitrogen demand yeast, can have on fermentation. Producers also inquired about the removal of copper following copper fining remediation treatments. This demonstrates the demand by the Australian wine sector for continued research and support in managing 'reductive' faults.

A brief summary of the benefits to the Australian wine industry and how the information was communicated to the sector, is provided below:

- The methods adopted by this project were also adopted by Affinity Labs and used for project work for commercial clients from the Australian wine sector.
- The latest results on managing the formation and fate of VSCs were presented to the sector in the form of two webinars delivered in 2018 and 2019. The response to the webinars was exceptionally good, with 105 and 150 registered attendees for each and with over 1,600 and 2,100 online views of the YouTube recording, respectively, for each webinar. Up to 83% of the respondents stated that they would reassess their current practices and implement the new learnings after attending the webinars. These are in the top 13% most popular webinars published by the AWRI in the last five years.
- The results of this project were communicated directly to the sector through presentations at industry workshops (2018 Oxidation in Winemaking workshop, Pernod Ricard; 2019 AB Biotek workshop) as well as communicated through 13 seminars presented as part of the AWRI roadshow seminar program to the Australian grapegrowers and winemakers in NSW, QLD, SA, and Victoria. During the 17th AWITC (2019) and the 18th AWITC (2022) two workshops were facilitated that were the first to sell out and had the maximum number of participants attending each workshop (approximately 60 attendees). The feedback from the attendees was exceptionally positive. Results of this project were also showcased in the Fresh Science plenary sessions at both AWITC conferences. Additionally, the wines produced for the remediation comparison trial (Section 7.5.1) were made available to the sector for tasting at the 17th AWITC in Workshop #15 and at seminars as part of the 2019 AWRI roadshow seminar program.
- The results of the projects were also shared at international conferences and workshops by invitation:
    - 2017 American Society of Enology and Viticulture: invited speaker for main conference as well as an invited speaker to 'New Understandings in Wine Oxidation Chemistry Symposium'
    - 2020 The International Chemical Congress of PacifiChem Basin Societies: invited speaker for a symposium entitled 'Advances in Wine and Beer Chemistry'
    - 2020 42nd South African Society of Enology and Viticulture Conference: invited to facilitate a workshop on managing 'reductive' aromas (cancelled due to COVID)
    - 2020 Tasmanian Winemaker Symposium: invited speaker; 2021 New Zealand Syrah Workshop: invited speaker.
- Overall, 12 peer-reviewed papers were published, two *Technical Review* articles, and four book chapters (see Appendix 1).
- The projects investigating the precursors and formation pathways of positive tropical sulfhydryls and negative 'reductive' sulfhydryls have identified target precursor compounds for winemakers to monitor to gauge a wine's risk for developing 'reductive' aromas. Commonly used wine additives that increase the release of H_2S from precursors have also been identified and winemakers were cautioned on how to use them to decrease the risk of liberating VSCs under specific conditions. The results of these trials have been published in six peer-reviewed papers (Bekker et al. 2018a, Kontoudakis et al. 2018, Bekker et al. 2018b, Bekker et al. 2019, Bekker et al. 2022, Espinase-Nandorfy et al. 2022).
- The projects investigating the role of microbial factors in VSC formation have identified specific commercially available yeast strains that winemakers can select to increase tropical sulfhydryl concentrations and decrease unwanted VSC concentrations in their wine. A novel yeast strain developed by the AWRI, and commercialised by AB Biotek in 2020, AWRI Rosa, was shown to produce lower levels of unwanted VSCs, and this strain can be used by winemakers to decrease VSC off-flavours in wine. The results of these trials have been published in three peer-reviewed papers (Cordente et al. 2019, Cordente et al. 2021, Cordente et al. 2022).
- The projects investigating practical strategies to remove VSCs, evaluated the commonly used remediation strategies and developed a novel remediation tool. This information

was conveyed to the sector through publication in three peer-reviewed journals (Bekker et al., 2020, Day et al. 2021, Mierczynska-Vasilev et al. 2022).

9. Recommendations

The results of this project have shown that a novel group of precursors, namely polysulfides, may contribute significantly to latent sources of VSCs present in wine post-bottling. Copper plays a critical role in modulating both the formation of these latent sources of VSCs as well as in binding and complexing with VSCs such as H_2S and removing the perception of 'reductive' aromas. Understanding the relationship between the formation of latent sources and the formation of metal-bound complexes will be crucial in developing assays to assess wine shelf life and the risk of producing 'reductive' faults post-bottling.

A large body of work has demonstrated that increased 'Total copper' is correlated to increased 'Total H_2S', however, the work described in Section 7.5.1 showed that although 'Total H_2S' increased, 'Free H_2S' remained low and as such the perception of 'reductive' faults were not affected by increased 'Total copper'. Future work should be directed at elucidating whether the fraction of 'Metal-bound' VSCs is irreversibly bound to copper and does not pose any risk of acting as a latent source of VSCs, or whether certain winemaking or ageing conditions may facilitate a shift in equilibrium from 'Metal-bound' to 'Free' VSCs which would have detrimental effects on wine aroma and perception of quality.

