Wine Australia

Charles Sturt University

Impact of metal speciation on the development, shelf-life and sensory properties of wine



FINAL REPORT TO WINE AUSTRALIA

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Impact of metal speciation on the development, shelf-life and sensory properties of wine.

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Abbreviations

BCA	Bicinchoninic acid
CDF	Cellulose-based Depth Filters
DCPIP	Dichlorophenolindophenol
DEDF	Diatomaceous Earth Depth Filters
DiBr-PAESA	4-(3,5-Dibromo-2-pyridylazo)-N-ethyl-N-sulfopropylaniline
DMS	Dimethyl Sulfide
EMS	Ethylmethylsulfide
FAAS	Flame Atomic Absorption Spectroscopy
GC-SCD	Gas Chromatography with Sulfur Chemiluminescence Detector
GDT	Gas Detection Tube
HPLC	High Performance Liquid Chromatography
ICPOES	Inductively Coupled Plasma with Optical Emission Spectrometry
LMWSC	Low Molecular Weight Sulfur Compounds
MeSH	Methanethiol
ME CCSP	Medium Exchange Constant Current Stripping Potentiometry
PES	Polyethersulfone
pFPD	Pulsed Flame Photometric Detector
PTFE	Polytetrafluoroethylene or Teflon
PVDF	Polyvinylidene fluoride
PVI/PVP	Polyvinylimadazole / Polyvinylpyrrolidone
UHPLC	Ultrahigh Pressure Liquid Chromatography
3SH	3-sulfanylhexanol
3SHA	3-sulfanylhexyl acetate
4MSP	4-methyl-4-sulfanylpentan-2-one

1 ABSTRACT

New methods were created for measuring total Cu and Cu fractions in wine, including Cu(II) organic acids (Cu fraction 1), Cu(I)-thiol complexes (Cu fraction 2), and copper sulfides (Cu fraction 3). Cu fraction 3 concentration could be lowered by exposing wine to oxygen, filtration approaches, fining with copolymers or bentonite treatment. Cu fractions 1 and 2 inhibited reductive aromas but during bottle aging their concentrations decreased, and their depletion was accelerated by increasing temperature, light exposure or metallic aluminium. Guidelines for Cu fraction 1 and 2 concentrations at bottling are proposed for minimising reductive aroma accumulation in wines during aging.

2 EXECUTIVE SUMMARY

Copper is known to participate in oxidative and reductive reactions in wine. Previous research provided a preliminary understanding of binding agents for Cu in wine and techniques to quantify certain broad categories of Cu using research orientated approaches.

This project advances the past research with the advent of novel measures of both total Cu and Cu fractions in wine, including fractions that have never been reported before. Importantly the techniques are more amenable for use in wineries given they use equipment commonly found in wineries (i.e., spectrophotometer). These measures enabled a better understanding of the role of Cu in oxidation processes, the reductive development of wine and how Cu can be more efficiently removed from wine. As well as providing new insights for these areas, the Cu measures allow a more strategic usage of Cu to improve the outcomes for wine after bottle aging.

The 2 bicinchoninic acid (BCA) colorimetric analysis of total Cu in white wine was an outcome of our previous Wine Australia-funded project. In this current project, a digestion procedure for red wine was devised to destroy red pigmentation and allow total Cu determination by BCA colorimetry. This colorimetric method was further modified allowing the determination of three difference fractions of Cu in white wine: Cu fraction 1 was associated with Cu(II) organic acids, Cu fraction 2 with weakly-bound Cu(I) thiol compounds, and Cu fraction 3 predominantly with copper sulfides but also some strongly-bound Cu(I) thiol compounds. The collection of permeate from wine passed through cellulose-based depth filters incorporated with diatomaceous earth, allowed the measurement of the combined Cu fraction 1 and 2 concentration in white wine, and Cu fraction 1 in red wine. Progress has been made automating the Cu measures but further work is required for full validation.

Cu fractions 1 and 2 were found to have a significant impact on rate of oxygen reaction in white wines containing ascorbic acid, but this was not the case in white wines without ascorbic acid and in red wines. For the latter, the total Cu concentration was more important in determining oxygen consumption rates than the specific distribution of Cu among the different fractions. The Cu fractions did not influence the amount of total sulfur dioxide consumed for a given amount of oxygen in any wine, but the presence of ascorbic acid in white wine could nearly halve this value.

Cu fraction 3 was shown not to have any direct benefit for wine, other than being a potential source of Cu fractions 1 and 2 (e.g., transition afforded with some oxygen exposure). In fact, with light exposure to white wine, Cu fraction 3 could act as a significant precursor for free H_2S. Most filtration methods were able to remove some Cu fraction 3. However, membrane filtration media only worked efficiently on small volumes and was found not to be effective at larger volumes. The most effective procedures for removal of Cu fraction 3 was the use of depth filtration with cellulose pads incorporating diatomaceous earth or fining with the copolymer PVI/PVP. Bentonite could also be used for white wines for Cu removal. PVI/PVP was unique for its ability to significantly decrease the concentration of all Cu fractions in white wine.

This project identified the most suitable gas chromatography (GC) methodologies for measuring the forms of H_2S and methanethiol that contributed directly to reductive aromas in wine. This was the free H_2S and free methanethiol measures by GC with a Sulfur Chemiluminescence Detector (SCD). Gas detection tubes were also suitable for measurement of free H_2S, albeit with some overestimation compared to GC-SCD, but still linked well to the sensory assessment of

reductive aroma. The link of Cu fractions to the inhibition of reductive aroma compounds was comprehensively established using studies involving 800 bottles of wine. Free H_2S did not accumulate in wines with combined Cu fraction 1 and 2 concentrations above 0.02 mg/L and free methanethiol did not accumulate in wines with Cu fraction 1 above 0.03 mg/L. An unfortunate ramification was that, as for the inhibition of free methanethiol (i.e., a thiol compound), Cu(II) addition also decreased the concentration of varietal thiol aroma compounds present in Sauvignon blanc. The effect of Cu(II) on varietal thiol compounds was immediate and lasted until the final timepoint measured in the bottle trial (i.e., 18 months). However, despite the lowered varietal thiol concentrations, the Cu(II) additions at bottling still often led to Sauvignon Blanc wines with more perceived tropical and fruit aromas than the control wines. This was attributed to the added Cu(II) removing masking compounds, such as free H_2S and methanethiol.

A key aim of this project was to ultimately provide guidelines for the concentration of Cu fractions at bottling that may limit the onset of reductive development in wines. This ideally required identification of those wines at risk of aging reductively and the establishment of Cu fraction 1 and 2 concentrations that would minimise both the risk of reductive development and the magnitude of the Cu(II) addition. To achieve this, it was necessary to first assess the stability of protective Cu fractions in wine. Cu fraction 1 and 2 were found to decrease during the aging of all wine, with a concomitant increase in Cu fraction 3. The rate of decrease in the protective Cu fractions varied from wine to wine and was accelerated by elevated temperature, light exposure or storage when in contact with metallic aluminium (i.e., cans). This meant that using kinetic studies and average rates of decay were not suitable to predict the time of depletion for the protective Cu fractions in wine. Instead, from the aging study of 43 wines over 12-18 months, it was found that the lag period between the loss of Cu fraction 1 and 2 and the emergence of the reductive aroma compounds was quite large (i.e., ~1-year). Consequently, the presence of Cu fraction 1 and 2 at bottling could still offer protection to wines even after depletion. Based on the aging studies in ideal conditions (i.e., 14-17 °C in darkness), free H_2S did not accumulate above 2 µg/L in wines with 0.115 mg/L for the combined Cu fraction 1 and 2 concentration at bottling, and free methanethiol did not accumulate above 5 µg/L when the Cu fraction 1 concentration exceeded 0.04 mg/L at bottling.

Progress was made on assessing a wine for its potential to age reductively. This involved bottling a wine in a similar manner to the final product (i.e., under screw cap and with similar total packed oxygen) and heating the bottled wine for 21-28 days at 40 °C. If the free methanethiol measured in the heated wine was above 5 µg/L then this was also likely to be the case for the wine after 12-18 months of bottle aging. Obviously a more rapid test would be beneficial and the subject of future work.

2.1 Acknowledgements

The activities and investigations described in this report were financially supported by Wine Australia on behalf of Australian grape growers and wine makers from levy funds with matching funds from the Australian Federal Government.

Much of the research conducted in this project (CSU 1702-4) would not have been possible without the assistance of many fantastic collaborators.

Dr Geoffrey Scollary, an adjunct appointment at The University of Melbourne, has overseen much of the pioneering research upon which this project (CSU 1702-4) was ultimately built. He continued to contribute mentorship, direction and assistance in publishing various works throughout this project.

Collaboration with the Australian Wine Research Institute personnel was performed for four different sections of work covered in this report:

i) Dr Eric Wilkes, Dr Kieran Hirlam and Dr Neil Scrimgeour for Section 6.2.4.1 (Cu removal with PVI/PVP),
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The cysteine-based and glutathione-based polysulfanes used in Section 6.5.4 were synthesised and purified at The University of Auckland by Dr Lisa Pilkington from Prof Bruno Fedrizzi's group. Dr Rebecca Deed was also involved in the collaboration for Section 6.5.4.

Multiple bottle aging trials were conducted onsite with Treasury Wine Estates and were critical to Sections 6.3.4, 6.5.3, 6.5.6 and 6.5.7 of this report. The key personnel involved was Ms Kylie Langford and with later assistance from Ms Vanessa Stockdale. We are greatly indebted to the wines and in-kind assistance provided by Treasury Wine Estates.

Wines were also provided for bottle aging trials and/or filtration trials by Warburn Estate, Medina Beverages, Casella Family brands, and De Bortoli Wines.

Collaboration with Dr Paul Bowyer from Blue H_2O Filtration contributed to the success of Sections 6.2.3 and 6.2.1.

Collaboration with Prof Kerry Wilkinson from The University of Adelaide occurred for Section 6.5.5 (Impact of light on wine). This portion of work was conducted by Charles Sturt University PhD student Ms Isara Vongluanngam as part of her PhD project, funded by the Australian Research Council Industrial Training Centre for Innovation Wine Production, and titled 'The impact of light on oxidative and reductive aging of wine'. Ms Vongluanngam's supervisors were the CSU-based personnel involved in CSU 1702-4, along with Prof Kerry Wilkinson.

Mr Guilherme Soares assisted in the large-scale wine treatments using PVI/PVP and depth filtration that are outlined in Sections 6.2.3. This work was part of his Master's studies through the University of Portugal.

3 BACKGROUND

Oxidative, reductive and colloidal phenomena are recognised as major contributors to wine faults. Copper can influence all these areas, impacting the rate of oxidation reactions, the concentrations of certain sulfur compounds, and contributing to haze formation.

Previously, the maximum total concentrations of metals in wine recommended to avoid metal-induced spoilage were ambiguous. Generally, recommendations of broad ranges (0.2-0.6 mg/L for Cu) rather than specific concentrations were provided (Kean and Marsh 1956, Amerine and Ough 1980, Peynaud 1984, Shields 1986, Rankine 2004, Cowey 2008, Iland et al. 2012). The recommendations also varied from author to author, due to the difficulty in linking an absolute total concentration to specific metal-induced effects in wine. This situation has been addressed in other industries, as for example, the total concentration of metals is often not used when assessing toxicity effects within environmental chemistry contexts. Instead, a specific form of a metal ion is measured and this provides a better assessment of the toxicity of the metal.

The precursor project (NWG 1401) to this current project (CSU 1702-4), also funded by Wine Australia, was the first to provide key insights into the different forms of Cu and Fe present in wines. After assaying different research-orientated techniques, a number of techniques were applied to a survey of wines (Kontoudakis et al. 2019b) and the testing of potential wine binding agents for Cu and Fe in wine (Kontoudakis et al. 2017b). This work provided two broad categories for Cu in wine consisting of sulfide-bound or non-sulfide bound Cu. Fe in wine was predominantly associated with organic acid or phenolic compounds.

Cu could readily transition between the sulfide-bound and non-bound forms in wine, with low oxygen conditions favouring the sulfide-bound form and high oxygen conditions favouring the non-sulfide bound form (Kontoudakis et al. 2017a; Kontoudakis and Clark, 2018). Consequently, after aging the wine in bottle, most Cu was found in the sulfide-bound form. Importantly, a link was found between the presence of non-sulfide bound Cu form and prevention of spoilage sulfur aroma compounds from accumulating (i.e., H_2S and MeSH) (Kontoudakis et al. 2019b).

In conjunction with this work, a Spanish research group (Franco-Luesma and Ferriera, 2014; 2016a) reported the release of H_2S from sulfide-bound Cu during the aging of wine in low oxygen conditions. This highlighted the potential danger of allowing high concentrations of residual sulfide-bound Cu in wine. Additional research within the precursor project (NWG1401), showed that during wine production the sulfide-bound Cu could be decreased in white wine containing protein with bentonite (Rousseva et al., 2016). Certain membrane filters were also observed to actively remove sulfide-bound Cu via adsorption mechanisms, such as PES and nylon, however the capacity for removal at high wine volumes was uncertain (Kontoudakis et al. 2019a).

Although the analysis techniques employed to measure the different Cu and Fe forms provided some useful insights, they were research-orientated techniques that did not lend themselves to ready adoption in wine production laboratories. For Cu, an electrochemical technique was utilised that was labour intensive and required the analytical delivery of samples and solvents via a programmable HPLC (Clark et al. 2016). For Cu and Fe, a solid phase extraction methodology involved initial fractionation and then quantitation via inductively coupled plasma with optical emission spectroscopy (ICP-OES) (Kontoudakis, Smith et al. 2017). This method was also labour intensive, slow and required the expensive ICP-OES instrumentation and supporting

infrastructure. Prior to the completion of the precursor project (NWG 1401), a colorimetric method for the determination of total Cu in white wine was designed to allow analysis in a winery containing a bench top-spectrophotometer (Kontoudakis et al. 2020). This technique utilised the colorimetric reagent bicinchoninic acid (BCA) and did require a 40 mm cuvette for sufficient sensitivity. The obvious extension of this research would be the application to red wine and the measurement of the different fractions of Cu.

4 PROJECT AIMS AND PERFORMANCE TARGETS

This project will utilise current knowledge on the forms of different metals (metal speciation) in wine to better understand the impact of metals on the development of wine in bottle. Furthermore, the knowledge of the metal speciation will provide more targeted evaluation of the optimum time and mechanisms to allow removal of specific forms of metal in a wine production setting. The overall outcome will be the production of wines whose metal profile can be measured, their subsequent impact on wine development, shelf-life and sensory properties predicted, and a number of wine production tools evaluated to limit detrimental influences of the metals. In addition, a technique allowing measurement in wineries will be designed to enable copper speciation measurements to be more readily conducted in red and white wines.

The overall aim of this project is to produce wine with improved bottle development by understanding the manner by which metal speciation can influence wine aging in bottle, and providing options to minimise detrimental influences of metals through wine production processes.

The objectives of this project are to:

1. Determine the influence of metal speciation and wine composition on the amount of sulfur dioxide consumed per mg/L oxygen in red and white wine.

2. Assess reversibility of key Cu speciation forms and their activity on mechanisms directly relevant to the development of red and white wines.

3. Establish the influence of ascorbic acid on the stability and activity of copper(I) sulfide.

4. Determine the impact of metal speciation and metal concentration ratios on mechanisms that contribute to colour and flavour development in wine.

5. Establish a link between metal speciation and steps in the wine production process that allow efficient removal of metals from wine.

6. Trial several large-scale applications of the most viable novel winery operations identified in small scale wine production.

7. Communicate the novel wine production techniques to winemakers, including the preparation of a ‘How to’ guide.

8. Prepare teaching notes for use in oenology subjects at Charles Sturt University and The University of Adelaide.

Year 3 deliverables

- Knowledge of copper speciation in wine, including its activity and transformations in various bottle storage conditions and its impact on wine development in bottle.
- Protocols on the use of ascorbic acid to influence the impact of metal speciation in wine.
- Wine production approaches to limit the impact of metal ions on the development of wine.
- Relationships between metal speciation and the extent of sulfide precursors in wine.
- The evaluation on a small-scale of a range of novel winery operations to modify the metal profile of a wine.

Year 5 deliverables

- The evaluation on a typical winery scale of novel winery operations to increase winery efficiency and stability.
- Establishing the influence of metal speciation and wine composition on the impact of sulfur dioxide consumption per mg of oxygen in wine consumed.
- The development of a 'How to guide' to implement the winery operations for the Australian wine industry.
- The development of teaching materials for use in the education of Australian winemakers and viticulturists.

In the tables below are the final approved outputs and activities. Modification of the outputs and activities were made throughout the lifetime of the project with the approval of Wine Australia. Once it became apparent that the winery-relevant colorimetric method for total Cu in white wine could be adapted to also measure Cu fractions, outputs and activities relevant to this research were added to the project. This was at the expense of outputs and activities focused on metal speciation measurements in juice, further assessment of Fe metal speciation effects and the impact of metal speciation on wine colour.

The project was originally scheduled for completion in June 2022 but was extended to December 2024 with no further funding costs due to salary savings throughout the project.

Table 4.1. The final approved project outputs and activities.

Financial Year 1: April – June 2018

	Output	Report Section	Activities
a	Data establishing the impact of copper speciation on the removal of Cu from red wine by membrane filtration.	6.2.1	Ten red wines adjusted to 1.0 and 0.3 mg/L copper, in the form of free Cu or Cu2S, and various membrane filtration media assessed for the efficiency of removal of the copper. Objective 5.
b	Data showing the feasibility of the Cu colorimetric measurement for determination of free Cu in white wine.	6.1.2	The free Cu concentration determined by colorimetric analysis in five white wines, at different free Cu concentrations. The results compared to those obtained by established electrochemical and SPE ICPOES methods. Objective 7.

Table 1. The final approved project outputs and activities (continued).

Financial Year 2: July 2018 – June 2019

	Output	Report Section	Activities
a	Determination whether free Cu accumulation in wine is indicative of depletion of potential sulfide sources.	6.5.2 6.4.1 6.4.2	Survey conditions for wine storage that favour transition of bound to free Cu(II) and determine the potential for re-binding of Cu by the wine and the rate at which this occurs. Objective 2.
b	Establish the impact of ascorbic acid on the oxidative stability of Cu_2S.	6.4.1	White wines, with and without ascorbic acid, and also with variable amounts of Cu2S are to be examined for Cu speciation during the consumption of oxygen. Objective 2.
c	Determination of the ascorbic acid and sulfur dioxide pair to induce transformation of free Cu to bound Cu.	6.5.2	Cu speciation measurements will be conducted over time in model and white wines, which will have variable ascorbic acid, sulfur dioxide, free Cu and total Fe concentrations. The impact of storage conditions will also be assessed. Objective 3.
d	Establish kinetics of transition between Cu species in wine.	6.5.3	Five red and/or white wines, with addition of free Cu(II), bottled and the rate of transition to bound established. Wines will be stored in different conditions (i.e., O2 concentrations) and compositional analysis performed. Objective 2.

Financial Year 3: July 2019 to July 2020

	Output	Report Section	Activities
a	Knowledge on wine production techniques to limit the impact of Cu and/or Fe on wine development.	6.2.2 6.2.3 6.2.4	The use of metal speciation measures in combination with wine production tools (bentonite, depth filtration media, PVP/PVI and protein fining) conducted on red and white wines to highlight efficient removal of reactive forms of the metals. Objective 5.
b	Data showing the feasibility of the Cu colorimetric measurement for determination of free and bound Cu in red wine.	6.1.1 6.1.3	The free and bound Cu determined by colorimetric analysis in twenty red wines. The results compared to those obtained by electrochemical methods. Objective 7.
c	Report to Wine Australia on stop/go milestone.	Previously submitted June 2020	Prepare report summarising the data obtained regarding the impact of metal speciation on wine stability and sulfur dioxide efficiency. This report will enable assessment of the viability continue the project into the final three years. Objectives 2, 3, 5 and 7.

Financial Year 4: June 2020 to July 2021

	Output	Report Section	Activities
a	Knowledge on the impact of metal speciation and/or other wine components on the oxygen to sulfur dioxide consumption ratio.	6.4.1 6.4.2	A bottle trial established for a white and red variety of wine, adjusted to different metal speciation profiles of Cu and/or Fe, and variable ascorbic acid concentrations. The oxygen to sulfur dioxide consumption ratios determined over a 6-7 month period for the wine in bottle. Objective 1.
b	Knowledge on the ability of the colorimetric method for Cu in white wine to distinguish between thiol-bound Cu and sulfide-bound Cu.	6.1.2	Investigate the ability of Ag(I), quinone and hydrogen peroxide, and different incubation times, to be used in conjunction with the colorimetric measure of Cu in wine to differentiate Cu binding to thiols and sulfide. Objective 7.
c	Knowledge on how wine compositional changes impacting Cu forms in wine can induce changes to free methanethiol.	6.3.3	A model, white and red wine will be examined for the impact of increased sulfide-binding or thiol-binding to Cu on the release of methanethiol from Cu (i.e., increase in free methanethiol). The thiols investigated will include glutathione, including its release from disulfide forms. Objective 2 and 4.
d	Teaching materials based on based on the use of oxygen, and metal speciation measures during wine production.	6.8	Develop a set of teaching materials for usage in oenology subjects at Charles Sturt University and The University of Adelaide. Such teaching materials would be adequate for either undergraduate or post-graduate courses. Objective 8.
e	Knowledge on the application of the most promising output (listed above) on large scale wine production.	6.2.3.2 6.2.4.2	The application of the most promising outputs listed above will be conducted on a large-scale volume (~500 – 1000 L) of red and/or white wine. Objective 6.

Financial Year 5: July 2021 to June 2022

	Output	Report Section	Activities
a	Establish links between colorimetric forms of Cu and reductive sensory characters in white wine.	6.3.1 6.3.2 6.3.3 6.5.3	This will involve a survey of 30-50 white wines to determine how colorimetric copper speciation (total Cu, Cu fractions I, II and III) relates to 'free' and salt-releasable H2S and MeSH, and in turn, how they all relate to 'reductive' character and sensory profile of white wines. This will be a collaborative project with Australian Wine Research Institute. Objective 4.

Financial Year 6: June 2022 to July 2023

	Output	Report Section	Activities
a	Part 1. Determine the effect of other metals on the forms of Cu in wine.	6.6	Zn(II) and Al(0)/Al(III) will be investigated for their ability to influence Cu forms in red and white wines. Objective 2 and 4.
b	Knowledge on predicting Cu binding in wine at different temperatures, and hence predicting the time-frame for its protection against free H2S accumulation.	6.5.5 6.5.7	Three red and white wines, with added Cu(II) at bottling, will be aged at variable temperature to allow determination of the activation energy for binding of Cu. This will allow potential prediction of Cu(II) binding in wine from bottling at different temperatures of storage. Objective 2 and 4.
c	Part 1. Automation of simplification of colorimetric measure of Cu in wine.	6.1.4 6.1.5	The translation of the current spectrometric measure of Cu forms in white wine to an automated analyser will be investigated. Objective 7.

Financial Year 7: July 2023 to June 2024

	Output	Report Section	Activities
a	Part 1. Knowledge of the influence of different Cu fractions on beneficial thiol aroma compounds during wine aging in bottle.	6.3.4	A bottle trial established for two white wines, with pronounced varietal thiol aroma, at different Cu concentrations. The change in Cu fractions and thiol aroma compounds will be determined periodically throughout 10-months of bottle aging. Sensory analysis on the wines will be conducted on the final analysis timepoint. Objective 4.
b	Established links between cross-flow filtration on the removal of Cu fractions from wine.	6.2.5 6.2.6	A white and red wine will undergo cross flow filtration at commercial scale (> 10,000 L) to determine its impact on Cu fractions in the wine. The extension to the project will allow the survey of a larger number of wines (i.e., five white wines and five red wines) to thoroughly evaluate the impact of cross flow filtration on total Cu and Cu fractions. Objective 6.
c	Part 1. Viability of a test to predict the reductive development of wine when stored under ideal conditions.	6.5.7	Seven white wines will undergo storage in bottle at 17C (10-months) and 40C (<2-weeks), in conjunction frequent measurement of Cu fractions and sulfhydryl aroma compounds, and final sensory analysis. Links between short-term storage at 40C, in conjunction with Cu fraction measures, with reductive development at 17C will be determined. Objective 2 and 4.
d	Part 2. Zn(II) and Al(0)/Al(III) will be investigated for their ability to influence Cu forms in red and white wines.	6.6	Apart from the completed experiments with Zn(II), Al(0) and Al(III) as variables in model, white and red wines matrices, a canned wine experiment was commenced in July 2024. The variables were Cu(II) concentration at bottling and the extent of damage to the internal liner of the cans. Extension of the project to 2024 will allow the following measurements on the canned wine (stored at 17 °C): - 6-month time point (January 2024). - 10-month time point (May 2024).
e	Part 2. Automation of simplification of colorimetric measure in Cu in wine.	6.1.4 6.1.5	Initial investigations into the use of an automatic analyser for the automated measurement of Cu in wine proved unsuccessful. However, CSU will soon purchase a new automated analyser (Thermo Fisher Konelab) which may provide more software and hardware flexibility and sensitivity to enable the Cu method to be automated. The feasibility of automating the colorimetric method for Cu (total and Cu fractions) on the new automated analyser will be conducted.

Financial Year 8: July to December 2024

	Output	Report Section	Activities
a	Part 2. Knowledge of the influence of different Cu fractions on beneficial thiol aroma compounds during wine aging in bottle.	6.3.4	In collaboration with Treasury Wine Estate (TWE, contact: Kylie Langford), a bottle trial was established for two Sauvignon Blancs, with pronounced varietal thiol aroma, each at three variable Cu concentrations. The bottle trials commenced separately on March 2023 and May 2023 for each wine, with the timing largely dictated by TWE based on the availability of appropriate wines. The change in Cu fractions (CSU) and thiol aroma compounds (AWRI) has been determined periodically throughout 10-months of bottle aging. Extension of the project to 2024 will allow the following measurements: • Sauvignon Blanc 1: 8 months (Jan 2024), 12 months (May 2024) and 18 months (Nov 2024). • Sauvignon Blanc 2: 12 months (March 2024), 18 months (Sep 2024). Sensory analysis will be conducted on both wines at 12-months.
b	Part 2. Viability of a test to predict the reductive development of wine when stored under ideal conditions.	6.5.7	Ten white wines (sourced from TWE, DeBortoli and Warburn Estate) haven undergone storage in bottles at 40 °C for 4-weeks (7-time point measurements) and are currently undergoing storage at 17 °C, with periodic measurement of Cu fractions and sulfhydryl aroma compounds. Links between short-term storage at 40°C, in conjunction with Cu fraction measures, with reductive development at 17°C will be determined. Extension of the project to 2024 will allow the following measurements for wines stored at 17 °C: • White wines A-D: 8 months (Jan 2024), 12 months (May 2024) and 18 months (Nov 2024) • White wines E-J: 12 months (March 2024), 18 months (Sep 2024). Sensory analysis will be conducted on wines at 12-months.
c	Metal speciation ‘How to’ guide for winemakers.	6.7	The ‘How to’ guide is best prepared after the final analysis time point of the bottle trials and other activities outlined above.

5 METHODS

5.1 Measurement of total Cu and its fractions in wine.

The following section outlines the methodology for the novel measures of total Cu and its various fractions in red and white wines developed as part of this project. For details on the measures of Cu that had previously been established, the following references can be consulted:

- Electrochemically-detected Cu by medium exchange constant current stripping potentiometry as described by Clark et al. (2016) and Clark and Kontoudakis (2018).
- Total Cu by inductively coupled plasma with optical emission spectroscopy (ICP-OES) as described by Rousseva et al. (2016).

5.1.1 Total Cu in red wine by bicinchoninic acid (BCA) colorimetric analysis.

Table 5.1. Optimised procedure for the addition of solutions for the determination of Cu in red wine by standard addition. All values are in units of mL.

Solution to add	Blank	Non-blank	Standard-1†	Standard-2†	Standard-3†
Water	0.55	0.05	0	0	0
Sample‡	10.00	10.00	10.00	10.00	10.00
80 g/L Ascorbic acid	0.05	0.05	0.05	0.05	0.05
Copper†	0	0	0.05	0.05	0.05
0.5 %(w/v) BCA	0	0.50	0.50	0.50	0.50

† The standard-1, -2 and -3 correspond to solutions of 20, 60 and 100 mg/L copper(II), respectively, and provided effective concentrations of 0.1, 0.3 and 0.5 mg/L Cu additions, respectively.

‡ For red wine the 'sample' is a digest of the red wine. That is, 50 mL of red wine, 5 mL of 30 %(w/v) hydrogen peroxide and 5 mL of 1.0 M sodium hydroxide heated to 80 °C for at least 6 hrs (overnight is also fine).

The measurement of total Cu in red wine by the bicinchoninic (BCA) colorimetric method was performed as described within Kontoudakis et al. (2020). The red wine underwent digestion, to remove red pigmentation, and then colorimetric analysis. The digestion involved the incubation of 50 mL of red wine with 5 mL of 1.0 M sodium hydroxide and 5 mL of 30 %(w/v) hydrogen peroxide. The digestion was conducted in 100 mL reagent bottles at 80 °C for 6 hours with the lid left loose for the first hour, to allow gas to dispel, and tightened thereafter. After digestion, the samples were left to cool to room temperature prior to colorimetric analysis. The 60 mL digest solution was then used to prepare a blank, sample and standard addition solutions (Table 5.1). The methodology of analysis was similar to that for white wine (Section 5.1.2 and Table 5.2), with three important modifications: 0.50 mL of 0.50 %(w/v) BCA was used rather than 0.25 mL of 0.05 %(w/v) BCA, no silver(I) addition was made, and the filtration and spectrophotometric measurement was conducted immediately after reagent addition, mixing and filtration (i.e., no 30 minute incubation period was utilised). Consequently, each solution (i.e., blank, non-blank etc…) in Table 5.1 was prepared individually and measured immediately, rather than prepared as

a group and measured with variable post-BCA-addition standing times. A benchtop Helios UV/Visible spectrophotometer was used for all spectroscopic analyses (including for the method outlined in Section 5.1.2). A spread-sheet (Appendix 6) provides automated conversion of absorbance measurements obtained from samles in Table 5.1 to total Cu concentration.

5.1.2 Cu in white wine by bicinchoninic acid (BCA) analysis: Total Cu and Cu fractions 1, 2, and 3.

Table 5.2. Volume of solutions to enable the colorimetric analysis of Cu fractions and total Cu in white wine. A 15 mL plastic centrifuge tube is used for each solution.

Determination:	**Cu fraction**			**Total Cu and Cu Calibration**			
Analysis parameter:	Wine F-blank	Wine F1	Wine F1+2	Wine blank	Wine +0Cu	Wine +0.1Cu†	Wine +0.3Cu†
Solution to add	*Volume (mL)*			*Volume (mL)*			
water	0.40	0.15	0.15	0.30	0.05	0	0
sample‡	10.0	10.0	10.0	10.00	10.00	10.00	10.00
80 g/L Ascorbic acid	0.05	0.05	0.05	0.05	0.05	0.05	0.05
1000 mg/L Ag(I)	0	0	0	0.10	0.10	0.10	0.10
20 or 60 mg/L Cu†	0	0	0	0	0	0.05	0.05
0.05 %(w/v) BCA	0	0.25	0.25	0	0.25	0.25	0.25
		Mix.				Mix.	
incubation time (min)	0	0	30	30	30	30	30
analysis	Syringe filter (0.2 or 0.45 µm) and measure absorbance (563 nm) using a 40 mm glass cuvette						

† The standard-1 and -2 require solutions of 20 and 60 copper(II), respectively, and provide effective concentrations of 0.1 and 0.3 mg/L Cu additions, respectively. ‡ The sample was adjusted to pH 4.0. +0Cu, 0 mg/L copper(II). +0.1Cu, 0.1 mg/L copper(II). +0.3Cu, 0.3 mg/L copper(II). BCA, 2,2'-bicinchoninic acid.

The method for colorimetric analysis of total Cu and its fractions in white wine was conducted as described by Clark et al. (2020). Table 5.2 shows the optimised protocol for determination of Cu fractions 1, 2 and 3, using BCA as well as calibration and total Cu determination. All additions described in Table 5.2 were made with 0.2, 1.0 and 10.0 mL Eppendorf Research micropipettes to plastic 15 mL centrifuge tubes, that were used as supplied without any further washing. The required solutions were all prepared fresh on the day of analysis, with ultrapure water, and consisted of L-ascorbic acid (80 g/L), 1 g/L silver(I) (from silver(I) nitrate), and 0.05 %(w/v) BCA. The stock solutions of Cu required during analysis were 20 and 60 mg/L and each was prepared from either 1000 mg/L Cu ICP standard or copper(II) sulfate pentahydrate. After additions were made, in the order indicated in Table 5.2, the final mixture was inverted in the capped centrifuge tube and syringe filtered (regenerated cellulose, pore size 0.20 µm, diameter 26 mm) immediately, or after 30 min, before spectrophotometric analysis. Prior to analysis, the first 1–2 mL of filtrate was used to rinse a 40 mm glass cuvette, and then the cuvette was filled with the remaining filtered mixture and the absorbance was measured at 563 nm. The time between BCA addition and the immediate absorbance measurement required around 0.5–1.0 min for filtration and rinsing of the cuvette. The addition of 0.05 mL of each of the standards during the

determination (Table 5.2) corresponded to a final Cu concentration of 0.1 and 0.3 mg/L for the calibration quantification. A spread-sheet (Appendix 6) provides automated conversion of absorbance measurements obtained from the samples in Table 5.2 to Cu concentration (i.e. fraction of Cu and total Cu).

5.1.3 Cu fractions in red wine by diatomaceous earth depth filtration

Beco Steril S80 diatomaceous earth depth filters (DEDF), 45 mm in diameter and 3.5 mm in depth, were sourced from Blue H2O Filtration (Oakleigh, Vic., Australia). The filters were used within Millipore (MA, USA) polypropylene Swinnex filter holders. The filters were conditioned with 50 mL of 1.0 %(w/v) citric acid, 20 mL of model wine and 10 mL of sample wine using a plastic 20 mL syringe to pass solutions through the filter. Afterwards, 100 mL of sample wine was passed through the conditioned filter, using a plastic 20 mL syringe, and the concentration of Cu in the filtrate was determined by ICP-OES. All wines were filtered in triplicate, using new, conditioned filters for each replicate. Filters were only used once and discarded after use.

5.1.4 Total Cu in white wine by DiBr-PAESA colorimetric analysis with an automated analyser.

Total Cu concentrations in white wines were determined utilising a KoneLab Arena 20XT Analyzer (Thermo Scientific, Waltham, MA USA) with 25x12 multicell cuvettes (7 mm pathlength). A commercially available automated analyser kit for Cu (984628, Thermo Scientific) was used for analysis, and was based on the colorimetric reagent 4-(3,5-dibromo-2-pyridylazo)-N-ethyl-N-sulfopropylaniline (DiBr-PAESA). The Cu-DiBr-PAESA complex was measured at 600 nm. Analysis parameters/settings for Cu determination were as per the operating instructions supplied with the Thermo Scientific Cu analysis kit. This included reagent 1 containing a pH 4.9 buffer and ascorbic acid, while reagent 2 contained the diBr-PAESA reagent and undisclosed stabilizers. All steps in the analysis were conducted at 37 °C. The test flow started with addition of 125 µL of reagent 1, dispensed with 20 µL extra reagent, then incubation for 18 seconds prior to addition of 12 µL of sample, dispensed with 40 µL extra. Note, the 'extra' dispensed goes to waste and is not measured as part of the analysis. This was then incubated for 5 minutes prior to addition of 12 µL of reagent 2, dispensed with 5 µL extra. This was incubated for another 5 minutes prior to absorbance measurement at 600 nm. The only deviation from the commercial operating instructions was the addition of 10 mg/L Ag(I) to certain wines prior to analysis. Calibration was performed by measuring a blank and four Cu standards in a 12 % aqueous ethanol solution buffered to pH 3.2 with tartaric acid. The Cu standards were 0.05, 0.2, 0.6 and 0.8 mg/L.

5.1.5 Cu fractions in white wine by DiBr-PAESA colorimetric analysis with an automated analyser.

Cu fractions in white wines were determined utilising the same KoneLab Arena 20XT Analyzer (Thermo Scientific, Waltham, MA USA) and cuvettes as described in the previous section. The photometric Cu kit was a Steroglass copper kit (119466) supplied by CIScal (www.ciscientific.com). Reagent 1 contained acetate buffer as well as undisclosed stabilisers and preservatives, and reagent 2 contained the DiBr-PAESA reagent. The test flow of analysis for

measurement of Cu fraction 1 and Cu fraction 1+2 is shown in Appendix 7. The only difference between the two measurements was the time of incubation after addition of the DiBr-PAESA reagent which was 20 seconds for measurement of Cu fraction 1 and 5 minutes for measurement of Cu fraction 1+2. Calibration was performed by measuring a blank and three Cu standards in a 12 % aqueous ethanol solution buffered to pH 3.2 with tartaric acid. The Cu standards were 0.05, 0.15 and 0.3 mg/L.

5.2 Depth filtration of wines: Industrial scale.

A plate and frame Seitz Enzinger Noll filter press was used with 200 mm x 200 mm plates. The unit was modified to only allow wine passage through two depth pads. Wine was pumped with a Liverani AC 220V flexible impeller pump.

The filter pads utilised were:

i. cellulose depth filters (CDF, Figure 6.17 A) with a thickness of 3.9 mm, and a nominal retention rate of between 0.1-0.4 µm (BECOPAD 170). The ash content (indicative of diatomaceous earth) was < 1.0 % and the bursting wet strength was >150 kPa and the water throughput was 77 L/m^2/min for a 100 kPa ΔP. The pads were cut to 200 mm x 200 mm from 400 mm x 400 mm sheets.

ii. diatomaceous earth-cellulose depth filters (DEDF, Figure 6.17B) with a thickness of 3.9 mm, and a nominal retention rate of 0.2 µm (BECO Steril S 80). The ash content (indicative of diatomaceous earth) was 50 % and the bursting wet strength was > 80 kPa and the water throughput was 46 L/m^2/min for a 100 kPa ΔP. The pads were cut to 200 mm x 200 mm from 400 mm x 400 mm sheets.

Prior to use pads were conditioned by passing a 1 %(w/v) citric acid solution, also containing 180 mg/L PMS, through the pads for 30-60 minutes at a flow rate of 2-3 L/min. A 20 L volume of the wine to be filtered was passed through the pads to wash off the residual citric acid solution. During filtration, the wine was at a temperature of 12.9 °C and remained within 1 °C of this temperature throughout the entire filtration process.

For the cellulose depth filters, triplicate filtration of 300 L was performed and for each 300 L the filtrate was collected in 50 L batches. The flow rate varied from around 3.3-2.5 L/min with a higher flow rate at the start of filtration compared to the final stages, and the maximum flow rate was dictated by the capabilities of the pump. Note that this flow would be around 10 times faster (i.e., 25-33 L/min) if the full pad capacity of the plate and frame unit was used (i.e., 20 pads rather than 2). The filtrate was analysed for total Cu and Cu fractions, as well as the analysis of a 300 L blend composed of an equal 500 mL mix of all individual filtrate fractions. All total Cu and Cu fraction measurements were conducted by BCA colorimetry for white wine and by DEDF ICP-OES for red wine.

For the diatomaceous earth depth filters, triplicate filtration up to 500 L was performed, and then duplicate filtration up to 950 L. The fractions collected are shown below:

fractions	Wine volume (L)
F1	0-58
F2	58-113
F3	113-171
F4	171-229
F5	229-295
F6	295-343
F7	343-400
F8	400-495
F9	495-600
F10	600-800
F11	800-945

The flow rate varied from around 1.8-2.6 L/min with a higher flow rate at the start of filtration compared to the final stages, and the maximum flow rate was dictated by the capabilities of the pump. The batches were analysed for total Cu and Cu fractions as well as the analysis of a 950 L blend composed of mix of all individual filtrate fractions in proportion to their overall volume.

5.3 Methods used in the instrumental analysis of reductive aroma compounds

5.3.1 Analysis of free and salt-treated H_2S and MeSH by GC-SCD

Analysis was conducted utilising an Agilent 355 SCD coupled to an Agilent 7890B gas chromatogram (Santa Clara, CA, USA) equipped with a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland) and an Agilent J&W DB-sulfur SCD 60m x 0.32 m x 4.2 µm analytical column. The GC settings were as described within Kontoudakis et al. (2017a).

The free forms of H_2S and MeSH were determined as described by Kontoudakis et al. (2017a) and as adapted from Franco-Luesma and Ferreira (2014). Immediately after a wine bottle was opened 10 ml of sample was taken with a glass 10.0 mL volumetric pipette and added into a 20 ml standard headspace vial (CrossLab, Agilent). 50 µL of ethylmethylsulfide (EMS) internal standard solution (20.2 mg/L) was added to achieve a final concentration of 100 µg/L. The vial was tightly sealed with a PTFE/silicone lined screw cap and immediately put in the sampler tray at 4 °C. Within 5-10 minutes of placement on the sample tray, the vial was incubated at 45 °C for 5 min with agitation at 400 rpm. Then 1000 µL of the headspace was taken, with a 1 mL heated headspace syringe (Agilent Technologies) thermostated at 60 °C, and injected into the injector, set at 150 °C, under a 1:8 split ratio. Stock solutions of both $Na_2S{\cdot}9H_2O$ (5.0 mg H_2S/L and 0.5 mg H_2S/L) and $NaSCH_3$ (5.0 mg MeSH/L and 0.5 mg MeSH/L) were prepared in chilled water (4 °C) and used with the EMS internal standard solution to make internal calibration standards in model wine. The

model wine consisted of 12 % (v/v) aqueous ethanol, 0.011 M potassium hydrogen tartrate and 0.008 M tartaric acid.

The salt-treated concentrations of H_2S and MeSH were determined as described by Kontoudakis et al. (2017a) and as adapted from Siebert et al. (2010). A 10 mL aliquot of sample and 50 µL of EMS internal standard (20.2 mg/L) were transferred to a headspace vial containing 2 g of NaCl (s) and placed on a sampler tray at 4 °C. Samples were left on the sampler tray anywhere from 1-15 h prior to analysis. The vial was incubated at 45 °C for 30 min and injected as described for the free forms. Internal calibration standards were prepared as described for free determination, except with the inclusion of 2 g NaCl (s) to each standard.

5.3.2 Analysis of free H_2S by gas detection tube (GDT)

Free H_2S analysis by GDT was performed largely as described within Allison et al. (2022), with modifications described below. The Gastec (The Canary Company, Lane Cove) GDTs utilised were 5L SO_2 (1.25-200 ppm) in series with 4LT H_2S (0.2-2.0 ppm), which were $HgCl_2$-type GDTs. The exception was for one wine (i.e., wine 21, Appendix 11) particularly high in H_2S, which required the alternate 4LL H_2S (2.5-60 ppm) GDT (i.e., $Pb(CH_3CO_2)_2$-type) rather than the 4LT. For several white wines with high molecular SO_2 concentration, it was necessary to use two 5L SO_2 GDTs in series prior to the 4LT GDT, to avoid SO_2 contamination of the 4LT tube. Upon opening wine bottles, 60 mL of sample was added to a round-bottom flask, and free H_2S was sparged with high purity nitrogen for 15 minutes from the flask into the 5L and 4LT GDTs in series. All H_2S GDTs were calibrated with 0, 1.7, 3.3 and 5 µg/L H_2S standards prepared in model wine.

6 RESULTS AND DISCUSSION

6.1 Measurement of total Cu and fractions

Prior to the commencement of this project, only total Cu in white wine could be measured by the colorimetric BCA method (Kontoudakis et al. 2020). The methodology for measurement is outlined in Table 6.1. The method utilises reagents and equipment that are either already present in wineries or can be sourced cheaply. The biggest impediment to the application of this method may be the capability of specific winery-owned spectrophotometers to fit a cuvette of 40 mm, but even with a cuvette of 10 mm, the method is still expected to have a limit of detection (i.e., 0.06 mg/L) below the total Cu concentration in most wines.

Table 6.1. Optimised procedure for the addition of solutions for the determination of total Cu in white wine by standard addition. All values are in units of mL.

Solution to add	Blank	Non-blank	Standard-1†	Standard-2†	Standard-3†
Water	0.55	0.05	0	0	0
Sample‡	10.00	10.00	10.00	10.00	10.00
80 g/L Ascorbic acid	0.05	0.05	0.05	0.05	0.05
1 g/L Silver(I)	0.10	0.10	0.10	0.10	0.10
Copper†	0	0	0.05	0.05	0.05
0.05 (w/v) BCA	0	0.50	0.50	0.50	0.50
Absorbance measurement at 563 nm after 30 minutes					

† The standard-1, -2 and -3 correspond to solutions of 20, 60 and 100 mg/L copper(II), respectively, and provided effective concentrations of 0.1, 0.3 and 0.5 mg/L Cu additions, respectively.

‡ For white wine the 'sample' is white wine pH adjusted to 4.0.

Although successful in white wine, there was no related simplified measure for total Cu in red wine or for the measurement of different forms of Cu in wine. The measurement of different Cu forms in wine could only be achieved with the research-orientated techniques of medium exchange constant-current stripping potentiometry or solid phase extraction with detection of fractions by inductively coupled plasma with optical emission spectroscopy (ICP-OES). Both techniques required expensive instrumentation and were labour intensive methods not generally available in winery laboratories. This section investigated the development of industry-friendly measures for Cu fractions in white wine, and total Cu and Cu fractions in red wine. Finally, based on the feasibility of such measures, their transition to automated measurement was also investigated.

6.1.1 **The colorimetric analysis of total Cu in red wine** (Kontoudakis et al. 2020)

While the BCA reagent was suitable for direct Cu analysis in white wine (Table 6.1), it was not suitable for red wine as the anthocyanins and polymeric pigments saturated the absorbance at the critical analysis wavelength of 563 nm. Preliminary investigations trialled the addition of BCA, or other colorimetric reagents, to red wine followed by solvent extraction of the coloured Cu-complexes for spectral measurement. However, all Cu quantitation attempts were deemed failures based on insufficient accuracy and/or precision of results.

Given the lack of success with extractive approaches, digestive procedures were investigated with an aim of maintaining the sensitivity of the BCA method as much as possible by minimising the dilution of the wine during digestion. The best result was obtained with the addition of 5 mL of 30 %(w/v) hydrogen peroxide and 5 mL of 1.0 M sodium hydroxide to 50 mL of red wine (i.e., a 1.2-fold dilution). Once the digesting reagents were added to red wine it was heated to 80 °C for at least 6-hours. Heating the digest overnight was also found to be suitable. After heating for 6 hours, the red wines often resembled the colour of oxidised white wines (i.e., brown), but after heating overnight the red wines were completely clear and transparent.

After digestion, samples were allowed to cool to room temperature, and the colorimetric analysis was conducted with the addition of solutions as indicated in Table 6.2. The methodology of analysis was similar to that for white wine (Table 6.1), with three important modifications: 0.50% (w/v) BCA was used rather than 0.05% (w/v), no Ag(I) addition was made, and spectrophotometric measurement was conducted immediately after reagent addition, mixing and filtration (i.e. no 30 min incubation period was utilised). Consequently, each solution, such as the blank and non-blank, in Table 6.1 was prepared individually and measured immediately, rather than prepared as a group and measured with variable standing times post-BCA-addition.

Table 6.2. Optimised procedure for the addition of solutions for the determination of Cu in red wine by standard addition. All values are in units of mL.

Solution to add	Blank	Non-blank	Standard-1†	Standard-2†	Standard-3†
Water	0.55	0.05	0	0	0
Sample‡	10.00	10.00	10.00	10.00	10.00
80 g/L Ascorbic acid	0.05	0.05	0.05	0.05	0.05
Copper†	0	0	0.05	0.05	0.05
0.5 %(w/v) BCA	0	0.50	0.50	0.50	0.50

† The standard-1, -2 and -3 correspond to solutions of 20, 60 and 100 mg/L copper(II), respectively, and provided effective concentrations of 0.1, 0.3 and 0.5 mg/L Cu additions, respectively.

‡ For red wine the 'sample' is a digest of the red wine. That is, 50 mL of red wine, 5 mL of 30 %(w/v) hydrogen peroxide and 5 mL of 1.0 M sodium hydroxide heated to 80 °C for at least 6 hrs (overnight is also fine).

The figures of merit for the measurement of total Cu in red wine, based on the procedure of Table 6.2, and white wine, based on the procedure of Table 6.1, were similar to each other (Table 6.3) and were sufficiently sensitive to allow routine analysis of Cu in the vast majority of red wines (> 0.01 mg/L). The limit of detection (LOD) and limit of quantification (LOQ) were slightly lower in red wine than white wine because the background colour in red wine was less than in white wine after the digestion. Despite the digestion step, the recovery of the Cu in red wine and white wine were similar.

Table 6.3. Figures of merit for the determination of total Cu in white and red wines using the BCA protocol. The uncertainty indicated is the standard deviation.

Validation parameter	White wine	Red wine
Procedure	Table 6.1	Table 6.2
Limit of detection (mg/L)	0.015	0.010
Limit of quantification (mg/L)	0.030	0.020
Repeatability RSD (%, n=10)	3 ± 1	3.8 ± 0.2
Reproducibility RSD (%, n=3)	6 ± 4	6.4 ± 5
Linear range (mg/L)	0.030 – 1.00	0.025 – 1.00
Recovery (%, n = 2)	102 ± 2	104 ± 3
Accuracy	Kontoudakis et al. (2020)	Figure 1

The accuracy of the methodology was assessed by comparison of determined results to that of ICP-OES (Figure 6.1). The results showed good agreement between the techniques, across the concentrations of Cu routinely found in red wines. There was a minor trend toward over-estimation by the colorimetric technique, which was reflected by the trend-line gradient in Figure 6.1 being below 1.000 (i.e., 0.9193). This may have been a consequence of some concentration of sample during the digestion step, but such losses were monitored and found to be generally negligible.