While this project has delivered a better understanding of some of the factors modulating the formation of the 'flinty' compound PMT during fermentation, the laboratory-scale results have yet to be confirmed under larger-scale winemaking conditions. In addition, there is still a knowledge gap in understanding of PMT evolution after alcoholic fermentation has been completed and how factors such as aeration or different wine maturation (ageing) processes might modulate the concentration of this 'flinty' compound. In addition, different preservation methodologies should be explored in more detail to aid in the preservation of PMT once wine has been bottled. A pilot-scale winemaking trial in Chardonnay is currently underway to validate some of this project's experimental findings.

Method development should be performed to optimise and focus the analysis of PMT and 2-FMT and omit the tropical sulfhydryls (3-SH, 3-SHA, 4-MSP) from the optimised method. To confirm the findings regarding the synergistic interaction between PMT and 2-FMT in modulating 'struck flint' aroma, a reconstitution and omission study in Chardonnay should be performed. This will confirm the influence of these sulfhydryls at different concentrations and combinations on the intensity and character of 'struck flint' aroma. Further flavour research should focus on the tentatively identified novel sulfhydryl compounds relating to PMT to determine whether these compounds are indeed associated with 'struck flint' aroma in wine.

This project provided a potential alternative to copper fining, the primary method for managing 'reductive' aromas in wine. Further avenues need to be explored for the commercialisation of, for example, AuNP-coated filtration devices, closures, packaging material, novelty decanters and benchtop wine aerators. Once successful commercialisation pathways are identified, scale-up and regeneration trials are needed to apply the technology on an industrial scale.

10. Appendix 1: Communication

Information has been extended to industry stakeholders through articles in international peer-reviewed journals, including the *Australian Journal of Grape and Wine Research*, trade journals, road shows, webinars, seminars to sector partners through invitation' as well as through

workshops, presentations, and posters at the 17th and 18th Australian Wine Industry Technical Conferences held in Adelaide in 2019 and 2022. The information has also been presented at international conferences and published in book chapters.

Papers published in peer-reviewed journals

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Technical Review articles

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AWRI roadshow seminar program

- Bekker, M.Z. Copper - the good, the bad and the ugly, 10 May 2017. Griffith, New South Wales.
- Bekker, M.Z. The beneficial style and performance effects of oxygen addition during fermentation, 10 May 2017. Griffith, New South Wales.
- Bekker, M.Z. Copper - the good, the bad and the ugly, 11 May 2017. Mildura, Victoria.
- Bekker, M.Z. Managing reductive aromas in wine, 7 November 2019. Stanthorpe, Queensland. Virtual seminar.
- Bekker, M.Z. Copper, the good, the bad and the ugly, 7 November 2019. Stanthorpe, Queensland. Virtual seminar.
- Bekker, M.Z. and Wilkes, E., Using copper sulfate without unwanted side effects, Clare Valley Seminar, AWRI Grape and Wine Roadshow, 12 November 2019, Clare SA, Australia.
- Bekker, M.Z. and Day, M.P., Beneficial style and performance effects of oxygen addition during fermentation, Clare Valley Seminar, AWRI Grape and Wine Roadshow, 12 November 2019, Clare SA, Australia.
- Bekker, M.Z. and Day, M.P., Managing 'reductive' aromas in wine, AWRI Grape and Wine Roadshow, 15 October 2019, Orange, NSW, Australia.
- Bekker, M.Z. Managing reductive aromas in wine, 19 November 2020, Griffith, New South Wales. Virtual seminar.
- Bekker, M.Z. Managing reductive aromas in wine, 2 February 2021, Stanthorpe, Queensland. Virtual seminar.
- Bekker, M.Z. Copper - the good, the bad and the ugly, 2 February 2021, Stanthorpe, Queensland. Virtual seminar.
- Bekker, M.Z. Managing reductive aromas in wine, 1 June 2022, Mornington Peninsula, Victoria
- Bekker, M.Z. Managing reductive aromas in wine, 2 June 2022, Geelong, Victoria

Webinars

- Bekker, M.Z. Managing 'reductive' aromas in wines 2019 AWRI webinar program, 7 November 2019, Adelaide, Australia.
- Bekker, M.Z. The formation and remediation of stinky sulfur aromas in wines 2018 AWRI webinar program, 31 May 2018, Adelaide, Australia.