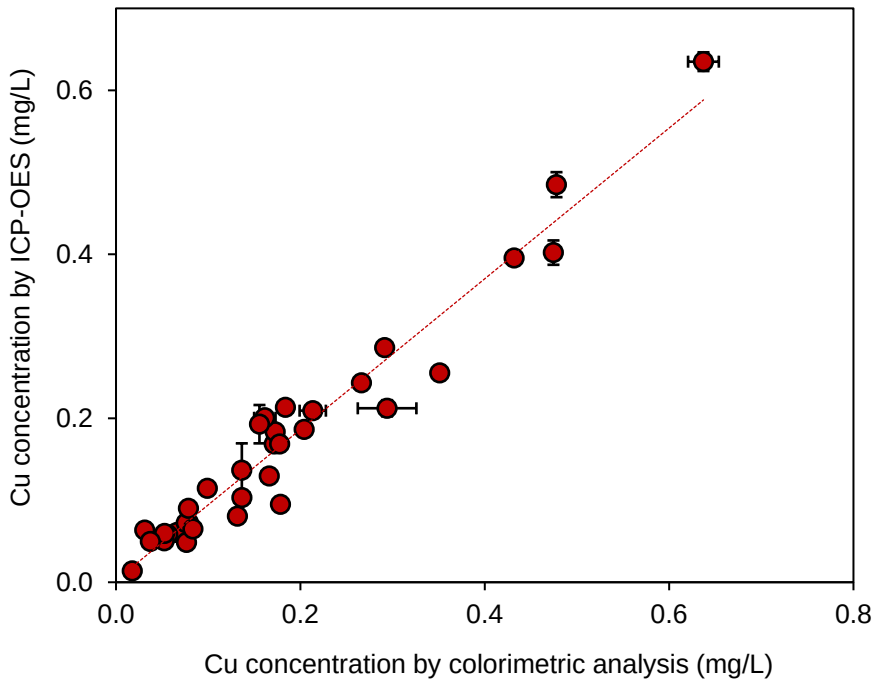


Figure 6.1. The correlation plot for the quantitation of Cu in 34 red wines by ICP-OES and the colorimetric technique. All samples were analysed in triplicate by each technique. The error bars represent the standard deviation (n = 3) and the equation for the trend-line was y = 0.919x + 0.002 (R^2 = 0.948).

6.1.2 The colorimetric analysis of Cu fractions in white wine

Our previous work (Kontoudakis et al. 2019b) showed that the majority of Cu in 49 red and white wines was not detectable by the electrochemical technique known as stripping potentiometry. When the same technique was applied to Cu in model wine systems with different wine components added individually (Kontoudakis et al. 2017b), it was found that only hydrogen sulfide could prevent detection of Cu by the electrochemical technique. Consequently, the electrochemically detected and non-detected forms were designated non-sulfide-bound and sulfide-bound forms, respectively (Kontoudakis et al. 2019b). A limitation of the binding study was the assessment of thiol compounds at concentrations more relevant to volatile thiol compounds in wine (Kontoudakis et al. 2017b), rather than at the higher concentrations relevant to involatile thiol compounds in wine (i.e., glutathione) (Kritzinger et al. 2013).

Table 6.4. Volume of solutions to enable the colorimetric analysis of Cu fractions and total Cu in white wine. A 15 mL plastic centrifuge tube is used for each solution.

Determination:	**Cu fraction**			**Total Cu and Cu Calibration**			
Analysis parameter:	Wine F-blank	Wine F1	Wine F1+2	Wine blank	Wine +0Cu	Wine +0.1Cu†	Wine +0.3Cu†
Solution to add	*Volume (mL)*			*Volume (mL)*			
water	0.40	0.15	0.15	0.30	0.05	0	0
sample‡	10.0	10.0	10.0	10.00	10.00	10.00	10.00
80 g/L Ascorbic acid	0.05	0.05	0.05	0.05	0.05	0.05	0.05
1000 mg/L Ag(I)	0	0	0	0.10	0.10	0.10	0.10
20 or 60 mg/L Cu†	0	0	0	0	0	0.05	0.05
0.05 %(w/v) BCA	0	0.25	0.25	0	0.25	0.25	0.25
		Mix.				Mix.	
incubation time (min)	0	0	30	30	30	30	30
analysis	Syringe filter (0.2 or 0.45 µm) and measure absorbance (563 nm) using a 40 mm glass cuvette						

† The standard-1 and -2 require solutions of 20 and 60 copper(II), respectively, and provide effective concentrations of 0.1 and 0.3 mg/L Cu additions, respectively. ‡ The sample was adjusted to pH 4.0. +0Cu, 0 mg/L copper(II). +0.1Cu, 0.1 mg/L copper(II). +0.3Cu, 0.3 mg/L copper(II). BCA, 2,2'-bicinchoninic acid.

To investigate the ability of the BCA-colorimetric method to discriminate against different Cu forms in wine, a model wine (pH 4) was prepared with various wine components added at white wine-like concentrations. The BCA analysis methodology was similar to that used for total Cu in white wine (Table 6.1) but without the addition of Ag(I) (Table 6.4). Additionally, 0.25 mL of the 0.05 %(w/v) BCA reagent was used (Table 6.4) rather than 0.5 mL (Table 6.1) as this was found to avoid excess precipitation of the BCA-Cu chromophore (Clark et al. 2020).

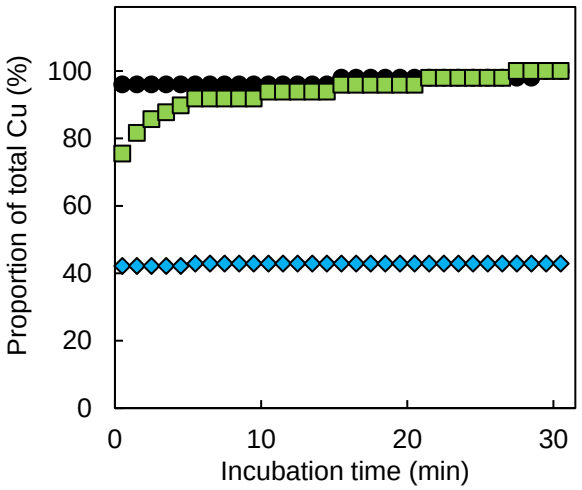


Figure 6.2. Proportion of total Cu detected during the colorimetric analysis of pH 4.0 model wine samples incubated with BCA over 30 minutes. The model wine samples all contained 0.1 mg/L Cu with either no other component (●), 50 mg/L glutathione (□) or 0.11 mg/L hydrogen sulfide (◆). No Ag(I) was added to the samples during BCA analysis. The BCA analysis was conducted 1.5 h after the samples were prepared and measurements were conducted every minute for 30 minutes.

Table 6.5. The concentration of determined Cu in model wine solutions (pH 4.00) after incubation for 0, 10 and 30 minutes with BCA during the colorimetric analysis.

Sample†	Added Cu (mg/L)	Storage time pre-analysis (h)	Cu concentrtion (mg/L) at different BCA incubation times		
			0 min	10 min	30 min
Control	0.1	2	0.10 ± 0	0.10 ± 0	0.10 ± 0
1000mg/L citric a.	0.1	2	0.10 ± 0	0.10 ± 0	0.10 ± 0
50mg/L catechin	0.1	2	0.10 ± 0	0.10 ± 0	0.10 ± 0
200mg/L caffeic a.	0.1	2	0.10 ± 0	0.10 ± 0	0.10 ± 0
400mg/L ascorbic a.	0.1	2	0.10 ± 0	0.10 ± 0	0.10 ± 0
120mg/L free SO_2	0.1	2	0.10 ± 0	0.10 ± 0	0.10 ± 0
50mg/L glutathione	0.1	0.7	0.079 ± 0.001	0.086 ± 0	0.097 ± 0.001
50mg/L glutathione	0.1	2	0.064 ± 0.001	0.068 ± 0.001	0.081 ± 0.001
50mg/L glutathione	0.1	5	0.045 ± 0.001	0.054 ± 0.001	0.061 ± 0.001
0.05 mg/L H_2S (1:1,H_2S:Cu)	0.1	2	0	0	0
0.11mg/L H_2S (1:1.5,H_2S:Cu)	0.3	2	0.13 ± 0.01	0.13 ± 0	0.13 ± 0

† Samples were prepared in duplicate for each measurement. ‡ The uncertainity refers to the standard deviation (n = 2). BCA, 2,2'-bicinchoninic acid.

Figure 6.2 shows that 0.1 mg/L Cu in the model wine, with no other added component (see ●), resulted in the near-instantaneous detection of all the Cu in the model wine. The same was the case when additional wine components were added to the model wine in isolation, including caffeic acid, (+)-catechin, L-ascorbic acid, sulfur dioxide, and citric acid (Table 6.5). The exception to this outcome was when either hydrogen sulfide or glutathione were added to the model wine

(Table 6.5). Figure 6.2 shows that in the hydrogen sulfide and Cu system with Cu in excess (mole ratio 1:1.5), the detected Cu was only around 40 % of total (i.e., 0.13 mg/L out of 0.30 mg/L Cu), and it remained constant over the entire 30-minute incubation period (see ♦). When the H_2S was added at a 1:1 mole ratio with Cu(II) then no Cu could be detected (Table 6.5). This demonstrated that BCA could not dissociate Cu from the copper(I) sulfide complex in the model wine within the 30 min incubation period. Therefore, like the electrochemical technique it appeared that the BCA colorimetric technique could provide some discrimination between the sulfide-bound and non-sulfide bound forms of Cu.

When 50 mg/L glutathione was present in the model wine (see ■ in Figure 6.2) some Cu was not able to be detected immediately, but after around 10 minutes, all the Cu was detected. This implied a weak interaction between glutathione and Cu that impeded instantaneous reaction between BCA and Cu, but after 10 minutes all the Cu could be sequestered by BCA. The reaction time between glutathione and Cu before colorimetric analysis was also found to be important, whereby reaction times of 0.7, 2 and 5 hours led to a decrease in the amount of Cu detected at all BCA incubation times (Table 6.5). Such a result is consistent with studies of thiol compounds (e.g., 6-sulfanylhexan-1-ol) and Cu in model wine solutions saturated with oxygen, whereby after initial reaction, further complex products and a precipitate eventuated (Kreitman et al. 2016). These results suggest that the colorimetric method may have the ability to discriminate between at least three different fractions of Cu. This includes Cu that can be sequestered by BCA immediately, that that can be sequestered during the 30 min incubation time, and finally the forms of Cu bound sufficiently strongly not to be dissociated within this time frame. The different measured species were subsequently referred to as Cu fractions 1, 2 and 3, respectively. Cu fraction 1 appeared most relevant to Cu-organic acid complexes, Cu fraction 2 to weakly bound Cu-thiol species and Cu fraction 3 attributed to the strongly-bound Cu associated with Cu-binding to sulfide, and also to any thiol-Cu complexes that are not dissociated by BCA within the 30-minute incubation period.

The results for the colorimetric method were compared to the stripping potentiometry method for 41 white wines. The results showed good agreement between Cu fraction 1, from the BCA colorimetric method, and the electrochemically detected Cu (Figure 6.3). In contrast, Cu fraction 2 and 3, as determined by the colorimetric method, both had poor agreement with the electrochemically detected Cu (data not shown). This implied that like Cu fraction 1 of the BCA analysis, the electrochemical technique was detecting Cu associated with Cu-organic acids and not Cu weakly-associated to thiol compounds or those strongly bound to thiols or sulfides. This was confirmed with further experiments in model wine systems containing Cu(II) and various wine-like concentrations of glutathione. Specific details on these results can be viewed in Clark et al. (2020), but they confirmed that with increasing glutathione concentrations, the electrochemically detected Cu in the model wine system decreased. Although past studies had investigated the impact of glutathione on electrochemically detected Cu, they had used concentrations of glutathione that were lower than often found in wine.

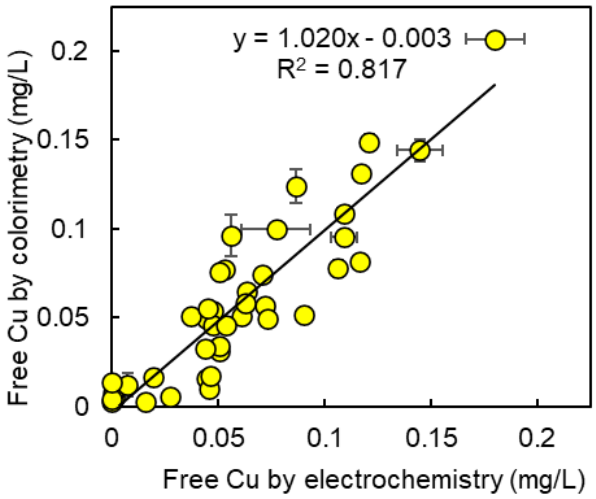


Figure 6.3. The comparison of Cu concentrations in 41 white wines as quantified by the BCA-colorimetric method and stripping potentiometry. The colorimetric method utilised samples adjusted to pH 4 and with a 0 minute incubation time. The error bars represent the standard deviation (n = 3).

The protocol outlined in Table 6.4 allows the quantitation of Cu fractions 1, 2, 3 and total Cu in white wine. The absorbance values obtained after spectroscopic measurement can be converted to concentrations using the '*BCA_Cu_calculator.xls*' file (Appendix 6). The total Cu concentration is quantified via standard additions, after subtraction of the corresponding blank (i.e., 'wine blank' in Table 6.4), and Cu fractions 1 and 1+2 are quantified via calibration graph after subtraction of their corresponding blank (i.e., 'F-blank' in Table 6.4). Cu fraction 3 can be found from the subtraction of Cu fractions 1+2 from the total Cu concentration. Cu fraction 2 can be found from the difference between Cu fractions 1+2 and Cu fraction 1.

6.1.2.1 General methodological tips for BCA colorimetric analysis

Several idiosyncrasies of the colorimetric technique are worthy of mention to aid accurate colorimetric determination.

The background reading of the spectrophotometer is the absorbance reading without the cuvette in the instrument. Once the spectrophotometer has warmed up (~20 min after turning the instrument on), it should be zeroed with water in the 40 mm glass cuvette at the wavelength of 563 nm. Close attention should then be paid to fluctuations in the background absorbance reading of the spectrophotometer throughout subsequent analysis (i.e., the absorbance when no cuvette is in the spectrophotometer and the sample compartment is closed). The background fluctuations will generally be instrument-dependent, and may impact Cu quantification if significant fluctuations occur between sample analysis and calibration determinations. Such fluctuations can be accounted for in quantification calculations if the background variation is recorded over time.

If more than 2 mL of sample/reagent filtrate is used to rinse the 40 mm glass cuvette prior to measurement then there may not be enough solution volume in the cuvette to enable accurate absorbance measurement. If the spectrophotometer light passes over the top of the solution or impinges on the solution/air interface then low or high readings, respectively, will result. In this case, if the glass cuvette is raised by 1–2 mm, for example by sitting it on its lid or an alternative flat spacer, then successful measurement can be achieved. If the cuvette is raised too high then the bottom of the cuvette will absorb light.

Other impediments to accurate absorbance readings include bubbles and a shimmering effect caused by the slow convective mixing of solutions of different viscosity. The latter can occur if the cuvette has been rinsed with water, and then is insufficiently rinsed with the analysis mixture, prior to addition of the bulk analysis mixture to the cuvette. If bubbles or solution shimmering occur, sufficient time should be given to allow these effects to dissipate. However, this delay may cause an overestimation of Cu fraction 1 in white wine samples. A short 1–2 s sonication of the analysis mixture, even within the glass cuvette, can remove both these effects rapidly. Bubbles can also be avoided by filtering samples slowly (i.e. under lower pressure), rather than quickly, and by not pushing any air through the filter in the final stages of filtering.

After measurement of the absorbance, the solution should be immediately removed from the glass cuvette and the cuvette rinsed with water. This avoids the gradual deposition of bicinchinonic acid (BCA)-precipitate on the cuvette that is otherwise difficult to remove. If the background absorbance reading of the spectrophotometer remains stable, but the reading for the blank (i.e. water filled cuvette) increases then this is generally evidence of precipitate on the cuvette face. If such precipitate forms, then the washing of the cuvette with a 1:1 mixture of conc. sulfuric acid and 30 %(v/v) hydrogen peroxide is necessary. Approximately two mL of each should be added separately to the cuvette, rinsed around the cuvette for 20–30 s and then tipped from the cuvette followed by rinsing with copious amounts of water. It should be noted that the cuvette will become quite hot during mixing of the sulfuric acid and hydrogen peroxide and care should be taken.

The gradient obtained for the standard addition trendline in white wine will usually be around 0.49 ± 0.02, but it will have some dependency upon the specific spectrophotometer used. Once users of the method are experienced with the technique and routinely achieve standard addition curves with a consistent gradient then a more rapid measure can be adopted. For the rapid measure of total Cu, then just the T-blank and +0Cu solutions need to be determined, while for the rapid measure of Cu fractions then just the F-blank, F1 and F1+2 solutions need to be determined. The blank corrected absorbance values can then be converted to concentrations by dividing them by the gradient value consistently obtained on the user's specific spectrophotometer. However, it is important to note that the full method outlined in Table 6.5 will be more analytically robust than the rapid analysis approach.

6.1.3 The analysis of Cu fractions in red and white wine by filtration

Previous studies showed that the sulfide-bound forms of Cu in white wine could be removed via adsorptive phenomena on various filter media (Kontoudakis et al. 2019a). This approach was investigated as an alternative means of measuring the non-sulfide-bound forms of Cu in white and red wines. Rather than using the membrane filters studied within Kontoudakis et al. (2019a), depth filters that incorporated diatomaceous earth within cellulose media were investigated given the previous application of diatomaceous earth to trap metal sulfides (Chen et al. 2017).

After conditioning of the depth filters, as described in the method section, the Cu in the filtrate was measured after passing 100 mL of wine through the filters. Figure 6.4a compares the Cu measured by ICP-OES in the filtrate with the electrochemically detectable Cu. From the results it is evident (i.e., trend-line gradient < 1) that the Cu in the filtrate tends to overestimate the electrochemically detectable Cu in white wine.

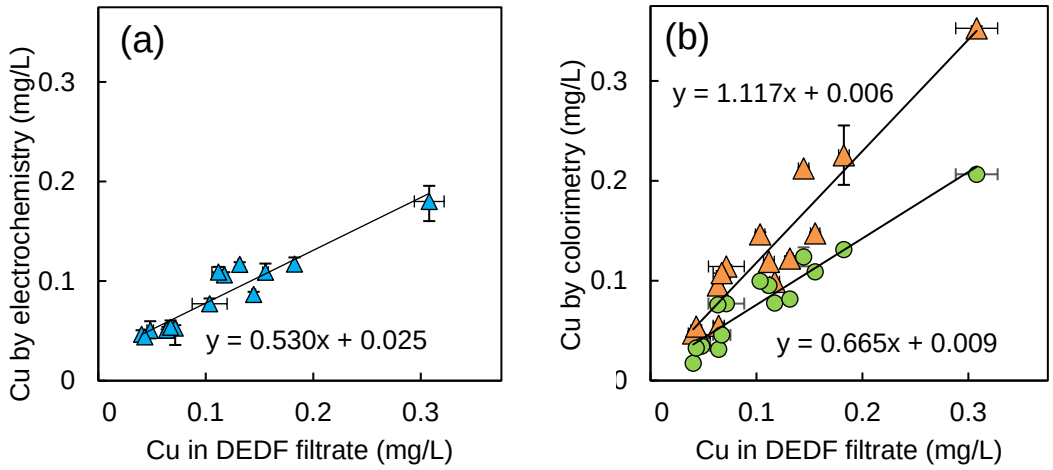


Figure 6.4. Comparison of (a) electrochemical detected Cu versus Cu in the diatomaceous earth depth filter (DEDF) filtrate, and (b) the colorimetric measure of Cu, with BCA incubation times of 0 minute (●) or 30 minutes (▲), versus the DEDF filtrate in 15 white wines.

Similarly, the Cu in the filtrate also overestimated the colorimetric Cu determined without BCA incubation (● in Figure 6.4b). However, Figure 6.4b shows good agreement between the Cu in the filtrate and the Cu measured by the colorimetric method at the longer incubation times (i.e., 30 min) (▲ in Figure 6.4b). This suggested that the filtration method was trapping the strongly bound Cu forms (Cu fraction 3), predominantly sulfide-bound Cu but also potentially aggregated and relatively inert-thiol Cu complexes, and allowing the Cu-organic acids (Cu fraction 1) and weakly-bound thiol-Cu (Cu fraction 2) to pass through the filter. It also suggested that the Cu-thiol complexes unable to be detected by BCA after 30 minutes in the colorimetric analysis may have been particulate and/or aggregated species due to their ability to be retained by the depth filters.

The direct colorimetric analysis of Cu forms in red wine was not possible due to the red wine pigments saturating absorbance at 563 nm, which was the wavelength of BCA-Cu absorbance. Digestion of the red wine allowed colorimetric determination of total Cu (see Section 6.1.1), but such an approach would perturb the Cu forms prior to measurement. Hence in red wine, the approach taken was to pass the wine through the diatomaceous earth depth filters and measure

the Cu in the filtrate by a conventional total Cu measure, such as ICP-OES or BCA colorimetric analysis.

The results in Figure 6.5 show that the electrochemically detected Cu and the Cu in the red wine filtrate compared well, in contrast to the results observed for white wine. A further study on 121 bottles of red wine showed that the relationship held for the larger data set of wines (Figure 6.6). These results suggest that the weakly-bound Cu interactions, associated with certain thiol-Cu complexes, may be less significant in red wine than white wine. Such an outcome is consistent with lower total thiol concentrations are expected in red wines compared to that in white wines, and is based on the more oxidative conditions encountered during the production of red wine compared to white wine.

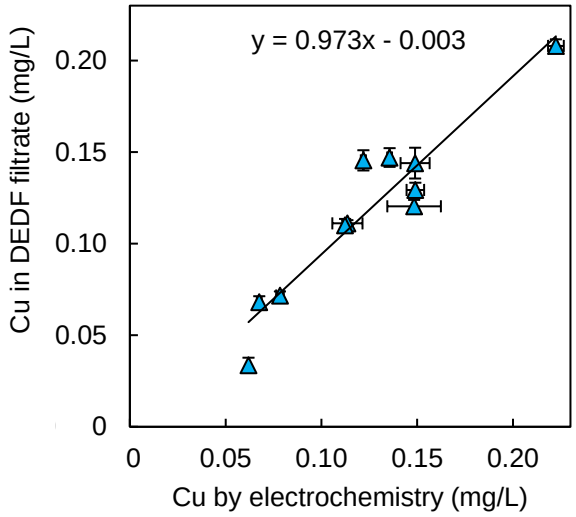


Figure 6.5. Comparison of the electrochemically detected Cu with Cu in the diatomaceous earth depth-filter filtrate determined in 11 red wines. The error bars represent the SD (n=3).

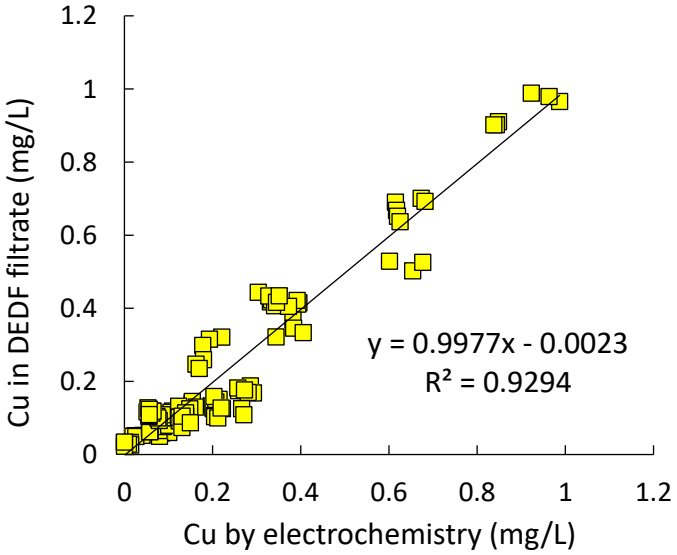
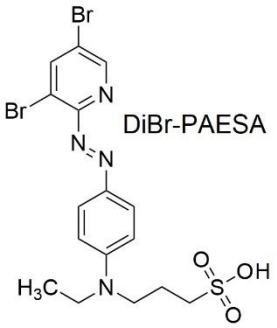


Figure 6.6. Comparison of the electrochemically detected Cu with Cu in the diatomaceous earth depth-filter filtrate determined in 121 bottles of red wines.

6.1.4 Automated analysers for the analysis of total Cu.

Many larger wineries have analysers that can automate colorimetric analysis. However, the transfer of the BCA methodology to the automated analyser at CSU proved unsuccessful. The impeding factors were lack of sensitivity, interference from turbidity and a lack of flexibility in the analysis parameter settings of the automated analyser. Despite numerous attempts at optimising various parameters, including temperature and pH, and attempting to acquire larger pathlength analysis cells, the BCA method did not appear feasible on the automated analyser.

A commercially available kit for total Cu analysis in wine, utilising an alternate colorimetric reagent, was available for use on an automated analyser. It used the reagent called 4-(3,5-dibromo-2-pyridylazo)-N-ethyl-N-sulfopropylaniline (DiBr-PAESA). The specific kit used was from ThermoScientific (984628). It must be noted that although the DiBr-PAESA reagent was more sensitive than BCA, it was not available for direct purchase as a colorimetric reagent throughout the lifetime of this project. This essentially meant it could only be practically used within automated analysers rather than bench top spectrometers due to its prohibitive cost.



The DiBr-PAESA kit was used with an automated analyser to measure 50 white wines and the results were compared to those from reference method of ICP-OES. The automated analyser provided good results for some wines (i.e., with data points near the trend line in Figure 6.7a) but for many other wines the total Cu concentrations were grossly underestimated. For example, for one white wine, the total Cu concentration determined by ICP-OES and the automated analyser were 0.53 ± 0.01 and 0.12 ± 0.01 mg/L, respectively.

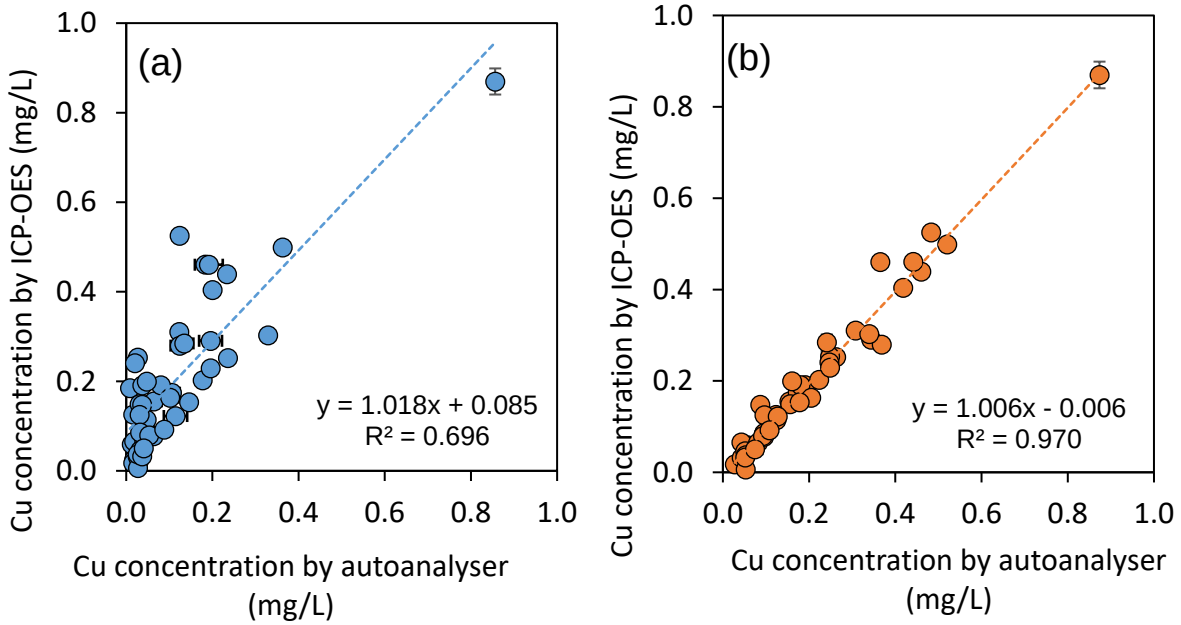


Figure 6.7. The correlation plot for the quantitation of Cu in 50 white wines by ICP-OES and with an automatic spectrophotometric analyser. The automated analyser method was conducted for white wine both without (a) and with (b) the inclusion of 10 mg/L Ag(I) in the samples. Samples were analysed in triplicate by each technique. The error bars represent the standard deviation (n = 3).

Given the success in utilising Ag(I) in the determination of total Cu with the BCA reagent, its influence on the accuracy of the automated method was also assessed. That is, it was proposed that the DiBr-PAESA would be better able to complex with all Cu in the white wine after Ag(I) first induced the release of Cu from any sulfide-bound forms. The results (Figure 6.7b) showed excellent agreement in the total Cu concentrations determined by ICP-OES and the automated analyser when 10 mg/L Ag(I) was added to the samples. Although the quoted determination limit for the Cu test kit was 0.2 mg/L, the results in Figure 6.7b show that when combined with Ag(I), it provides results comparable to ICP-OES at much lower concentrations. This demonstrated the benefit of Ag(I) addition to wine for general colorimetric analysis of total Cu, rather than just for the BCA methodology.

After this work was conducted in 2019, the ThermoScientific DiBr-PAESA Cu analysis kits were no longer commercially available. An alternative photometric Cu kit (Steroglass and supplied by CIScal) was eventually sourced by mid-2024. It used the same DiBr-PAESA colorimetric reagent as the ThermoScientific kit but in combination with other unspecified reagent formulations. Based on Abe et al. (1989), the typical reagent formulations for DiBr-PAESA colorimetric analysis include stabilisers and preservatives, such as ascorbic acid, anionic surfactants (e.g., sodium dodecyl sulfate) and pH buffers. However, preliminary experiments showed that unlike the ThermoScientific kit (Figure 6.7b), the Steroglass Cu kit responded directly to Ag(I). For example, using the Steroglass Cu kit, an absorbance (at 575 nm) of 0.161 ± 0.005 was measured in a model wine and this increased to 0.35 ± 0.08 when 10 mg/L Ag(I) was added to the model wine. Despite the two kits using the same DiBr-PAESA colorimetric reagent, the ThermoScientific reagent formulation seemed to preclude interference from Ag(I) while the Steroglass kit did not. Therefore, further work is required to assess if the currently available DiBr-PAESA-based Cu detection kits can determination total Cu accurately in white wines without the use of Ag(I).

6.1.5 Use of automated analysers for the analysis of Cu fractions.

The Steroglass Cu kits were first optimised on a plate reader (Figure 6.8) to assess their feasibility in measuring Cu fractions in a similar manner to using BCA. Figure 6.9a shows the 575 nm absorbance readings after the addition of the Steroglass photometric Cu reagents to 12 % aqueous ethanol tartaric acid solutions (i.e., model wines) with and without 0.8 mg/L Cu(II). It is evident that a significant absorbance increase occurred in the sample with added Cu(II) (Figure 6.9a) and that the increase was virtually instantaneous (i.e., time 0 minutes in Figure 6.9a). This



Figure 6.8. The colour of model wine and wine samples after the addition of the DeBr-PAESA reagent. The lighter coloured samples are model wine with no added Cu and the darker samples are after addition of 0.8 mg/L Cu(II) to either model wine or real wine samples.

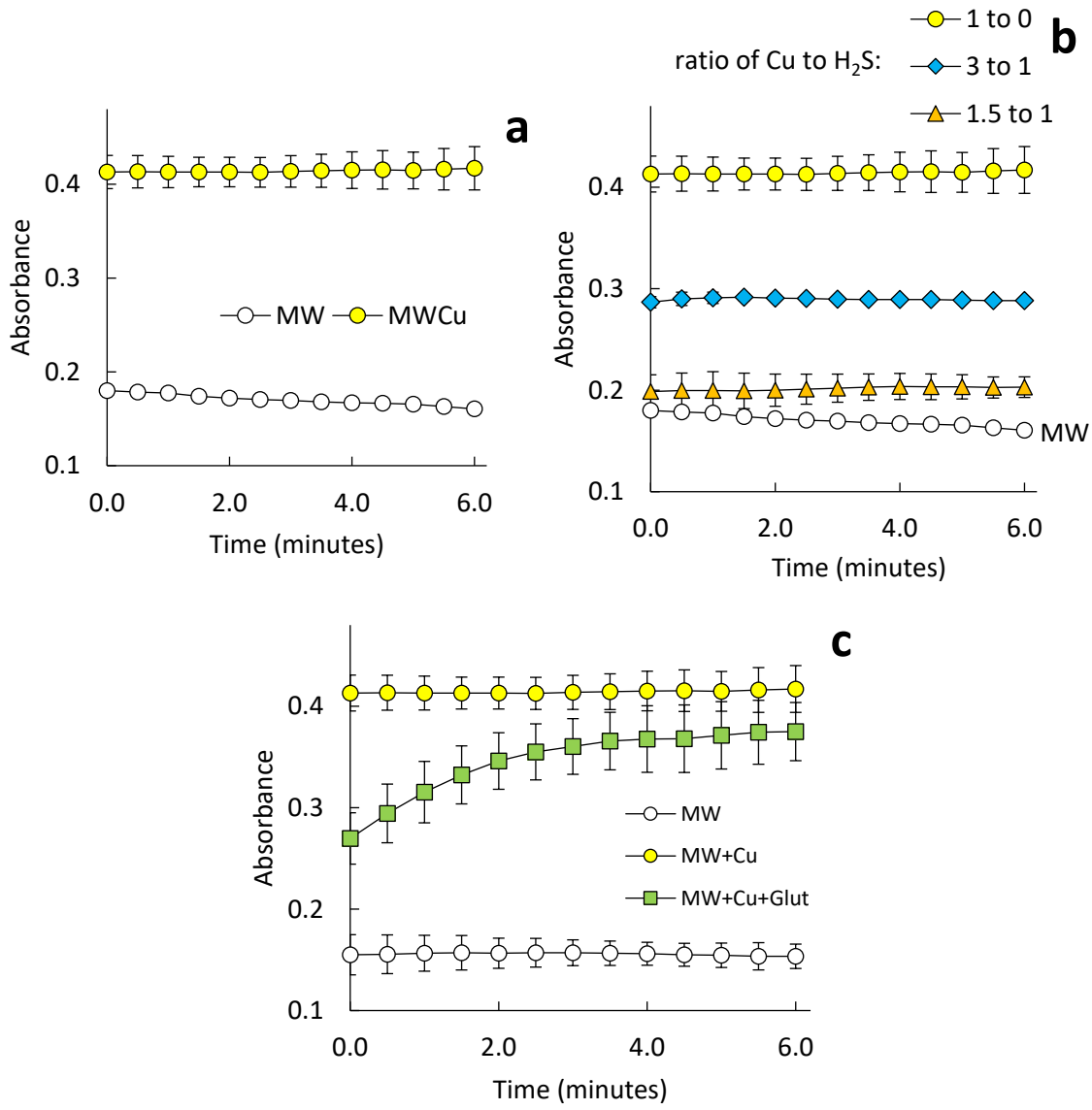


Figure 6.9. The 575 nm absorbance readings of different model wine samples after analysis with the Steroglass photometric Cu kit that utilised the DiBr-PAESA reagent. Cu(II) was added at a concentration of 0.8 mg/L (a-c), glutathione (Glut) at 300 mg/L (c) and H_2S at the stated mole ratios to 0.8 mg/L Cu (II) (b). All model wines were at pH 4.0. MW: model wine consisting of 12 % aqueous ethanol and 0.011 M potassium hydrogen tartrate and 0.008 M tartaric acid (pH 4).

implied that the Cu(II) tartrate complexes in this system were not slowly releasing Cu(II) to the DiBr-PAESA reagent, but instead DiBr-PAESA could access the Cu(II) rapidly. The model wine was also measured with 0.8 mg/L Cu(II) and either 300 mg/L glutathione or increasing concentrations of H_2S. The H_2S was added at a mole ratio of Cu:H_2S of 1:0, 3:1 and 1.5:1, which is an increasing amount of H_2S relative to Cu. Figure 1b shows that the presence of H_2S affords a fraction of Cu that is not detected, and Figure 1c shows that glutathione can induce a fraction of Cu that is not detected instantaneously but can be detected after around 4-5 minutes of incubation. These results showed good parallels with the results obtained by BCA in model wine systems (Figure 6.2, Table 6.5) and provided encouraging evidence that DiBr-PAESA may fractionate Cu in the same manner as the BCA reagent. That is, the potential DiBr-PAESA determination of Cu fraction 1 (Cu(II) organic acid complexes), Cu fraction 2 (Cu(I) thiol complexes) and subsequent calculation of Cu fraction 3 (copper sulfides or aggregated Cu(I) thiol complexes). Although analysis with a plate reader allowed insights into the DiBr-PAESA methodology, it was not sufficiently sensitive to afford the fractionation of Cu at the concentrations routinely found in wine. For this reason, the transfer of the method to a more sensitive automated analyser was necessary.

Separate methodologies were used on the automated analyser for measurement of Cu fraction 1 and Cu fraction 1+2 (Appendix 7). The methods only differed in the time of incubation after addition of the DiBr-PAESA reagent, which was 20 seconds for Cu fraction 1 and 300 seconds for Cu fraction 1+2, and in both cases the incubation temperature was 37 °C. The shorter incubation time meant that the DiBr-PAESA reagent would be more likely to just have access to Cu from Cu(II) organic acid complexes and not from copper(I) thiol complexes or copper sulfides. The longer incubation time meant that the DiBr-PAESA reagent would be more likely to dissociate Cu from both organic acids and thiol compounds, as was the case in Figure 6.9c. With some preliminary optimisation experiments, it was found that analysis by the DiBr-PAESA reagent had better agreement with the BCA-colorimetric measurement if the pH of the wine was not adjusted to pH 4 (data not shown).

By this stage of the DiBr-PAESA experiments, which were being conducted in December 2024, the overall project was coming to an end. However, a preliminary comparison between the DiBr-PAESA-automated analyser and BCA-benchtop-spectrophotometer methodologies for measurement of Cu fractions in wine was undertaken. This involved fourteen commercial white wines being opened in anaerobic conditions and Cu(II) additions made to ensure that the wines would contain Cu fractions 1 and 2. The wines were re-sealed and left overnight prior to triplicate analysis by both techniques. Figure 6.10a shows the relationship between the two techniques for Cu fraction 1 and Figure 6.10b shows the comparative results for Cu fraction 1+2. The results show reasonable correlation between the methods for measurement of Cu fraction 1 and Cu fraction 1+2 (i.e., R^2 >0.87). The measurement of Cu fraction 1 provided good agreement between the trend-line for the comparison data (dashed red line, Figure 6.10a) and the ideal trendline (solid black line, Figure 6.10a). Alternatively, there was some underestimated for Cu fraction 1+2 by the DiBr-PAESA method (Figure 6.10b) but more so at the higher concentrations measured as evident in the gap between the trendlines in Figure 6.10b. Several parameters within the test flow (e.g., endpoint ‘measurement type’, reagent 2 ‘dispense with’ settings) were found to be particularly important for measurement repeatability and were improved during the analysis of the 14 wines (Appendix 7 has optimised settings).

The DiBr-PAESA methodology shows promise for the measurement of similar Cu fractions to the BCA colorimetric method in white wine but with the advantage of automation. However, further work is required to fully validate the automated DiBr-PAESA method. This includes performing a larger survey of white wines to comprehensively compare the Cu fraction results between the BCA and DiBr-PAESA methods. Also, additional work is necessary to provide the figures of merit for the DiBr-PAESA automated analyser method, including the limit of detection and limit of quantification. Finally, the potential to utilise the DiBr-PAESA method for determining Cu fractions in red wine should be investigated.

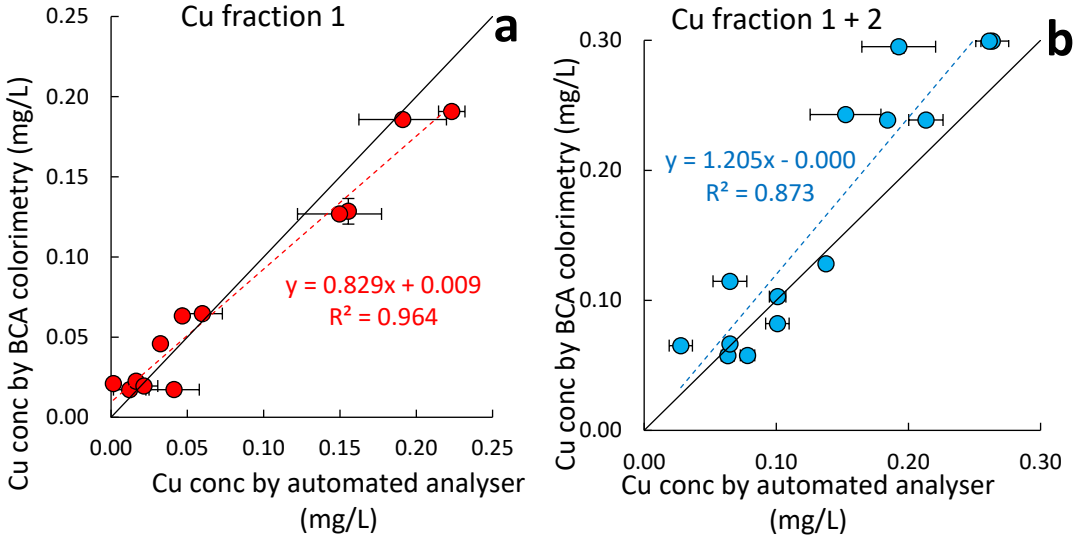


Figure 6.10. The comparison of Cu fraction 1 (a) and Cu fraction 1 + 2 concentrations (b) by BCA colorimetry and the DiBr-PAESA automated analyser. The dashed coloured lines are the trend-lines for the comparison data, while the solid black line is the ideal trend-line with an intercept of 0 and gradient of 1.

6.1.6 Overview of measurable Cu fractions in wine.

Figure 6.11 provides a summary of the different fractions of Cu in red and white wine measured by the independent techniques of stripping potentiometry (i.e., electrochemistry), BCA-colorimetry and diatomaceous earth depth filtration (DEDF). In white wine, three Cu fraction were determined:

- Cu fraction 1, attributed to dissolved Cu(II)-organic acid complexes (e.g., Cu(II)-tartrate),
- Cu fraction 2, attributed to dissolved Cu(I)-thiol complexes (e.g., Cu(I)-glutathione), and
- Cu fraction 3, attributed predominantly to suspended copper sulfides (e.g., Cu(I) sulfide, Cu(II) sulfide). Cu fraction 3 could also consist of some strongly-bound and/or aggregated Cu(I)-thiol species (Clark et al., 2020).

Although, the techniques do not provide characterisation of the identity of the complexes formed, they allow insight into the general forms of the measured species, such as sulfhydryl-free and sulfhydryl-bound Cu. Such assignment must be taken from the perspective of the operationally-defined nature of the instrumental measure. It does not discount that 'sulfhydryl-free' portion of Cu (i.e., Cu fraction 1) may still interact with thiol compounds, but merely that such interactions are not sufficiently strong to preclude the detection of this Cu by the specific technique.

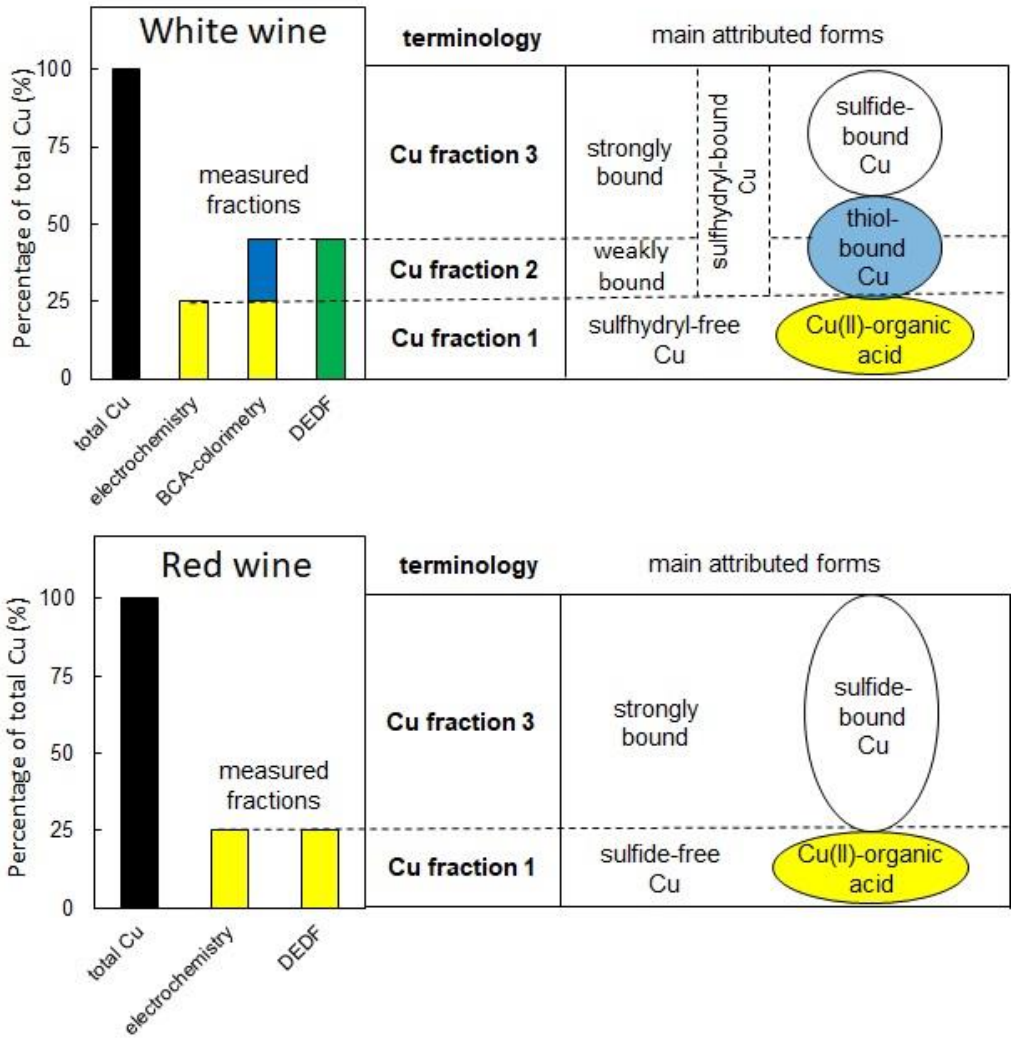


Figure 6.11. Overview of the measured fractions of Cu in wine and their main attributed forms in white and red wine. The percentages shown in the figures for the Cu fractions are just examples.

In white wine, Cu fraction 1 could be measured by stripping potentiometry (i.e., electrochemistry) or BCA-colorimetry (without an incubation period), while the combined Cu fraction 1 and 2 could be measured by BCA-colorimetry (with a 30-minute incubation time) or DEDF. The subtraction of the combined Cu fraction 1 and 2 measure from total Cu allowing the calculation of Cu fraction 3. Preliminary work has been conducted on the application of the DiBr-PAESA reagent for the automated measurement of Cu fractions in white wine. However, further work is required to confirm that the Cu fractions measure agree with those from the other techniques shown in Figure 6.11, and to fully validate the method.

In contrast to white wine, only Cu fractions 1 and 3 were observed in red wine without any evidence for Cu fraction 2. This was presumably a consequence of the lower concentrations of thiol compounds, such as glutathione (Dienes-Nagy et al. 2022), in red wines compared to white wines due to the conditions being more oxidative during red wine production. For this reason, in red wine the fractions of Cu measured by DEDF compared well with the electrochemically-detectable Cu (Figure 6.11). The analysis method used with DEDF in our study was ICP-OES, but the Cu in the filtrate could be measured by the total Cu BCA-colorimetric method (Kontoudakis et al. 2020) or any other routine measure of total Cu in wine (Clark et al. 2015b).

The development of novel measures for Cu have been achieved as part of this project. The colorimetric methodologies for determination of total Cu and Cu fractions allow ready adoption in most standard winery laboratories, with low-cost reagents and simple instrumentation (i.e., spectrophotometer). The future sections of this report will investigate the implications of the different Cu fractions on wine development, shelf-life and sensory properties.

6.2 The removal of Cu and its fractions from wine.

The previous section describes different approaches for the measurement of Cu fractions in red and white wines (Figure 6.11). Later sections of this report will demonstrate a beneficial role of Cu fractions 1 and 2 for preventing the accumulation of reductive aroma compounds in wine. In contrast, the Cu fraction associated with suspended copper sulfide particles (i.e., Cu fraction 3) has no demonstrated benefit for wine. Indeed, there has been some evidence that such copper sulfides may break down over time and release hydrogen sulfide that contributes to undesirable off-odour and reductive characters (Franco-Luesma and Ferreira 2016a,b). As of yet, a mechanism for the release of sulfide from the copper sulfides is yet to be established. This section focusses on the identification of wine production tools for the removal of Cu from wine, and importantly whether Cu fraction 3 can be removed whilst affording minimal changes to Cu fractions 1 and 2.

The total Cu concentration in wine can be lowered by a variety of approaches. Traditionally, when brass-induced Cu contamination issues were commonplace in wineries, blue fining was widely used. This method utilised the formation of an insoluble cupric ferrocyanide salt that was racked and/or filtered off (Ribéreau-Gayon 1935). Due to potential cyanide contaminating the wine, blue fining is now illegal in many countries and its use is highly regulated in others. Other approaches for lowering Cu, include the use of bentonite or the copolymer PVI/PVP can also be used to lower Cu concentrations in wine (Rousseva et al. 2016), but their impact on the different fractions of Cu (Figure 6.11) is not certain.