National and International Conferences and Seminars

- Bekker, M.Z. Myths and Facts about the Role of Precursors in the Formation of "Reductive Aromas" in Wines Postbottling, 68th American Society of Enology and Viticulture National Conference, Enology – Wine Aroma Session, Bellevue, Washington, USA, 26-29 June 2017.
- Bekker, M.Z. The effects of oxygen on 'reductive aroma' compounds during fermentation, remediation, and post-bottling, 68th American Society of Enology and Viticulture National Conference, New Understandings in Wine Oxidation Chemistry Symposium, Bellevue, Washington, USA, 27 June 2017.
- Bekker, M.Z and Day, M.P. What happens as wine ages? The Complete Wine Science Spectrum, Big Science Adelaide, The Store, Adelaide SA, Australia. 15 August 2017.
- Bekker, M.Z. Post-bottling formation of stinky sulfur aromas in wines and remediation solutions, Oxidation in Winemaking Workshop, Pernod Ricard Winemakers, Rowland Flat, South Australia. 18 October 2018.
- Kulcsar et al. Evaluating the effectiveness of five commonly used strategies for the remediation of 'reductive' aromas, 11th Oeno IVAS Conference, Talence, Bordeaux, France. 25 – 18 June 2019.
- Bekker et al. Are dicysteinyl polysulfanes responsible for post-bottling release of hydrogen sulfide?, 11th Oeno IVAS Conference, Talence, Bordeaux, France. 25 – 18 June 2019.
- Cordente, A.G.: Yeast modulation of wine flavour and aroma. National Science week. Adelaide, South Australia. August 2019.

- Cordente, A.G. Yeast modulation of wine flavour and aroma. AB Biotek, Sydney, New South Wales. September 2019.
- Leskovic et al. Is copper the solution to fixing reductive wines? 42nd SASEV Conference, 3 - 5 November 2020.
- Siebert et al. 'Did someone light a match?' Struck match/struck flint and other fun sulfur compounds in Chardonnay, 2020 Tasmanian Winemaker Symposium. Virtual seminar. 11 November 2020.
- Cordente, A.G. Modulation of 'fruity' thiol production by yeast in red wines. Crush Conference 2021. Adelaide, South Australia. 19 June 2021.
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- Bekker et al. Managing 'Reductive' Aromas in Syrah Wines, New Zealand Syrah Workshop, "Syrah people talking Syrah", Bayview Chateau Tongariro. 3-5 November 2021.
    - Bekker et al. Factors contributing to the formation of latent sulfur sources and sulfur traps in wine, International Chemical Congress of Pacific Basin Societies, Virtual Conference, 16 - 21 December 2021.17th Australian Wine Industry Technical Conference, Adelaide Convention Centre, 21-24 July 2019.
        - 17th AWITC Workshop.
            - Bekker, M.Z., Zhang, X., Clark, A., Garcia Cordente, T., Espinase Nandorfy, D. AWITC Workshop #15 Fruity vs stinky: Impacts of viticulture and winemaking practices on wine aroma. The workshop was one of the first to sell out, attended by the maximum number of attendees. Received overwhelmingly positive reviews.
        - 17th AWITC Presentation:
            - Kulcsar et al. Stopping the stink: An evaluation of common reductive aroma remediation strategies. Fresh Science Presentation in Session B.
        - 17th AWITC Posters:
            - Kulcsar et al. Stopping the stink: An evaluation of common reductive aroma remediation strategies.
            - Bekker et al. Polysulfanes as a new class of precursors to hydrogen sulfide.
    - 18th Australian Wine Industry Technical Conference Adelaide Convention Centre, 26-29 June 2022.
        - 18th AWITC Workshop:
            - Bekker, M.Z., Zhang, X., Clark, A., Hixson, J., Siebert, T., Garcia Cordente, T., and Espinase Nandorfy D. AWITC Workshop #17 Modulating 'flint' and 'tropical' aromas and controlling 'reductive' aromas in premium Australian wines, The workshop was the first to sell out, attended by the maximum number of attendees. Received overwhelmingly positive reviews.
                - 18th AWITC Posters:
                    - Espinase Nandorfy et al. Compositional correlations with the sensory properties and consumer preference of duelling styles of Chardonnay.
                    - Cordente et al. Modulation of 'fruity' thiol release during fermentation of red musts by wine yeast.
                    - Siebert et al. 'Struck flint' aroma in Chardonnay wines: what causes it and how much is too much?
                    - Kulcsar et al. Sneaky stinkies: the challenges associated with analysing volatile sulfur compounds.
                    - Bekker et al. Factors affecting the formation and preservation of 'struck flint' aroma in wine.
                    - Bekker et al. How do tannins clean up stinky wine?

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11. Appendix 2: Intellectual property

Program IP has been communicated through presentations and publications and resides in the public domain. Copyright in any reports, posters or presentations is held by the AWRI. Copyright in publications remains with the publisher. No patentable discoveries have been identified from the work. No Program IP has been commercialised at the time of writing this report. Plasma polymer coated surfaces were developed that may have future potential for commercialisation based on their effectiveness in removing 'reductive' sulfhydryls. Commercialisation evaluation has been conducted and identified a need for further scale-up and regeneration trials.

12. Appendix 3: References

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13. Appendix 4: Staff

Dr Marlize Bekker, Dr Toni Garcia Cordente, Allie Kulcsar, Deanna Langone, Dr Tracey Siebert, Dr Agnieszka Mierczynska-Vasilev, Flynn Watson, Lisa Pisaniello and Dr Alicia Jouin.

Sensory analyses were conducted by Damian Espinase Nandorfy, Desiree Likos, Eleanor Bilogrevic, Dr Leigh Francis and the sensory panellists.

14. Appendix 5: Other supporting material (including raw data, if appropriate)

Not applicable