The use of filtration may provide a non-invasive and practical means of Cu removal just prior to bottling. However, removal of copper sulfides by filtration is complicated by their sufficiently small particle size allowing them to often pass through 0.2-0.45 µm pore-size membrane filters (Kontoudakis et al. 2019a). Selection of the filter medium is a critical issue for effective removal of Cu fraction 3. Initial studies of Cu removal in aqueous ethanol solutions, buffered to pH 3.2 with tartaric acid, showed that regenerated cellulose, nylon and polyethersulfone (PES) membrane filters could efficiently adsorb Cu fraction 3 (Kontoudakis et al. 2019a). When applied to a white wine, however, polysaccharide and protein macromolecules were identified as the components of white wine that hindered Cu fraction 3 adsorption by membranes. In this case, regenerated cellulose membranes could no longer remove Cu fraction 3 and PES and nylon had reduced efficiency compared to their action in model wine. These studies were conducted on an analytical filtration scale, with 4.7 cm^2 (cross sectional area) syringe filter membranes and 10 mL volumes of wine. The capacity of these membranes to adsorb Cu from larger volumes of wine was uncertain, as was their ability to remove copper sulfides from red wine. Depth filters (~3.5 mm depth) incorporating a component of diatomaceous earth are known to specifically adsorb Cu fraction 3 from 100 mL volumes of red or white wine (see Section 6.1.3 of this report) (Clark et al. 2020). The capacity of the depth filters to remove Cu fraction 3 from larger volumes of wines has not yet been studied.

6.2.1 Cu removal with membrane filtration

The membrane filters used were regenerated cellulose, nylon, PES, polytetrafluoroethylene, polyvinylidene fluoride (PVDF) and glass fibre. All membrane syringe filters were of 26 mm

diameter and 0.14 mm thickness (Figure 6.12). Two pore sizes, 0.2 µm and 0.45 µm, were used for all membranes except for PVDF (0.45 µm only) and glass fibre (1.2 µm only).

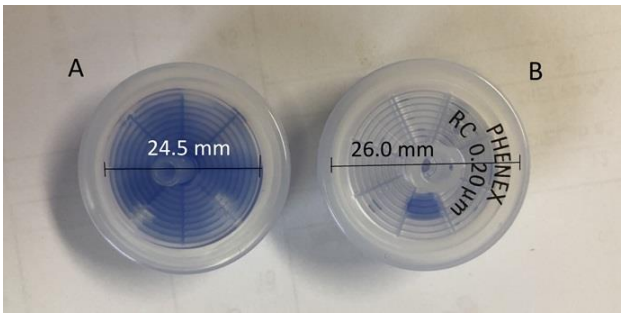


Figure 6.12. The back and front of the 26 mm diameter membrane-syringe filters. The filters have been stained with a blue dye to show the active filtering surface area which has a diameter of 24.5 mm.

Initially, assessment of the membrane filters on small wine volumes (10 mL) was conducted and this was later extended to larger volumes. The impact of dissolved oxygen on the removal of Cu fraction 3 was investigated as oxygen is known to allow conversion of Cu species between Cu forms and may also alter the redox form of Cu in copper sulfides (i.e., copper(I) sulfide or copper(II) sulfide). The ability of PES, nylon and regenerated cellulose membranes to remove Cu fraction 3 from five red and five white wines at different oxygen concentrations is shown in Figure 6.13. The concentration of oxygen in these samples were 0.1 ('anaerobic'), 1-1.5 ('bottle') and 5.5-7 ('saturated') mg/L. Prior to filtration, the wines had 4.7 µM (or 0.3 mg/L) Cu(II) added with 9.4 µM H_2S (as Na_2S), which was a mole ratio for H_2S to Cu ratio of 2:1, to ensure that all the added Cu would be in the form of copper sulfide.

Figure 6.13 shows that the oxygenation of wines prior to filtration had little impact on the Cu removed by the different membranes. This was the case regardless of expressing the removed Cu as a percentage of total Cu (Figure 6.13) or in absolute concentration units (i.e., mg/L Cu removed, Appendix 8). Significant removal of Cu from both red and white wine by membrane filtration was achieved (Figure 6.13) although there were some differences in the relative efficiencies of Cu removal by the different membranes in white wine versus red wine. For instance, the PES and nylon membrane media provided increased Cu removal from white wine compared to the regenerated cellulose, while for red wines the nylon membrane tended to remove more Cu than PES and regenerated cellulose membranes.

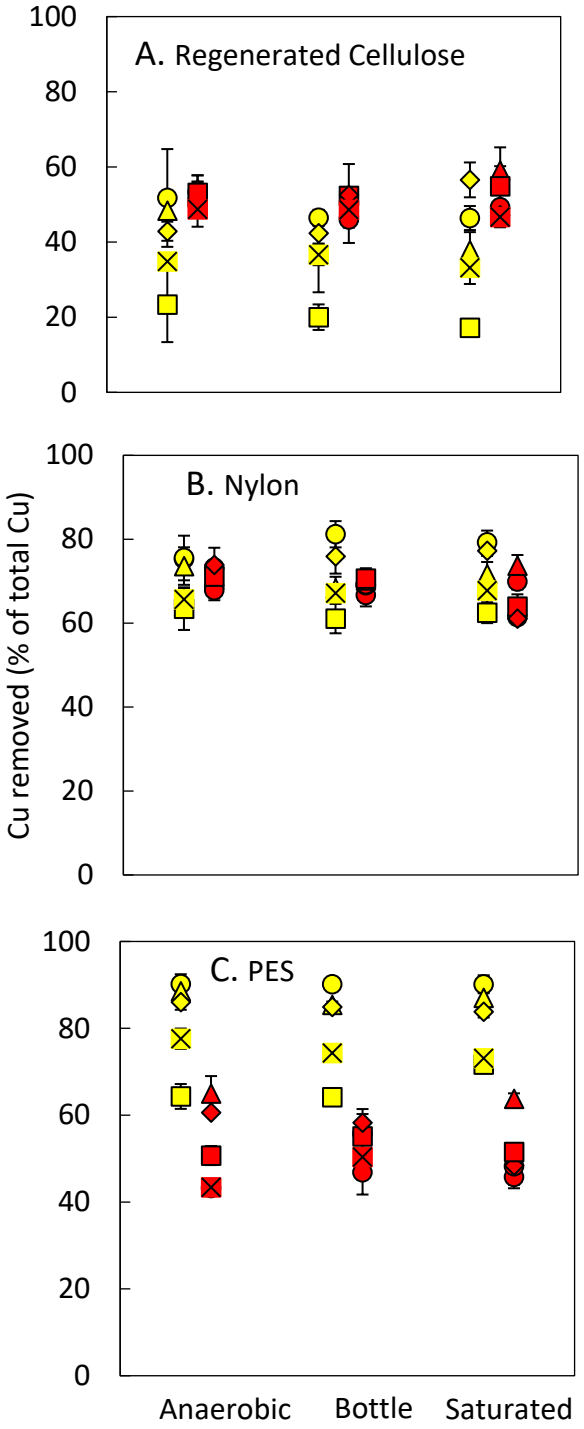


Figure 6.13. The Cu removed (% of total) from five white (yellow symbols) and five red (red symbols) wines with three different oxygen conditions (anaerobic, directly from wine bottle and saturated) and three different membrane filtration media: A) regenerated cellulose, B) nylon and C) polyethersulfone (PES). All filters had a pore size of 0.45 µm. The symbols for the different white and red wines are: wine 1 (○), wine 2 (△), wine 3 (□), wine 4 (◇) and wine 5 (×).

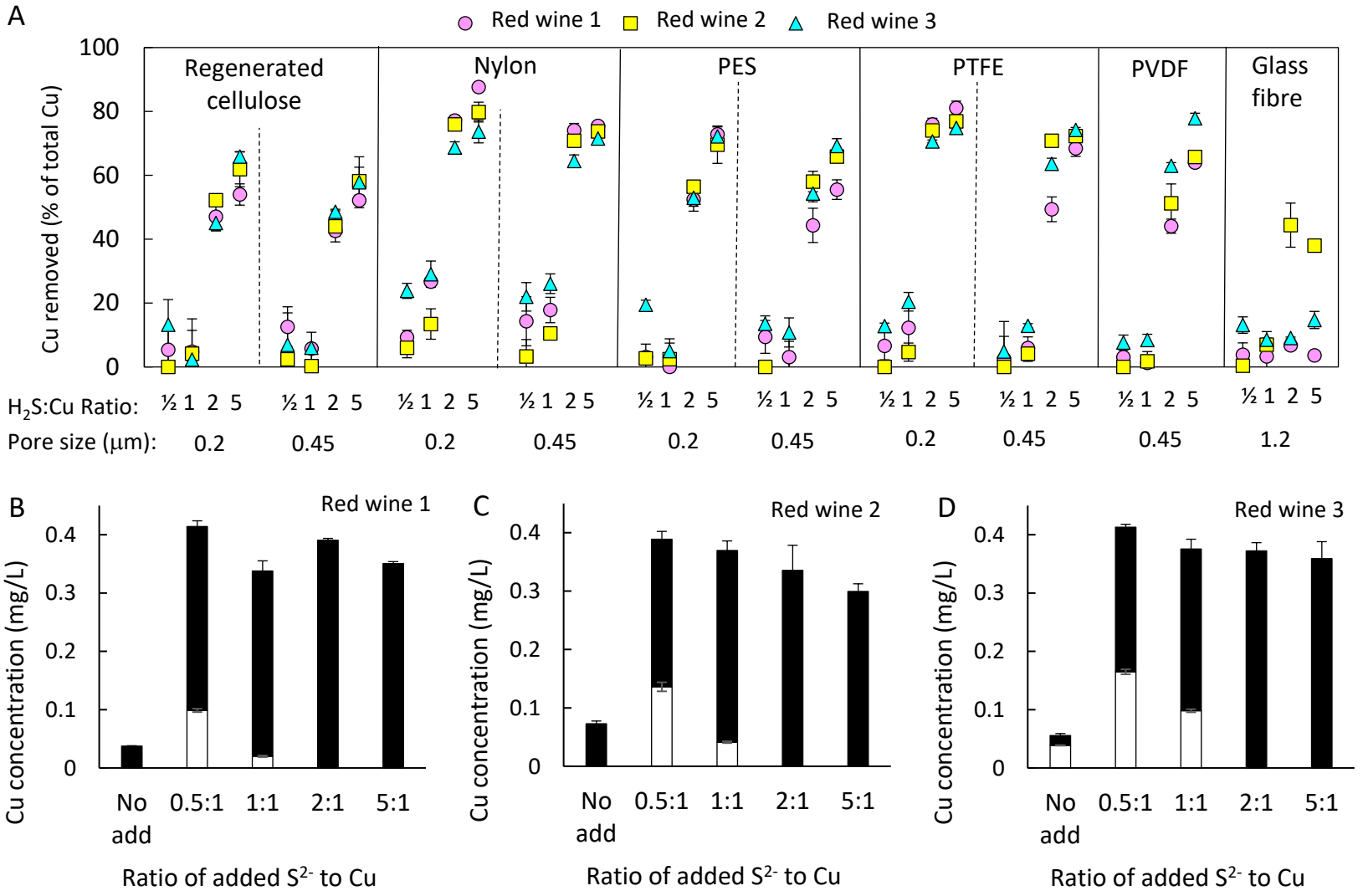


Figure 6.14. The (A) Cu removed (% of total Cu) from three red wines with various membrane filters, pore sizes and ratios of added H_2S to Cu. Also shown are the concentrations of Cu fraction I (□) and III (■) in three red wines (B-D). The 'No add' in panels B-D refers to the wine before addition of Cu(II) and sodium sulfide.

The impact of varying the sulfide to Cu(II) mole-addition ratio (hereafter abbreviated as S:Cu ratio) on the removal of total Cu by membrane filtration has been previously studied in white wine (Clark et al., 2015a), where it was evident that increased adsorptive removal of Cu occurred at higher S:Cu ratios. Given the compositional differences between white and red wines, including pH and phenolic concentrations and profiles, it was necessary to ascertain if a similar impact for S:Cu ratio and Cu removal existed for red wine as in white wines. The results in Figure 6.14 show that the S:Cu ratio also influences the capacity for a membrane filter to remove Cu from red wine. At a S:Cu ratio of 2:1, Cu removal was significant (> 40 % of total Cu) for all membrane types, apart from glass fibre, while at a S:Cu ratio of 1:1 the removal was generally much less than 30 % of total Cu. It would appear that surface characteristics of the sulfide-bound Cu differ when formed in Cu-rich or sulfide-rich environments, with the latter enhancing adsorption onto membrane filters. Consequently, wine conditions favouring complete binding of Cu to sulfide, such as found in many bottle aged wines (Kontoudakis et al., 2019b), would be conducive to efficient removal of sulfide-bound Cu by adsorption onto membranes. Alternatively, in wines with appreciable concentrations of Cu fraction I, implying an excess of Cu(II) in the wine compared to sulfide, may be less efficient in allowing membrane adsorption of sulfide-bound Cu. This latter case would be more likely for wine with recent Cu additions.

The nylon membrane consistently provided the highest percentage of Cu removal across all red wines followed by polytetrafluoroethylene (Teflon or PTFE) and then PES (Figure 6.14). In terms of membrane pore size, there were slight increases in Cu removal with 0.2 µm pore size filters compared to 0.45 µm filters for most wines and filtration-types, but the differences were generally small (Figure 6.11). PVDF provided similar Cu removal to PTFE (both 0.45 µm), while regenerated cellulose provided Cu removal that was generally the lowest, apart from the 1 mm glass fibre filters.

Figure 6.15A and B shows the proportion of total Cu removed from three red and three white wines, to which sulfide and Cu(II) had been added at a mole ratio of 2:1, as a function of increasing filtration volume. As is evident from Figure 6.15A and B, the capacity of the membrane filters to remove Cu from these wines rapidly diminishes with increasing filtration volume. Even the best performing membranes across both red and white wine (i.e., nylon and PES) showed less than 30 - 40 % of total Cu removed after filtration of 200 mL of wine. If 24.5 mm diameter membrane filters (area: 4.7 cm^2) can only remove ~35 % of total Cu from 200 mL of wine, this could be extrapolated to industrial membranes (typically of surface area 1.8-2.4 m^2 per 30" cartridge) removing ~35 % of Cu from 850 L (i.e., 0.2L x 2 m^2 / 0.00047 m^2). It is also likely that the adsorption capacity of the membranes would be much lower if they were previously used for other wines and had already adsorbed significant components of copper sulfides. Therefore, although adsorptive removal of Cu by membrane filtration may be significant for the filtration of small volumes of wine, which would be relevant to wine filtration prior to instrumental analysis of Cu, the results suggest the adsorptive capacity of membrane filters is not adequate for treatment of bulk wine.

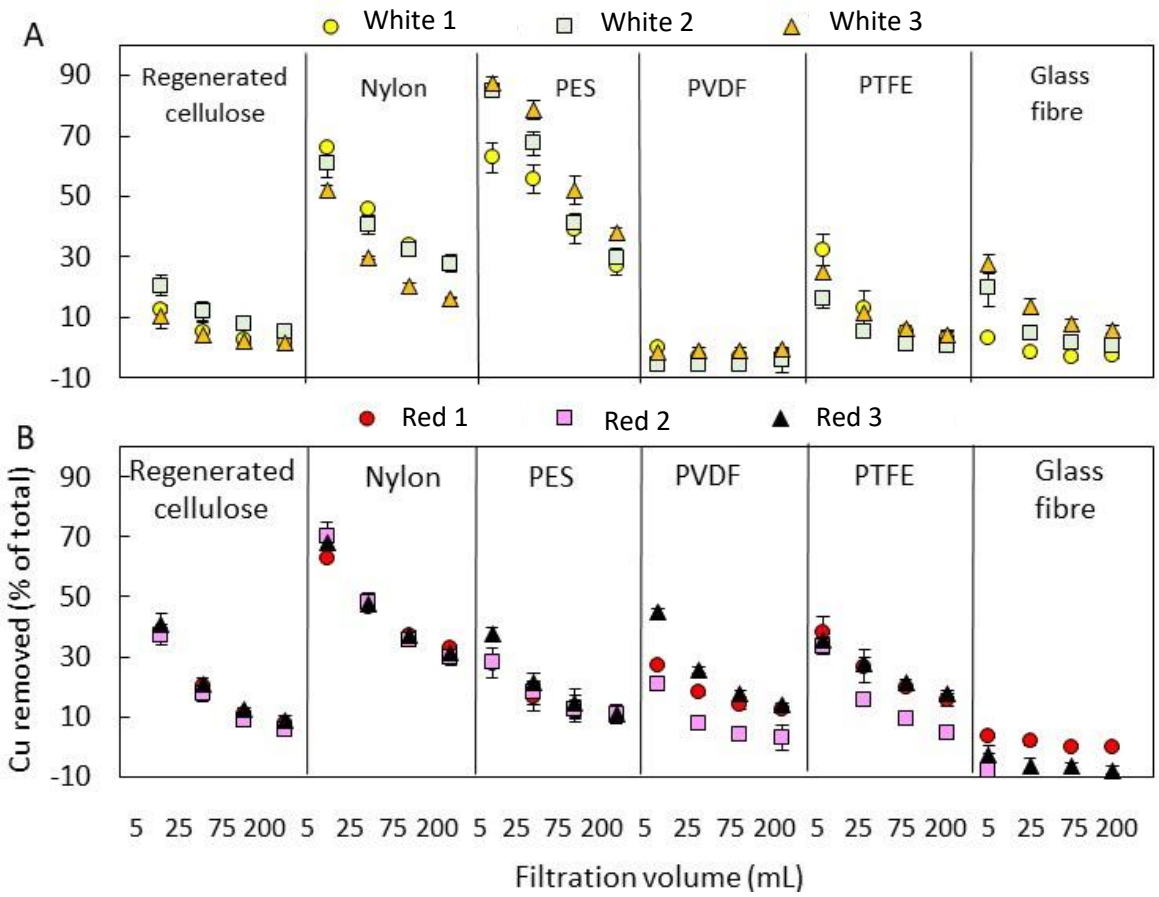


Figure 6.15. The Cu removed (% of initial total Cu) from white wines (A) and red wines (B) with various membrane filters (at a pore size of 0.45 μm, apart from glass fibre (at 1 μm). The wines were prepared at an added H_2S to Cu mole ratio of 2:1.

6.2.2 Cu Removal by bentonite.

Previous research showed that bentonite can lower Cu concentrations in white wine (Catarino et al. 2008, Rousseva et al. 2016, Clark et al. 2015a), but the forms of Cu being removed and the impact of wine protein on the efficiency of Cu removal was uncertain. The main role of bentonite during wine production is for the removal of grape-derived protein that can be otherwise form problematic hazes during white wine aging. Bentonite can also interact with the protein used as a fining agent during wine production. Bentonite fining at 0.2 g/L has been proposed by Iland et al. (1993) to assist in settling the copper sulfide precipitate formed during copper fining.

This study utilised three commercial white wines that were all were protein stable (i.e., passed heat test) prior to this study. The Riesling and Chardonnay 1 were used without any Cu addition, as they already contained 0.2-0.25 mg/L total Cu, while 0.3 mg/L Cu(II) was added to Chardonnay 2 as it originally had less than 0.01 mg/L total Cu. Some H_2S was added (as Na_2S) to Chardonnay 2 to ensure that some portion of its Cu existed as Cu fraction 3 (i.e., copper sulfides). The H_2S was added to Chardonnay 2 to afford a H_2S to Cu ratio of 0.67 : 1. Each wine was split into four batches corresponding to:

i. Control (coded: 'No-B')
ii. Bentonite (coded: 'B')
iii. bentonite + egg white (coded: 'B+egg')
iv. bentonite + isinglass (coded: 'B+isinglass')

The egg white and isinglass fining agents were chosen as they contain protein with contrasting tertiary structures, that is, globular and fibrous, respectively. The calcium bentonite (0.5 g/L), egg white (0.4 g/L) and isinglass (0.1 g/L) were added at rates often used in wine production. All wine samples were prepared in anaerobic conditions in triplicate. After addition and mixing, samples were stored in darkness overnight, allowing the bentonite to settle, and afterwards all samples were filtered (including the control) with 0.2 µm regenerated cellulose filters, leaving behind any bentonite lees. The regenerated cellulose filters were used as they were known to have a minimal capacity to adsorb Cu fraction 3 when used for white wine (Figure 6.15A).

Figure 6.16 shows show a significant decrease in total Cu concentration in all the wines after bentonite treatment. In fact, the bentonite treatment removed nearly half the total Cu in all three wines. There was some further Cu removed with the use of bentonite and egg white combined, particularly for Chardonnay 2, but in all cases the action of bentonite alone provided the largest magnitude of Cu decrease. Isinglass combined with bentonite provided minimal Cu removal compared to bentonite treatment alone. Casein (0.25 g/L) was also trialled for Chardonnay 2 and it provided results similar to that of isinglass, whereby it offered no enhancement of Cu removal compared to bentonite alone (data not shown).

In terms of Cu fractions, the bentonite had the biggest impact on Cu fraction 3 with only minor impacts on Cu fractions 1 and 2. Therefore, it appeared that the bentonite was most efficient at removing the insoluble and particulate Cu sulfides, and related aggregated species, more so than the dissolved forms of Cu bound to organic acids or thiol compounds. This combined with the non-critical role of protein in the removal of Cu, suggests that as observed for the filtration

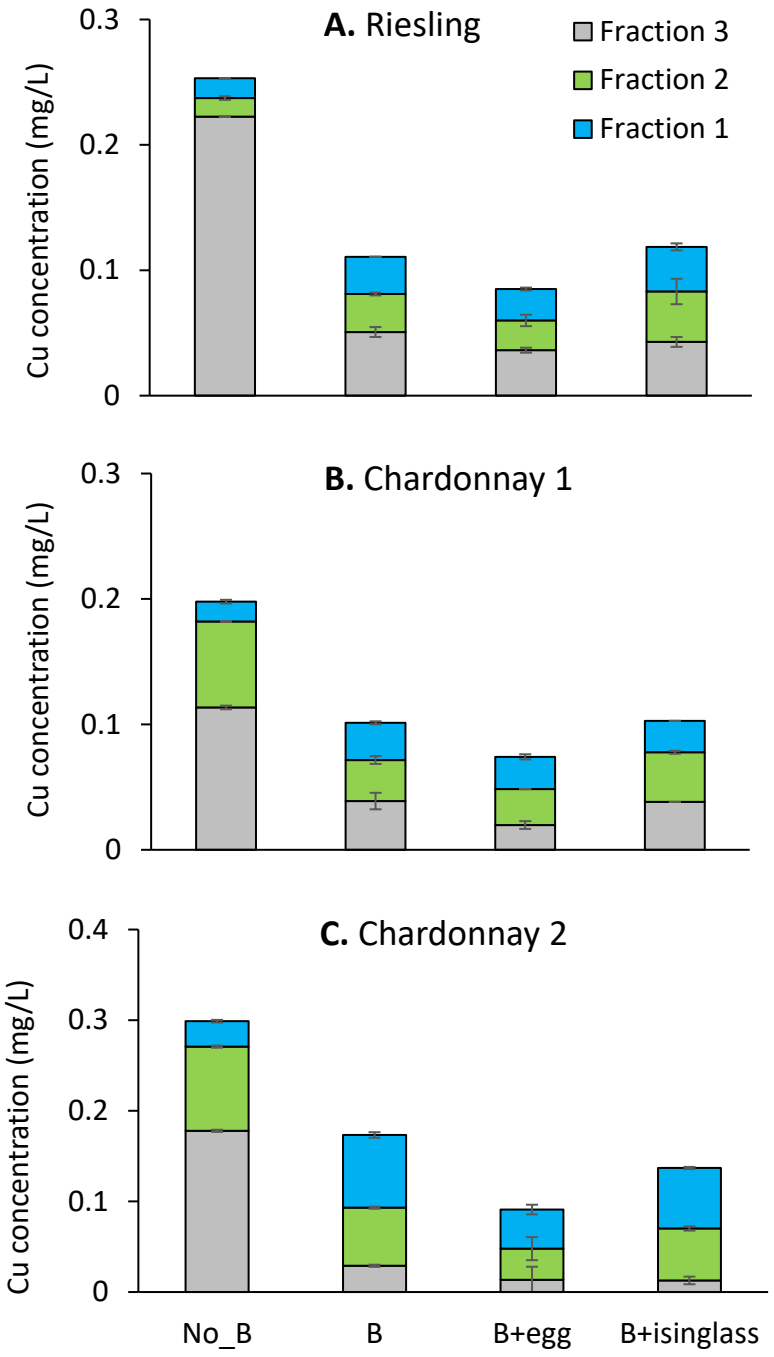


Figure 6.16. The influence of no treatment (No_B), bentonite alone (B) or bentonite in combination with egg white (B+egg) or isinglass (B+isinglass) on the concentration of Cu fractions in three white wines. B = bentonite.

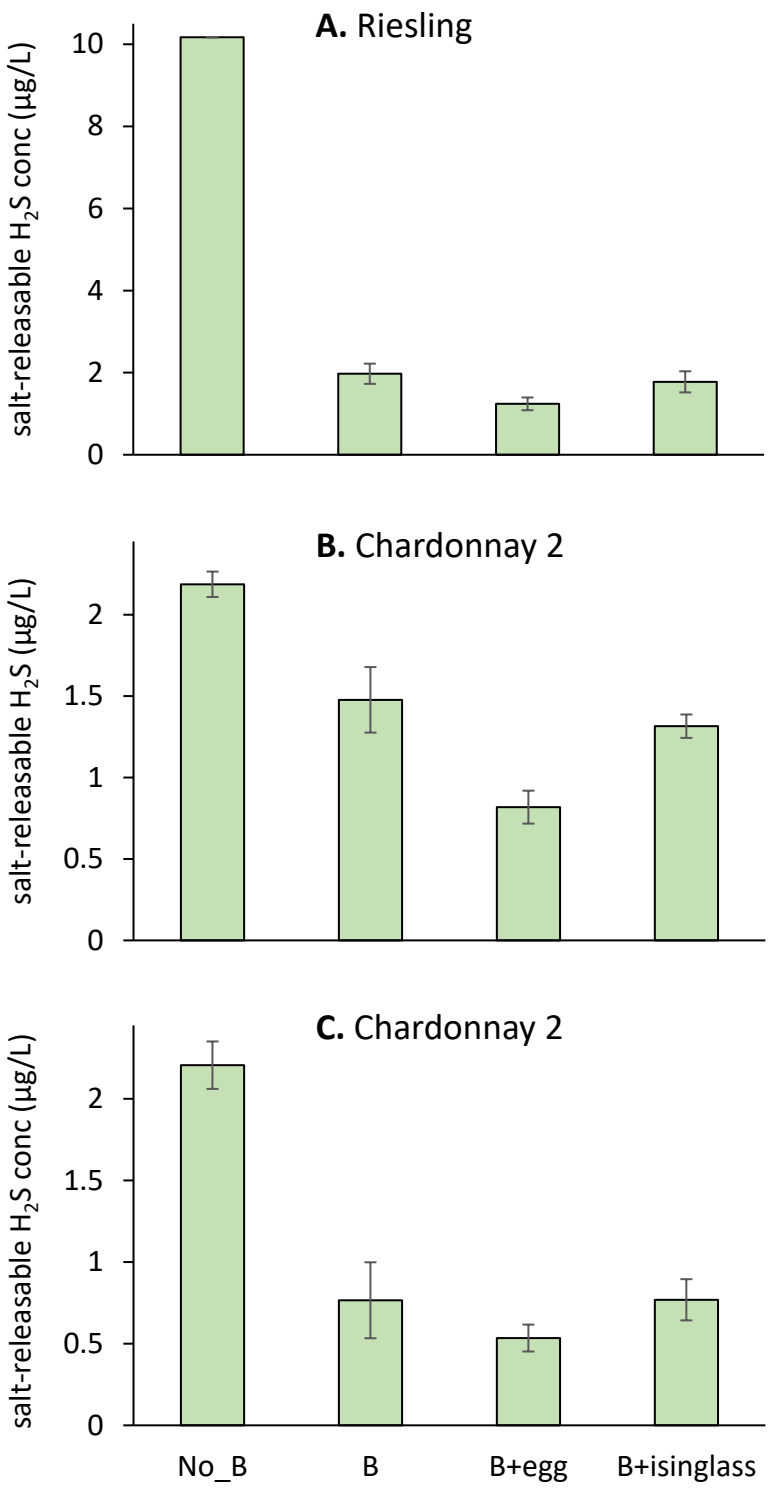


Figure 6.17. The influence of no treatment (No_B), bentonite alone (B) or bentonite in combination with egg white (B+egg) or isinglass (B+isinglass) on the concentration of Cu fractions in three white wines. B = bentonite.

studies, the removal of the copper sulfides by bentonite may be more due to adsorptive characteristics rather than cation exchange processes.

The metal-complexed sulfide in samples (i.e., salt-releasable H_2S by GC-SCD) was measured to confirm that sulfide was removed with Cu fraction 3. The results in Figure 6.17 show a decrease

in the salt-releasable hydrogen sulfide in the wines after the treatment of the wines with bentonite. The relative concentration for salt-releasable hydrogen sulfide among the different samples (Figure 6.17) was similar to the relative Cu fraction 3 concentrations (Figure 6.16). This included the egg white in combination with bentonite having the highest loss of salt-releasable hydrogen sulfide (Figure 6.17). As for Cu fraction 3, bentonite had the biggest impact on salt-releasable hydrogen sulfide. The results in this section show that bentonite can be used to lower the total Cu concentration of white wine, and that the decrease is predominantly due to removal of Cu fraction 3 (copper sulfides).

6.2.3 Cu removal with depth filtration.

6.2.3.1 Depth filtration on a laboratory scale

Depth filters, as the name implies, have a thicker filter body compared to membrane filters and this usually allows superior adsorption capacity combined with an increased residence time of wine being within the filtration media during filtration. Although nylon and polyethersulfone were the most successful membrane filters used in the previous section, these were not available commercially as depth filters. Hence, just cellulose-based depth filters (CDF) and those incorporating diatomaceous earth (DEDF) were utilised in this study.

Depth filters of three nominal pore sizes were used. These were designated as coarse, medium and tight, corresponding to nominal particle size retention of greater than 2-3, 1-2 and 0.1-0.3 µm, respectively. The filters were 3.5 mm thick and 45 mm in diameter, although when placed in the filter holder, the effective diameter was reduced to 41.0 ± 0.5 mm (see Figure 6.18).

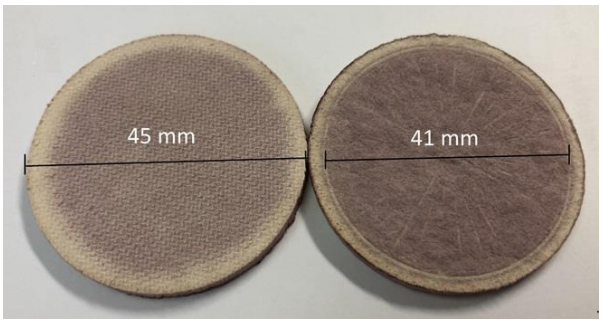


Figure 6.18. The back (left) and front (right) of the depth filters after filtration of red wine. The overall (left) and effective (right) diameters of the respective filters are shown.

A preliminary trial to check the effectiveness of depth filters with and without incorporated DE was carried out with 200 mL of white wine containing only Cu fraction 3, and no Cu fractions 1 or 2. The CDF could only remove copper from a small volume of wine: less than 35% was removed from 200 mL of wine, irrespective of the nominal pore size of the filter. However, the DEDF removed the Cu with much more effectively with a 50, 80 and 90 % decrease in total Cu with coarse, medium and tight filters, respectively. This agreed with the earlier observations for DEDF to adsorb Cu fraction 3 (Clark et al. 2020).

The DEDF were then applied to the filtration of 3 litres of six red and six white wines using the tight and medium filters. For each wine, a bulk sample was prepared by combining and mixing the contents of sufficient bottles, and filtration was performed using a manual syringe system.

Figure 6.19 presents the amount of Cu fraction 3 removed from the twelve wines for the experiments with the tight pore-sized filters. The average Cu fraction 3 removed from 3 litres of the twelve wines was 80 ± 20 %, dropping to 50 ± 20 % for the medium pore-sized filters. We know from separate experiments that wines with a higher sulfide to copper ratio show more effective Cu fraction 3 removal, most probably as a consequence of sulfide altering the surface characteristics of the copper sulfide, leading to better adsorption characteristics (Zhang et al. 2021). However, it was pleasing to see that two wines (white wine 2 and red wine 2) with a high concentration of Cu fractions 1 and 2 (i.e., 0.085±0.007 and 0.154±0.003 mg/L, respectively) still showed 70-80 % removal of Cu fraction 3. The change in Cu concentration with the amount of red and white wine filtered (i.e., up to 3.0 L) can be viewed in Appendix 9.

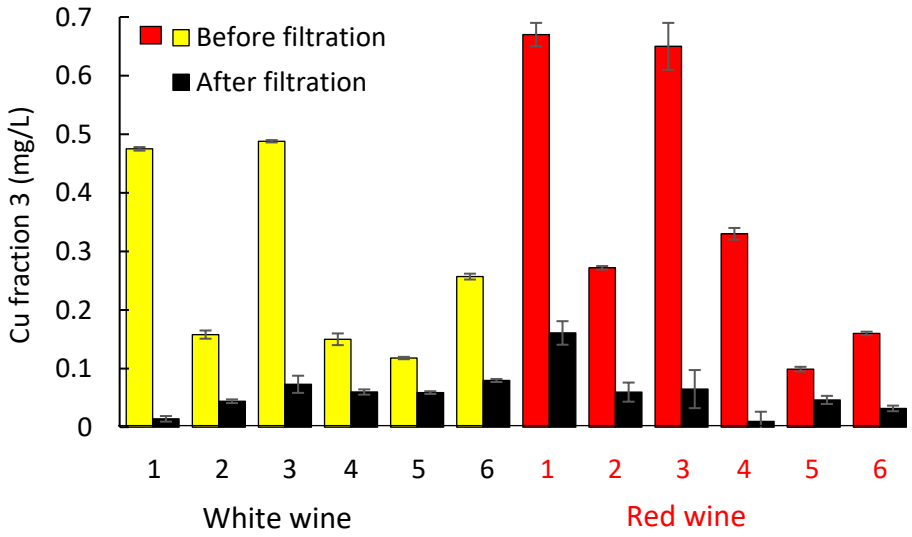


Figure 6.19. The Cu fraction 3 concentration in white and red wines before and after filtration of 3.0 L of wine with tight cellulose-depth filters incorporating diatomaceous earth. Wines 1 and 2 are the same wine except with Cu and H_2S added to wine 1 to generate more Cu fraction 3 in this wine prior to filtration.

The results shown in Figure 6.19 indicate the potential of the approach for a winery scale filtration. The volume of the DE-containing depth filters used was 4.6 cm^3. A typical industrial sized depth filter sheet (400 × 400 × 3.5 mm) would have a volume of 560 cm^3, becoming 11,200 cm^3 if twenty sheets are used in parallel. Assuming a linear extrapolation of adsorption, the data in Figure 6.19 would equate to the treatment in excess of 7,000 litres for 20 standard sheets used. From an industry perspective, the other critical factor is the filtration flow rate. The flow rate with the manual syringe system used here was 25.0 mL/min, corresponding to a residence time in the filtration material of 11 seconds, based on an overall flow rate of 1140 L/(m^2·h). When used in wineries, the suggested flow rates for these depth filters are about 4 to 5 times slower (220 – 320 L/(m^2·h)) than the rate used in this study, and this would result in a longer residence time of wine in the depth filter for wineries and hence potentially more efficient Cu fraction 3 removal.

6.2.3.2 Depth filtration on an industrial scale

A current vintage Chardonnay (4000 L) was used with the specifications as shown in Appendix 10. The 4000 L of wine was pre-filtered prior to the main experiment with cellulose-based depth filter

(CDF) pads, of nominal rating 0.1-0.3 µm, to ensure that the base wine was free from turbidity. Cu(II) addition was made to the wine to ensure that the initial Cu concentrations was around 0.6 mg/L prior to filtration. Na_2S was also added to the wines, at a ratio of 1.8 : 1 for Na_2S to Cu(II), thereby ensuring that the majority of the Cu would be in the form of Cu fraction 3.

Filtration of the wine was conducted with a depth filter pads (Figure 6.20A,B) in a 20 x 20 cm plate and frame filter unit (Figure 6.20C). The filtration unit was modified so that wine would only flow through two filter pads, thereby allowing the experiment to assess the filtration capacity of two filter pads during the passage of up to 1000 L of wine. Fresh pads were used for replicate volumes of 1000 L (refer to the method section for technical details for the filter pads and filtration parameters). These DEDF pads were identical to those used within the laboratory-scale filtration study (Figure 6.19) and for those previously described for measurement of Cu fractions (Figure 6.11). The cellulose-based pads (CDF) were used to filter 0-300 L of wine in triplicate, while the DEDF were used to filter 0-500 L in triplicate and 0 – 950 L in duplicate.



Figure 6.20. The cellulose (A) and diatomaceous earth-cellulose (B) depth filter pads and the plate and frame filter unit (C and D). Plastic inserts forced the flow of wine through the first two pads on the left of the unit, which are the whitest coloured pads in (C). The other pads are just required to ensure the filter unit is sufficiently sealed.

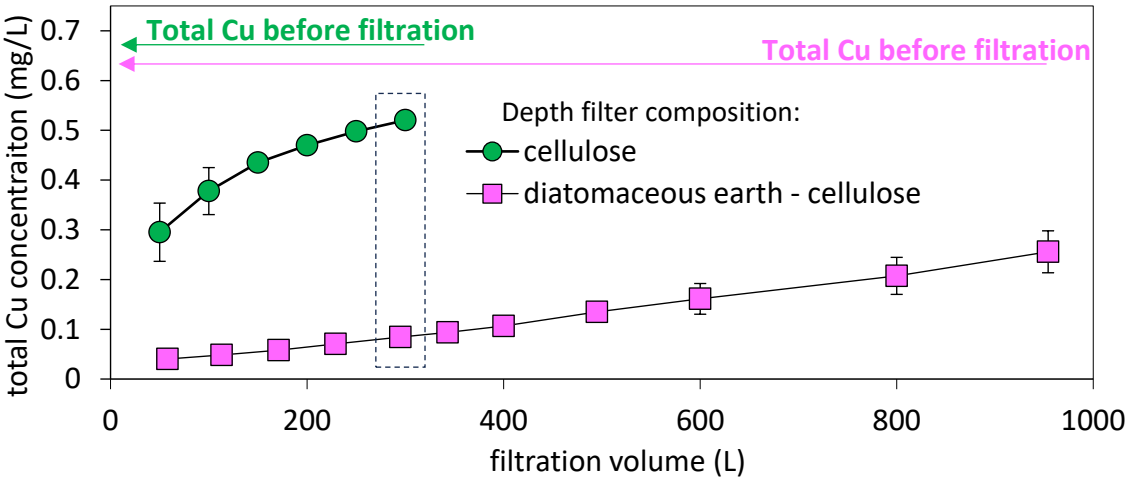


Figure 6.21. The total Cu concentration in wine after filtration through cellulose (CDF) or diatomaceous earth – cellulose depth (DEDF) filters. The original total Cu concentration in the wine prior to filtration is indicated.

Figure 6.21 shows the total Cu concentration in filtrate after the filtration of 0-300 L with CDF and 0-950 L with DEDF. This graph is the calculated Cu concentration that would be in the wine with continuous filtration of the wine into one tank. Of note was the repeatability of measured total Cu concentration across the filtration replicates as evidenced by the relatively small error bars in Figure 6.21 (i.e., often smaller than the data point symbols). The results are consistent with those observed for the laboratory scale study (Figure 6.19, Appendix 9), whereby the DEDF is more efficient at removing Cu from wine than the CDF. The CDF was able to remove 56 % of the initial Cu after filtration of 50 L, but after the filtration of 300 L the amount of Cu removed had dropped to 24 % of the total Cu. Alternatively, after filtration of 300 L of wine, the DEDF showed an 87 % removal of Cu and even after the filtration of 950 L of wine, the DEDF allowed 60 % removal of Cu, which was still greater removal than the CDF for 50 L.

Given that the Cu removed in Figure 6.21 was using two pads of 20.0 cm x 20.0 cm x 3.5 cm dimension (or 1400 cm^3 volume) some further insight can be provided on the potential capacity of Cu removal for an industrial scale plate and frame unit. For example, large scale plate and frame units can use pads of 40.0 cm x 40.0 cm x 3.5 cm dimension (or 5600 cm^3 volume) and a capacity to fit 80 filter pads, which would equate to 160-times (= [5600 cm^3 x 80)/(1400 cm^3 x 2)]) the wine volume treated with the set up in figure 6.20. This means the 300 L of wine treated in Figure 6.21 would equate to 48,000 L with a large-scale plate and frame unit, and the 950 L treated would equate to 150,000L. The 50 L of wine treated by the CDF that removed around half the total Cu in Figure 6.21 would equate to the treatment of 8,000 L of wine with a large-scale plate and frame unit. Therefore, although the CDF is much less efficient at removing Cu than the DEDF, it can still afford the reduction in Cu concentration by around 50 % if a smaller wine volume is filtered with fresh CDF pads (i.e., 8,000 L of wine through 80 40.0x40.0x3.9 cm pads).

Figure 6.22 shows distribution of Cu fractions in the wine before and after filtration, as well as in each of the collected filtrate volumes. The initial wine was dominated by Cu fraction 3, with minor amounts of Cu fraction 1 and 2. For both the CDF and DEDF experiments, the major form of Cu removed was Cu fraction 3, consistent with the results of the laboratory scale studies (Figure 6.19). Signficant decrease in Cu fractions 1 and 2 were also evident in the DEDF filtrate volumes up to around 230 L, but the amount of these fractions removed was relatively low.

The equivalent experiments were repeated in Shiraz but the increased back pressure associated with the filtration of the red wine led to the buckling of plates and the subsequent tearing of filtration pads. This occurred dispite the pre-filtration of the Shiraz wine to low turbidity (< 1 NTU), prior to the start of the experiment, and it seemed to be a consequence of the copper sulfides accumulating on the filter pads. Filtration of the red wine containing high Cu fraction 3 concentration commenced with flow rates at 2.3 L/min but it rapidly decreased to < 1 L/min before reaching 500 L. At this filtration rate it was not possible to filter the 1000 L of wine without burning out the pump and without tearing the pad filters. If the plate and frame filter unit was used in the typical manner, with 20 x pads, then the filtration of the red wine could still be performed albeit at a slower rate than for white wine. However, due to the back pressure issues, the repeat experiment for red wine with the methodology used for white wine (Figure 6.20) was not feasible.

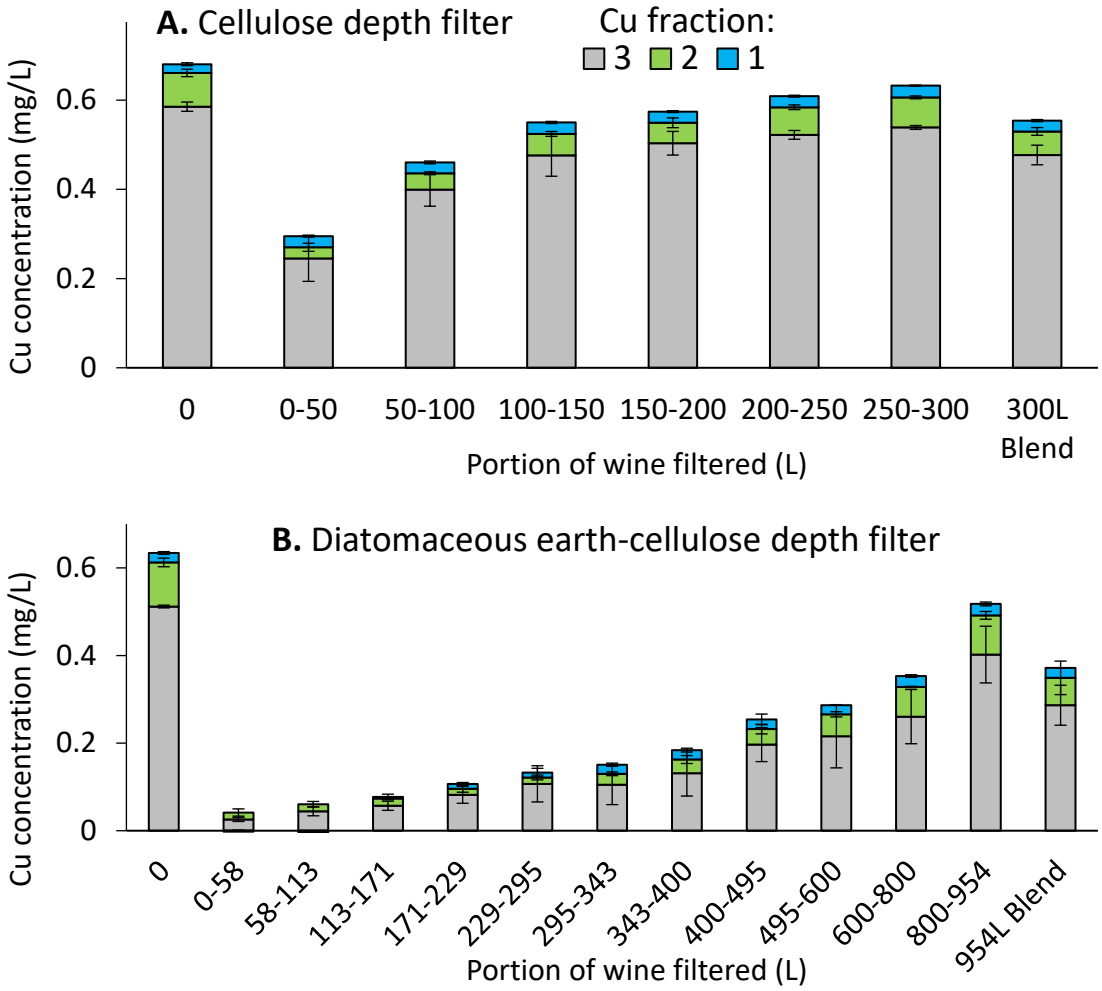


Figure 6.22. The Cu fractions in white wine before filtration (see '0 L') and in the filtrate of cellulose (A) and diatomaceous earth-cellulose (B) depth filters during the treatment of 300 L and 954 L respectively. The final column shows the Cu fractions in the bulk wines after blending of all filtrate volumes together and is consistent with the final data point in Figure 6.21. The error bars are the standard deviation.

Overall these results suggest that plate and frame filtration can be used to lower the Cu concentration of white wine and more specifically the Cu fraction 3 concentration, and that this can be achieved on an industrial wine filtration scale. The decrease in Cu fraction 3 can be achieved with either cellulose or diatomaceous earth-cellulose pad filters but the latter are much more efficient for larger volumes of white wine. Although similar experiments were not able to be conducted on red wine, the results at laboratory scale suggest a similar result would eventuate as for white wine, although it is likely that a slower filtration rate would have to be used given the higher back-pressure encountered for red wines with high Cu fraction 3 concentration.

6.2.4 Cu removal with PVI/PVP.

PVI/PVP (Figure 6.23) is already known to efficiently remove Cu from wine, but it is not certain which Cu forms it most effectively removes. Indeed, many studies (Friedenberg et al. 2018; Mira

et al. 2007) have used PVI/PVP on wines with recent and large Cu(II) additions that may favour the removal of one form of Cu over another (i.e., Cu-organic acid versus Cu-sulfide complexes). Furthermore, it is not certain if PVI/PVP removes Cu alone or in combination with its complexing agents. For example, if sulfide was released from Cu during PVI/PVP treatment, then the accumulation of the uncomplexed H_2S would be detrimental to wine quality. The following sections outline the action of PVI/PVP on a laboratory scale (i.e., 300 mL scale) and then on a larger commercially relevant scale (i.e., 1000 L scale). The laboratory scale work was performed in collaboration with Dr Eric Wilkes, Dr Neil Scrimgeour, and Dr Kieran Hirlam from AWRI.

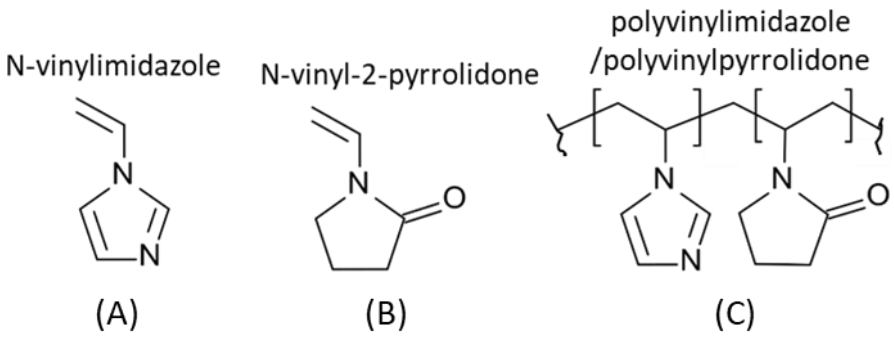


Figure 6.23. The monomer units N-vinylimidazol (A) and N-vinyl-2-pyrrolidone (A) used to manufacture the copolymer PVI/PVP (C).

6.2.4.1 PVI/PVP on a laboratory Scale

The complete experimental details and additional discussion of results for this section can be found within Zhang et al. (2021).

Impact of pH on Cu removal from wine by PVI/PVP

Previous reports showed that PVI/PVP removed Cu more efficiently from white wines rather than red wines. The results from the 38 wines in the Hirlam et al (2019) study showed poor correlation ($R^2 < 0.3$) between total Cu removal and wine pH (data not shown). However, many of the wines in this dataset had relatively low total Cu concentrations prior to the PVI/PVP treatment and the results from these wines seemed to have confounded the overall results. Additionally, the practical application of PVI/PVP treatment in wineries for Cu removal would not be conducted on wines with such low total Cu concentrations. Consequently, the data of Hirlam et al. (2019) was further analysed using only the data from wines with more than 0.15 mg/L of total Cu. To place this selection criteria into context, a previous study showed only 13 out of 49 commercial white and red wines showed total Cu concentration below 0.15 mg/L (Kontoudakis et al., 2019b). As shown in Figure 6.24, a good relationship ($R^2 = 0.726$) was evident between wine pH and Cu removal (% of total Cu) with more efficient Cu removal evident at lower wine pH. Of course the red wines typically had a higher pH than the white wines.

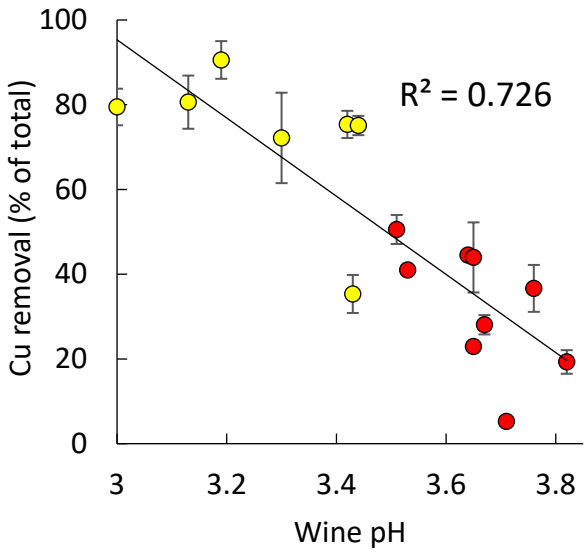


Figure 6.24. The total Cu removed from 7 white wines ( ) and 9 red wines ( ) in relation to their pH values. All wines had total Cu concentrations of 0.15 mg/L or higher prior to PVI/PVP addition, and the data utilised is from Hirlam et al. (2019). Error bars indicate the standard deviation (n=2).

It is suspected that at lower pH, the competitive binding and/or adsorption of Cu by PVI/PVP is favoured, whilst at higher pH, the wine matrix can repress such Cu removal mechanisms. However, the deviation from a perfect linear fit of the data in Figure 6.24 certainly suggests that other factors besides wine pH are influencing the Cu removal efficiency by PVI/PVP across these wine samples.

Impact of PVI/PVP on the removal of different fractions of Cu from wines

Two types of PVI/PVP products, Stabyl MET (ST) and Claril HM (CL), both sourced from Enartis (Malvern East, Australia), were utilised in this study. The former product is composed of the PVI/PVP co-polymer and silicon dioxide, while the latter contains the co-polymer and chitosan. Prior to application, 0.16 g of the PVI/PVP product was hydrated with 3.2 mL of the 12 % (v/v) aqueous ethanol solution on a laboratory rotator for 60 min.

The entire preparation and storage of samples was conducted in an anaerobic hood at low oxygen conditions. The hydrated solution of PVI/PVP was added to 320 mL samples of wines or model wine to afford a concentration of 0.5 g/L. After addition of the PVI/PVP to samples, they were stirred for 5 h at room temperature, and then the PVI/PVP sediment was left to settle overnight.

The model wine (3.5 L) was prepared using the 12 % (v/v) aqueous ethanol solution (pH 3.2) with the addition of 200 mg/L caffeic acid, 170 mg/L white wine polysaccharide and 50 mg/L sulfur dioxide. The polysaccharide was added to ensure that the model wine would behave like white wine whereby minimal sulfide-bound Cu would be removed by adsorption onto regenerated cellulose membrane filters prior to instrumental analysis (Kontoudakis et al., 2019a). Four white and four red commercial wines (Appendix 11) were utilised for this study. In order to provide a range of Cu forms in the wines under study, white wine 4 (W4) and red wine 4 (R4) both had an addition of 0.3 mg/L Cu(II) prior to sample preparation, while no Cu addition was made to the other wines.

In line with previous studies (Friedenberg et al., 2018; Hirlam et al., 2019; Mira et al., 2007), total Cu concentrations significantly decreased after the PVI/PVP treatment in model, white and red wines (Figure 6.25). There was notable variation in the efficiency of Cu removal from wine to wine, particularly among the red wines. Higher total Cu removal was observed in model wine and four white wines compared to in the four red wines, irrespective of the PVI/PVP type, consistent with previous report (Hirlam et al., 2019) and higher pH values of the red wines (pH 3.5 ± 0.1) compared to the white wines (pH 3.1 ± 0.2). The type of PVI/PVP (i.e., CL versus ST) gave similar results apart from the model wine and one white wine (W1) with increased Cu removal by the CL-type (Figure 6.25).

The Cu fractions in the model wine were measured by stripping potentiometry (i.e., electrochemistry), the white wine was measured by BCA colorimetry, and the red wine by DEDF and ICP-OES (Figure 6.11). With regard to the activity of PVI/PVP on the removal of different Cu fractions, it was evident that PVI/PVP could effectively decrease all fractions of Cu in the white wines (Figure 6.25). Similarly, model wine showed clear concentration decreases in Cu fraction 1 and 3, implying the removal of both sulfide-bound (Cu fraction 3) and organic acid-bound Cu (Cu fraction 1). For the red wines R1 – R3, Cu fraction 3 showed the largest concentration reduction upon PVI/PVP treatment, while the impact on Cu fraction 1 was modest (Figure 6.22). The exception was red wine 4, which had a significant portion of Cu fraction 1 removed. It should be noted that red wine 4 was the only red wine that had a Cu addition prior to analysis and this possibly contributed to the PVI/PVP being able to remove higher portion of Cu as fraction 1. Importantly, these results demonstrated that PVI/PVP is active in removing the fraction of Cu associated with sulfide-binding (i.e., Cu fraction 3), despite this Cu fraction interacting with the strongest binding agent in wine. Similar to the action of filtration media for removal of sulfide-bound Cu (Kontoudakis et al. 2019a), it is likely that adsorption is an important mechanism for the removal of Cu fraction 3 by PVI/PVP. The ability of PVI/PVP to efficiently decrease the concentration of Cu fraction 2, which is the fraction associated with Cu-thiols complexes (Figure 6.11), in white wines was not unexpected given that the BCA colorimetric reagent could sequester this fraction of Cu during a 30 min incubation step. The more efficient removal of Cu from white wines compared to red wines has already been linked to wine pH (Figure 6.24), but also appears to be a consequence of Cu fraction 1 being more easily removed from white wines than red wines (Figure 6.25).

This work confirms the ability of PVI/PVP to effectively reduce total Cu and various fractions of Cu in wine. However, the following section investigates the extent to which binding agents for Cu in fraction 2 and 3 (i.e., thiols and sulfides, respectively) remain in the wine after the removal of Cu by PVI/PVP treatment.

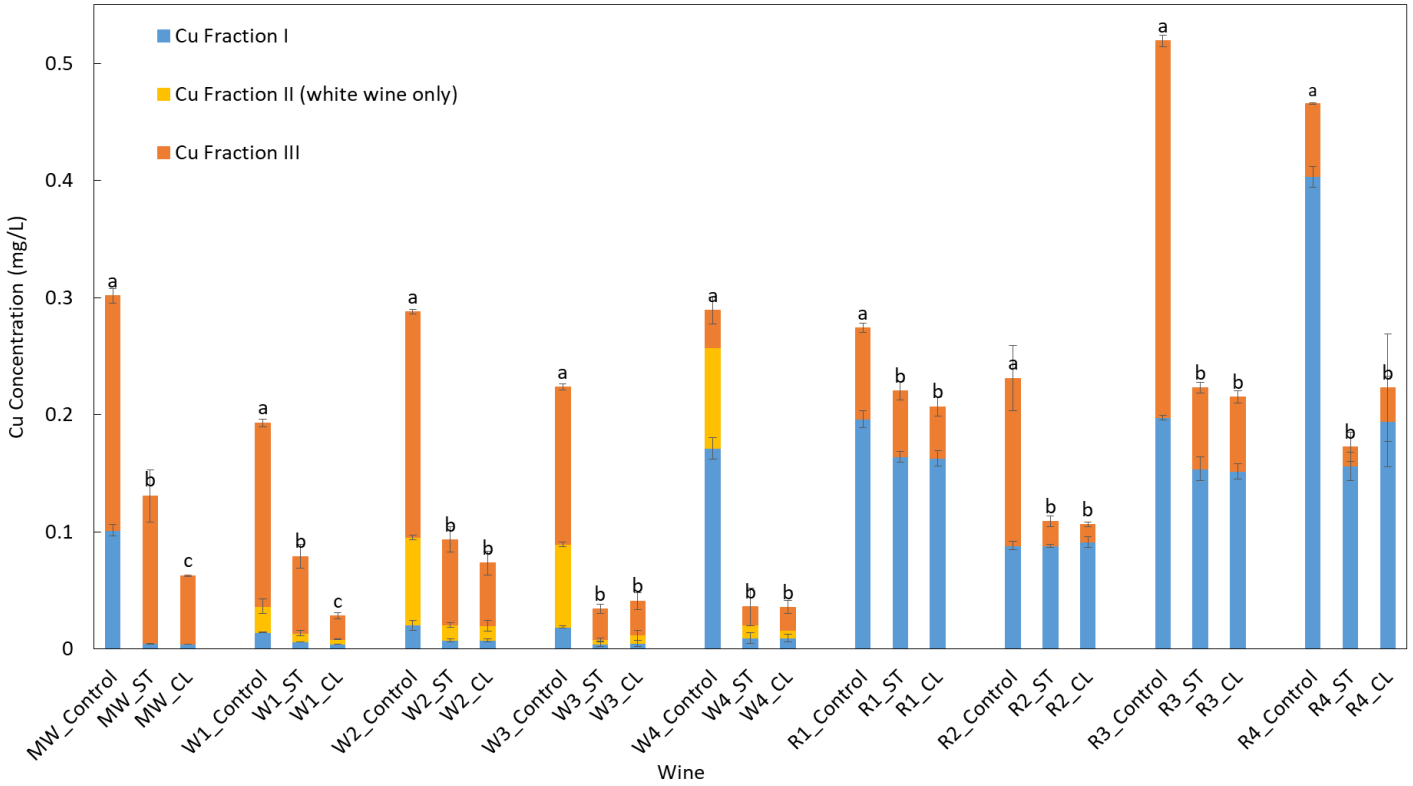


Figure 6.25. Concentrations (mg/L) of different Cu fractions in model wine (MW), white wines (W1-W4) and red wines (R1-R4), without (control) and with (ST and CL) the PVI/PVP treatment. The control results were obtained from samples after 5 h of stirring and filtration without PVI/PVP addition. Error bars indicate the standard deviation (n=3), and the indicated significant differences (letters above bars) for effects within a given sample are for total Cu.

To confirm whether any sulfide was left behind in wine after PVI/PVP treatment, 0.3 mg/L Cu(II) was added back into PVI/PVP treated wines, and if sulfide had been left behind as uncomplexed H_2S, then the added Cu(II) would readily reform copper sulfides (i.e., Cu fraction 3). Alternatively, if PVI/PVP had removed the Cu-sulfide complex as a whole, then the subsequently added Cu(II) would largely exist as Cu fraction 1 and/or 2, that is in Cu-organic acid and/or Cu-thiol complexes. As shown in Figure 6.26, the Cu(II) added to the PVI/PVP-treated wines formed minimal amount of Cu in fraction 3, and mainly presented as Cu fraction 1 and/or 2. This result implied that copper sulfides were removed from the wines without leaving unbound H_2S in the wine. It is consistent with an adsorptive removal mechanism of the sulfide-bound Cu by PVI/PVP whereby the co-polymer adsorbs the entire Cu-sulfide complex rather than its chelating groups sequestering Cu from the complex.

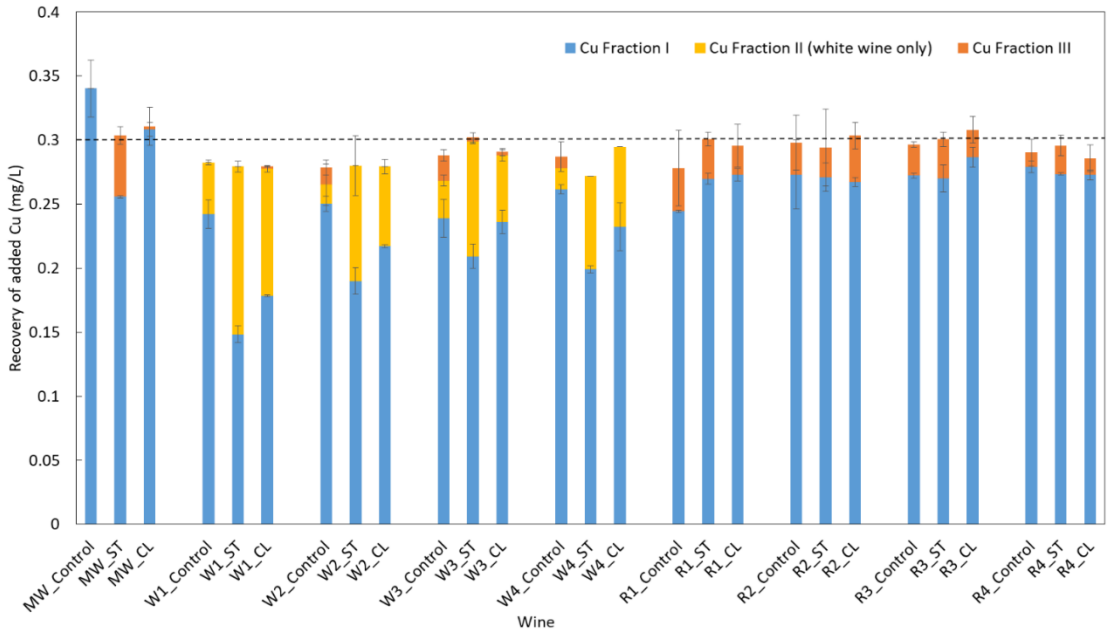


Figure 6.26. The Cu fractions of 0.3 mg/L Cu(II) after its addition to model wine (MW), white wines (W1-W4) and red wines (R1-R4), after PVI/PVP treatment (ST or CL) or with no PVI/PVP treatment (Control). Error bars indicate the standard deviation (n=3).

GC-SCD analysis of the PVI/PVP treated and non-treated wines also indicated a reduction in salt-releasable H_2S concentration in the model wine and white wines upon PVI/PVP treatment. This provided further evidence that both Cu and its binding agent sulfide had been removed from the wine (Figure 6.27A). The concentration of salt-releasable H_2S measured in the red wines before and after PVI/PVP treatment displayed mixed results. Before any treatment, only wines R1 and R3 showed appreciable salt-releasable H_2S concentration, and a significant decrease in this concentration was evident after PVI/PVP treatment (Figure 6.27A). It is suspected that the low measured salt-releasable H_2S concentrations measured in R2 and R4 may been a function of incomplete recovery of H_2S from copper sulfides when utilising the salt-saturation HS-GC analysis methodology for red wines that contained high concentrations of Cu in fraction 1.

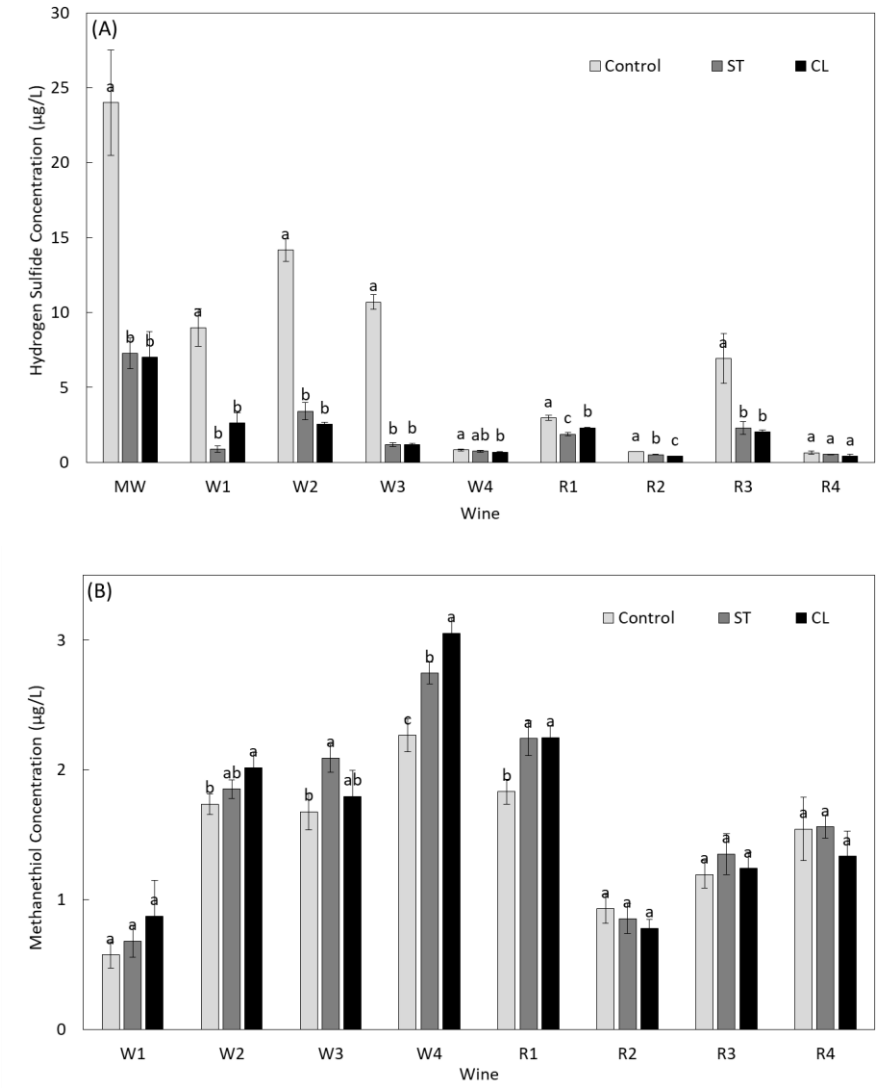


Figure 6.27. Concentrations (µg/L) of salt-releasable hydrogen sulfide (A) and methanethiol (B) in model wine (MW), white wines (W1-W4) and red wines (R1-R4), without (control) and with (ST and CL) PVI/PVP treatment. Error bars indicate the standard deviation (n=3) and different letters on the top of bars indicate a significant difference for treatments on a wine.

The ability of PVI/PVP to impact the thiol binding capacity of the white wines was also assessed. Figure 6.26 shows that after adding Cu(II) to all the different wines of Figure 6.25, a larger portion of the added Cu(II) existed as Cu fraction 2 (associated with Cu-thiol complexes) in the PVI/PVP treated samples compared to the control. These results suggested that the Cu was sequestered from the Cu-thiol complex by PVI/PVP without significant removal of the thiol compounds from the wine, and that the remaining thiol was still available for rebinding upon Cu(II) addition. These results are also consistent with the lack of any reduction of the methanethiol concentration before and after the PVI/PVP treatment of wines (Figure 6.27B). Although removing an off-flavour thiol compound, such as methanethiol, would be regarded as advantageous, there are many other thiol compounds in wine that are beneficial for wine aroma, such as 3-sulfanylhexanol and 3-sulfanylhexyl acetate, or for their antioxidant abilities, such as glutathione. Hence, the ability of PVI/PVP to remove sulfide-bound Cu complexes from wine but only sequester Cu from Cu-thiol complexes is most likely a net benefit for wine flavour provided that the volatile form of MeSH left behind is not too high in concentration.

Overall, these results show the effective removal of different forms of Cu from wines by PVI/PVP treatment, but importantly that a potential source of H_2S can be simultaneously removed as well. Furthermore, no significant release of H_2S into the wine was observed during the PVI/PVP treatment of wines containing sulfide-bound Cu. Finally, the PVI/PVP treatment appears to sequester Cu from thiol-binding agents rather than removing these ligands with Cu. This outcome is beneficial to wines with dominant varietal thiol aroma compounds.

6.2.4.2 PVI/PVP on an industrial scale

PVI/PVP was applied to triplicate 1000 L batches of Shiraz and Chardonnay wine (Appendix 10). The wines were adjusted to around 0.6 mg/L Cu and Na_2S was added at a ratio of 1.5-1.8 : 1 for Na_2S to Cu to ensure a reasonable portion of Cu fraction 3 in the wines. Claril HM PVI/PVP (Enartis) (500 g) was hydrated by in 10 L of water for 20 minutes prior to addition to 1000 L of wine given an addition rate of 0.5 g/L. The wine was circulated for 1 hour, using a flexible impeller pump (Liverani), to the aid mixing of the wine and PVI/PVP.

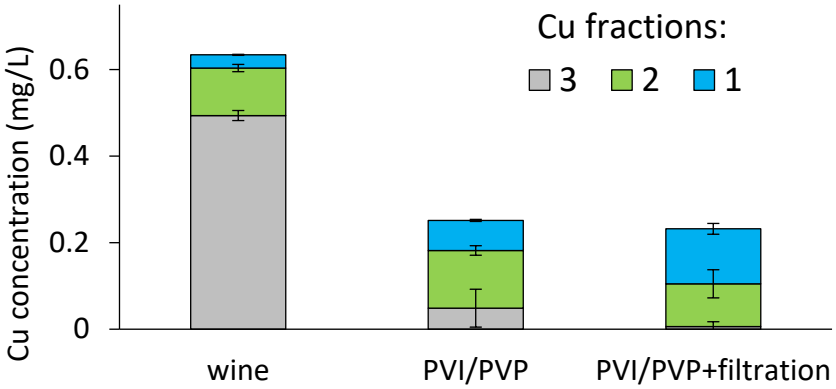


Figure 6.28. The concentration of Cu and its fractions in Chardonnay before and after the application of 0.5 g/L PVI/PVP either with or without filtration of the sample prior to analysis. Triplicate 1000 L volumes of Chardonnay were treated with PVI/PVP. Cu fractions were measured by BCA colorimetry. Error bars indicate the standard deviation (n=3).

For the Chardonnay, the wine was allowed to settle for 3-days after PVI/PVP addition. Samples (200 mL) were taken for BCA-colorimetric analysis, which were filtered (0.2 µm regenerated cellulose) or collected without filtration. Figure 6.28 shows that the wine initially had most Cu in the form of Cu fraction 3, with minor amounts of Cu fraction 1 and 2. After treatment with PVI/PVP, the total Cu concentration was around a third of its original magnitude and filtering the sample prior to measurement of total Cu had negligible difference on the total Cu concentration. The amount of Cu removed was generally less than observed in the smaller scale experiments (Figure 6.25) and most likely due to less efficient mixing of the PVI/PVP with wine on the large scale compared to the smaller scale. Cu fraction 3 was the main fraction removed by the PVI/PVP and some increase in Cu fraction 1 with PVI/PVP treatment may have been a consequence of the oxidative conversion during the 3-day settling period.

For the Shiraz, samples were taken 1 hour after mixing the wine with PVI/PVP and then another sample was taken after 3-days to allow settling of the PVI/PVP. All samples (~200 mL) were filtered with 0.2 µm regenerated cellulose filters and measured by DEDF ICP-OES to allow determination of Cu fractions and ICP-OES for total Cu. Figure 6.29 shows that the wine initially had most Cu in the form of Cu fraction 1. After treatment with PVI/PVP, the total Cu concentration was around half of the original concentration at the 1-hour time-point and only marginally lower after 3 days. As for the white wine, amount of Cu removed from the red wine was generally less than observed in the smaller scale PVI/PVP experiments (i.e., 'CL' Figure 6.25). The PVP/PVI was able to remove both fractions of Cu from the red wine but after 3 days a larger proportion of initial Cu fraction 3 was removed than for Cu fraction 1 (i.e., decrease of 70 % and 57 %, respectively).

One consideration for PVI/PVP treatment is the cost at $240 per kg (2024 price), which is considerably more than for the bentonite used in section 6.2.2 (approximately $3 per kg). This means that the treatment of 1000 L of wine at a rate of 0.5 g/L PVI/PVP would cost around $120.

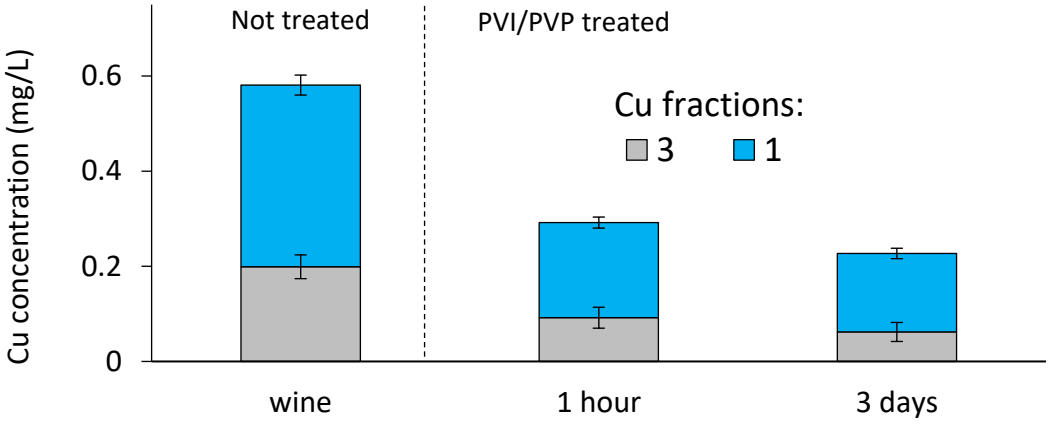


Figure 6.29. The concentration of Cu and its fractions in Shiraz before and after treatment with 0.5 g/L PVI/PVP. Triplicate 1000 L volumes of Shiraz were treated with PVI/PVP. Cu fraction 1 was measured by stripping potentiometry and total Cu was measured by ICP-OES.

6.2.5 Cu removal by cross flow filtration: up to 50 kL

The earlier sections of this report have already reported on the impact of membrane and depth filtration on Cu concentrations in wine. However, crossflow filtration is often more commonly used for large volume filtrations (i.e., > 20,000 L) than these other approaches. Crossflow filtration has wine flowing tangential to the filtration media and the retentate is recirculated while the permeate is collected in a receival tank or directed to other production steps. The filtration media used for crossflow filtration is typically composed of ceramic or fibre-based (i.e., cellulose) material. The impact of crossflow filtration on the concentration of Cu in three red and three white wines was investigated onsite at a commercial winery.

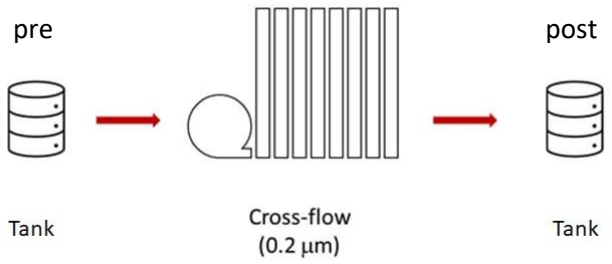


Figure 6.30. Cross flow filtration of wine between wine tanks. The wine was sampled from tank before cross flow filtration (pre), and once the bulk wine was crossflow filtered to specification, a final sample was taken from the permeate in the destination tank (post).

Wines were sampled from tank before crossflow filtration, and these samples were coded 'pre' (Figure 6.30). The wines then underwent crossflow filtration, through ceramic media with 0.2 µm nominal rating, and the permeate was collected in a separate tank. The flow rate of wine varied from 7 to 12 kL/hour. Once the wine was filtered to the required specification of the winery, a sample of the filtered wine (or bulk permeate) was taken from the second tank and coded 'post'. The volume of wine filtered varied between 20,000 to 55,000 L and the pH and alcohol percentage is shown in Figure 6.31. Given the large volumes of wine involved, and the commercial on-site setting of the experiment, it was not possible to replicate the filtration for a given wine, but instead the collected samples were measured in duplicate for total Cu and Cu fractions. The total Cu concentration was measured by ICP-OES and the Cu fractions in the white wine were measured by BCA colorimetry and in the red wine by DEDF-ICP-OES.

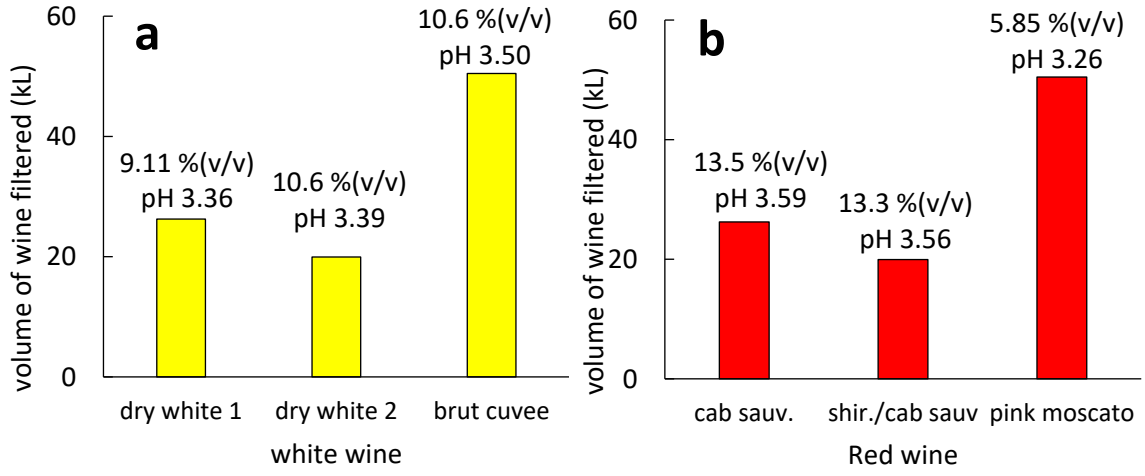


Figure 6.31. The volume (kL) of white (a) and red (b) wines filtered via cross flow and their corresponding alcohol concentration and pH.

Between wines, the crossflow filtration unit was cleaned in the standard manner for the winery, with 50 % sodium hydroxide solution, followed by a 1 % citric acid solution and then a water rinse and drying with nitrogen gas.

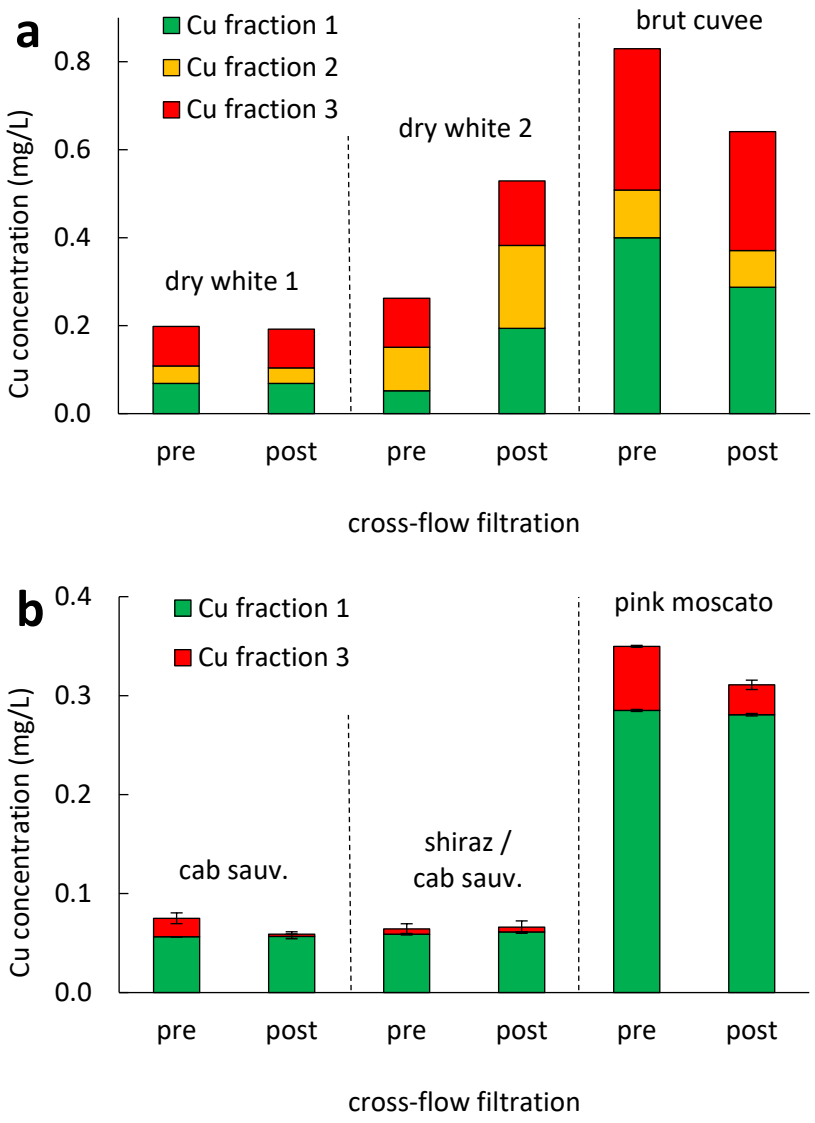


Figure 6.32. The concentration of Cu fractions in white (a) and red (b) wines pre- and post- cross flow filtration. The error bars indicate the standard deviation of analytical replicates and not the uncertainty associated with filtration replicates.

Prior to filtration, the white wines had considerable portions of each fraction of Cu, while the red wines were dominated by Cu fraction 1 (Figure 6.32). After filtration, the white wines exhibited all possible total Cu changes, including a 23 % decrease in total Cu for the brut cuvee, a 100 % increase in total Cu for the dry white 2 and no change for dry white 1. For the red wine, there was no change for either the Cabernet Sauvignon or the blend (i.e., Shiraz / Cabernet Sauvignon), while the pink Moscato had an 11 % decrease in total Cu. The Cu removed from the pink Moscato was predominantly Cu fraction 3 (copper sulfides), which was the same fraction of Cu removed by depth filter filtration

and membrane filtration (Sections 6.2.1 and 6.2.3). Alternatively, for the brut cuvee it was mainly Cu fraction 1 (Cu organic acids) that was removed, which contrasted the other filtration studies in this report. In fact, it was only the chelating copolymer PVI/PVP that removed Cu fractions 1 and 2 from wine (Section 6.2.4). The dry white 2 that showed a substantial increase in total Cu had large increases in Cu fraction 1 and 2, and this is difficult to explain. One possibility is that the increase in Cu was caused by carryover from the crossflow filter, but the comprehensive cleaning procedure between wines would decrease this likelihood. It is also possible that sampling complications from such large wine volumes may also have contributed to the apparent increase in Cu. Appendix 12 shows the concentrations for other metal ions (i.e., Al, Fe, Mg, Mn, Zn) in these wines but none show any dramatic change with cross flow filtration, other than a slight decrease in Al for the brut cuvee.

These results show that cross flow filtration can have variable impact on the concentration of Cu in wine and on its fractions. In terms of Cu removal, the magnitude of Cu removed was marginal with a maximum of 23 % of total Cu removed, but there was one anomaly where the Cu concentration doubled in one white wine after cross flow filtration. Future work is required to demonstrate this was most probably due to erroneous sampling and repeat experiments would be best to implement replicate wine sampling before and after filtration. It would also have been beneficial to source some red wines with larger concentrations of Cu fraction 3 to provide insight into whether this would have contributed to increased Cu removal.

6.2.6 Cu removal by cross flow, lenticular and membrane filtration in series prior to bottling: up to 272 kL.

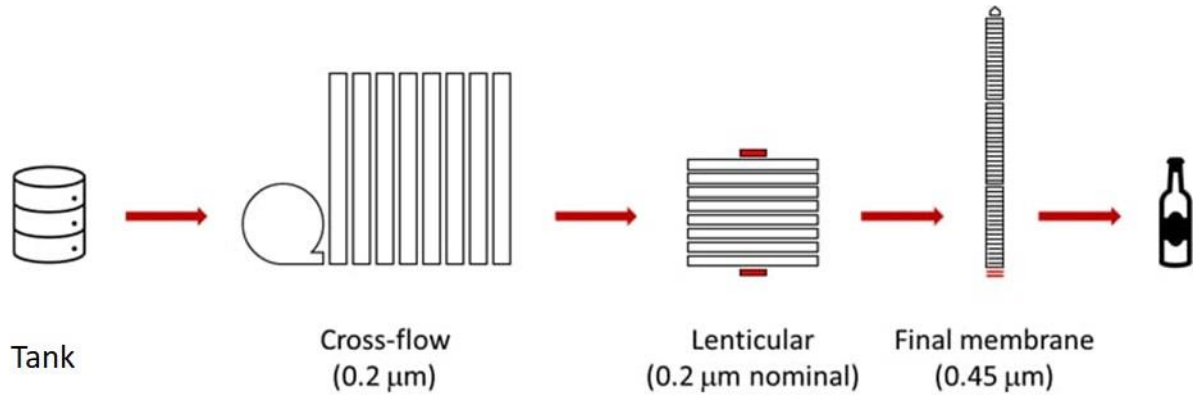


Figure 6.33. Bulk wine was filtered through the three different types of filters shown prior to the bottling of the wine. Samples were collected from tank, prior to the filtration steps, and then from bottles after the filtration. One bottle was taken from early in the bottling run, and a second bottle was taken towards the end of the bottling run.

Prior to bottling, commercial wineries often use multiple filtration processes in series. The impact of such sequential filtration on the concentration of total Cu and Cu fractions was explored. In this case, the wine passed sequentially through three filtration set-ups as shown in Figure 6.33. The first was a 0.2 µm nominal rated ceramic crossflow filter, the second was a 0.2 µm lenticular filter (cellulose fibre composition), and the final was a 0.45 µm membrane (PES composition). Wine was sampled from the tank prior to filtration and then also from bottled wine. To assess any changes in the efficiency of Cu removal during the bottling procedure, samples were taken from bottled wine at the start and end of

the bottling run. The volume of five white and five red wines filtered varied between 55,000 to 275,000 L and the pH and alcohol percentage is shown in Figure 6.31. The same measurement techniques were used as in the previous Section (6.2.5). Due to the commercial setting of the experiment, each wine was filtered once and not able to be replicated.

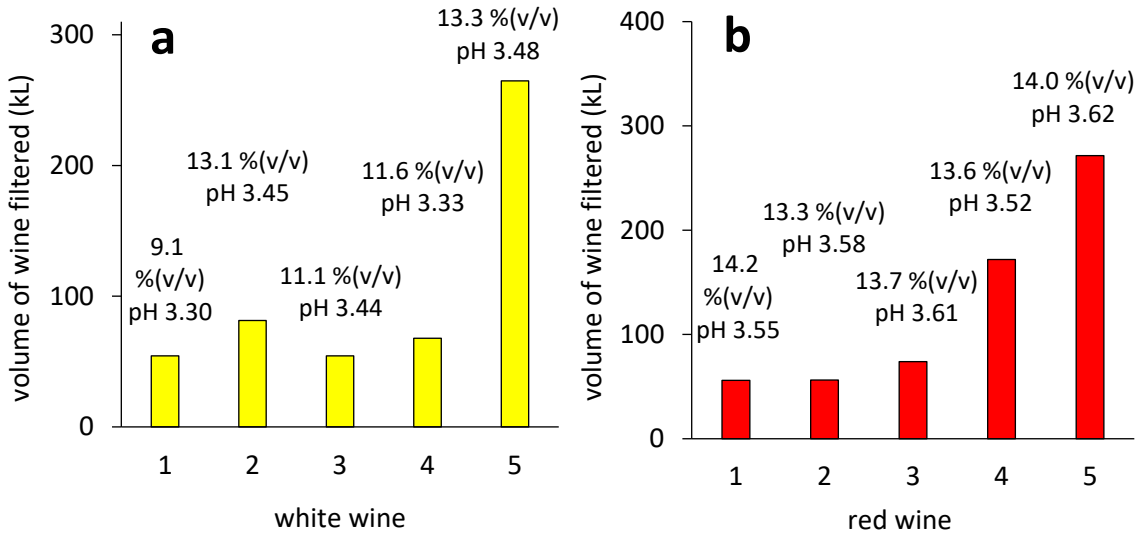


Figure 6.34. The volume (kL) of white (a) and red (b) wines filtered prior to bottling (Figure 6.33) and their corresponding alcohol concentration and pH.

Prior to filtration, the wines generally had a larger amount of Cu fraction 3 than any other fraction (see the 'pre' samples in Figure 6.35). After filtration, the white wines had every possible outcome for changes in total Cu, including three wines with decreases, one wine with an increase and another with negligible change (Figure 6.35a). For the red wine, there was a decrease in total Cu for all wines, albeit with a larger magnitude for some compared to others. In the cases, where Cu was removed from wines, it appeared to be mainly due to a decrease in Cu fraction 3. In contrast, there were many wines with slight increases in Cu fractions 1 and 2 after filtration. This may have been indicative of some oxidative- or volatile-losses of hydrogen sulfide which allowed the dissociation of copper sulfides and therefore formation of the other fractions from Cu fraction 3. The largest decrease in total Cu was in the white wine 4 that had a decrease in total Cu of 68 ± 3 % and a decrease in Cu fraction 3 by 83 ± 1%. There was no large difference between Cu concentrations for wine sampled early in the bottling run ('post early') compared to later in the bottling run ('post late') (Figure 6.35).

For the white wine 1, which had the substantial increase in total Cu, Cu fraction 2 increased more than any other fraction. The total Cu increased in this wine by 38 ± 4 % after the filtration process, while the Cu fraction 2 concentration increased by 210 ± 30 %. Although the reason for the Cu increase is uncertain, it was similar to the increase observed for dry white 2 in the previous section of this report (Section 6.2.5, Figure 6.32). Appendix 12 shows the concentrations for other metal ions (i.e., Al, Fe, Mg, Mn, Zn) in these wines and while a slight decrease in Al for white wine 1 was noted, no other sizeable change with crossflow filtration was observed.

In summary, the sequential filtration processes outlined in Figure 6.33 had a variable impact on Cu in wine. Generally, there was either no change in Cu concentration or a decrease in total Cu concentration, with Cu fraction 3 the form commonly removed from the wine while Cu fractions 1 and 2 often had slight increases. The amount of Cu removed from one white wine was particularly large

(i.e., 68 % decrease in white wine 4). Once again, there was one apparent anomaly, with one white wine increasing in Cu concentration and it was Cu fraction 2 that increased more than any other fraction.

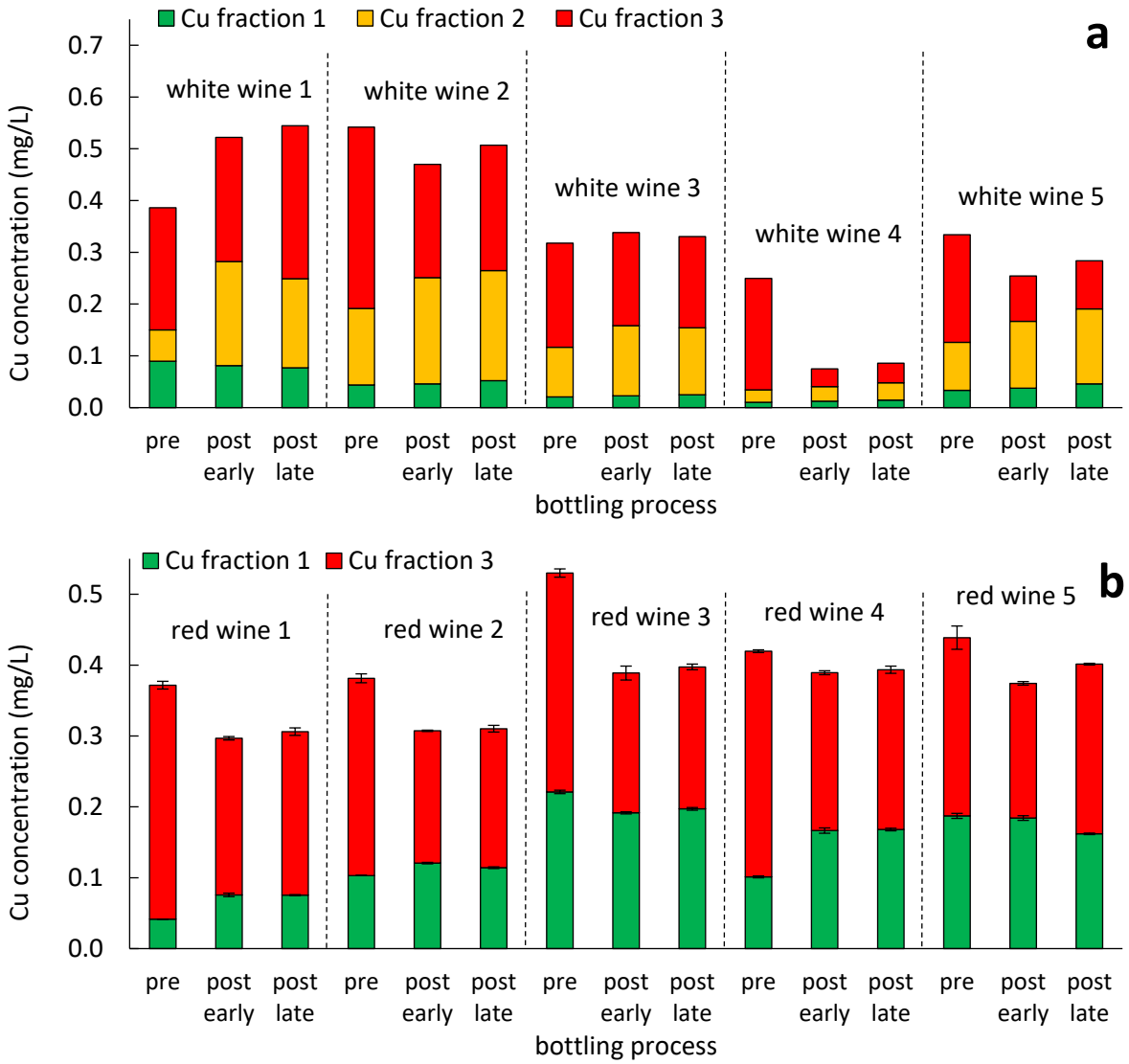


Figure 6.35. The concentration of Cu fractions in white (a) and red (b) wines pre- and post- cross flow filtration. The 'bottling process' involves all the steps depicted in Figure 6.33.

6.3 Impact of Cu species on wine aroma.

This section examines the relationship between Cu fractions (Figure 6.8) and sulfur aroma compounds in wine. This includes the influence of Cu fractions on those sulfur compounds that may be either detrimental or desirable for wine aroma.

6.3.1 Links between Cu species, H_2S, MeSH and the sensory perception of reductive wine character.

The most common culprit compounds thought to be directing 'reductive' wine off-odours are hydrogen sulfide (H_2S) and methanethiol (MeSH), and to lesser extent ethanethiol (EtSH) (Siebert et al. 2010). However, the accurate quantitation of H_2S and MeSH in wine is complicated by their inherent volatility, ready participation in redox reactions, capacity to complex metal ions, and the presence of unstable precursors (Kreitman et al. 2019).

Analytical methodologies have been developed to distinguish between the volatile forms of both H_2S and MeSH from their involatile metal complexes (Table 6.6 and the references therein). Measurement of the volatile or 'free' forms requires direct analysis of wine with sufficiently 'mild' extraction procedures to limit extra production from precursors and metal complexes. Analysis with GC, using either a sulfur chemiluminescence detector (SCD) or pulsed flame photometric detector (pFPD), has the advantages of high sensitivity, high selectivity, and simultaneous determination of H_2S and MeSH, as well as other sulfhydryl, thioether and disulfide compounds. Analysis by gas detection tubes (GDT) has the advantage of inexpensive equipment but only enables quantitation of H_2S, and interference from volatile thiol compounds can occur if the more sensitive $HgCl_2$-based GDTs are utilised (Allison et al. 2022).

The quantitation of involatile metal complexed forms of H_2S and MeSH is performed via the chloride-induced dissociation of complexes (methods 5-9, Table 6.6). This is achieved by diluting wine with brine or saturating wine with NaCl (s) (i.e., 2-3 g in 10 mL), prior to an extraction step (Table 6.6). Subtracting the free concentrations from those treated with brine or salt yields the brine-releasable or salt-releasable forms, respectively.

The following sections compare the H_2S and MeSH levels in 50 white wines using free and salt-treated GC-SCD measures and free H_2S alone with GDT (methods 2,4 and 6, Table 6.6). It involved measuring free and salt-treated concentrations of both compounds with GC-SCD, and H_2S alone with GDT. These measures were modelled with sensory ratings to identify the best predictors of sulfidic off-odours, and links to Cu fractions were examined. The full details of these experiments can be consulted in Zhang et al. (2024) and was conducted as part of a collaboration between Charles Sturt University and the Australian Wine Research Institute.

Table 6.6. Analytical methodologies for the analysis of the volatile and involatile forms of the aroma compounds responsible for 'reductive' faults in wine.

terminology for analysis	targeted form measured	wine pre-treatment	analytes	method no.	analysis instrumentation	extraction protocol[reference]
free	volatile (non-metal complexed)	none	H_2S, MeSH, EtSH	1	GC-pFPD	10 min. at 60 °C (Franco-Luesma and Ferreira 2014)
				2	GC-SCD	5 min. at 45 °C (Kontoudakis et al. 2017a)
				3	GC-SCD	15 min. at 30 °C (Ontañón et al. 2019)
			H_2S	4	GDT	10 min. at ~20 °C + N_2 sparging (Allison et al. 2022)
salt-treated[a]	volatile and metal-complexed	2-3 g NaCl (s) addition to 10 mL wine	H_2S, MeSH, EtSH	5	GC-SCD	2 g NaCl, 30 min at 45 °C (Siebert et al. 2010)
				6	GC-SCD	3 g NaCl, 30 min at 45 °C (Kontoudakis et al. 2017a)
brine-treated[b]	volatile and metal-complexed	50-fold brine dilution	H_2S, MeSH, EtSH	7	GC-pFPD (SPME)	20 min. at 35 °C (López et al. 2007)
		10-fold brine dilution	H_2S, MeSH, EtSH	8	GC-SCD	25 min. at 70 °C (Ontañón et al. 2019)
			H_2S	9	GDT	10 min. at ~21 °C + N_2 sparging (Allison and Sacks 2021)

[a] salt-releasable is the difference between the measures of free and salt-treated and corresponds to a measure of the metal complexed form of the sulfhydryl compounds.
[b] brine-releasable is the difference between the measures of free and brine-treated and corresponds to a measure of the metal complexed form of the sulfhydryl compounds.

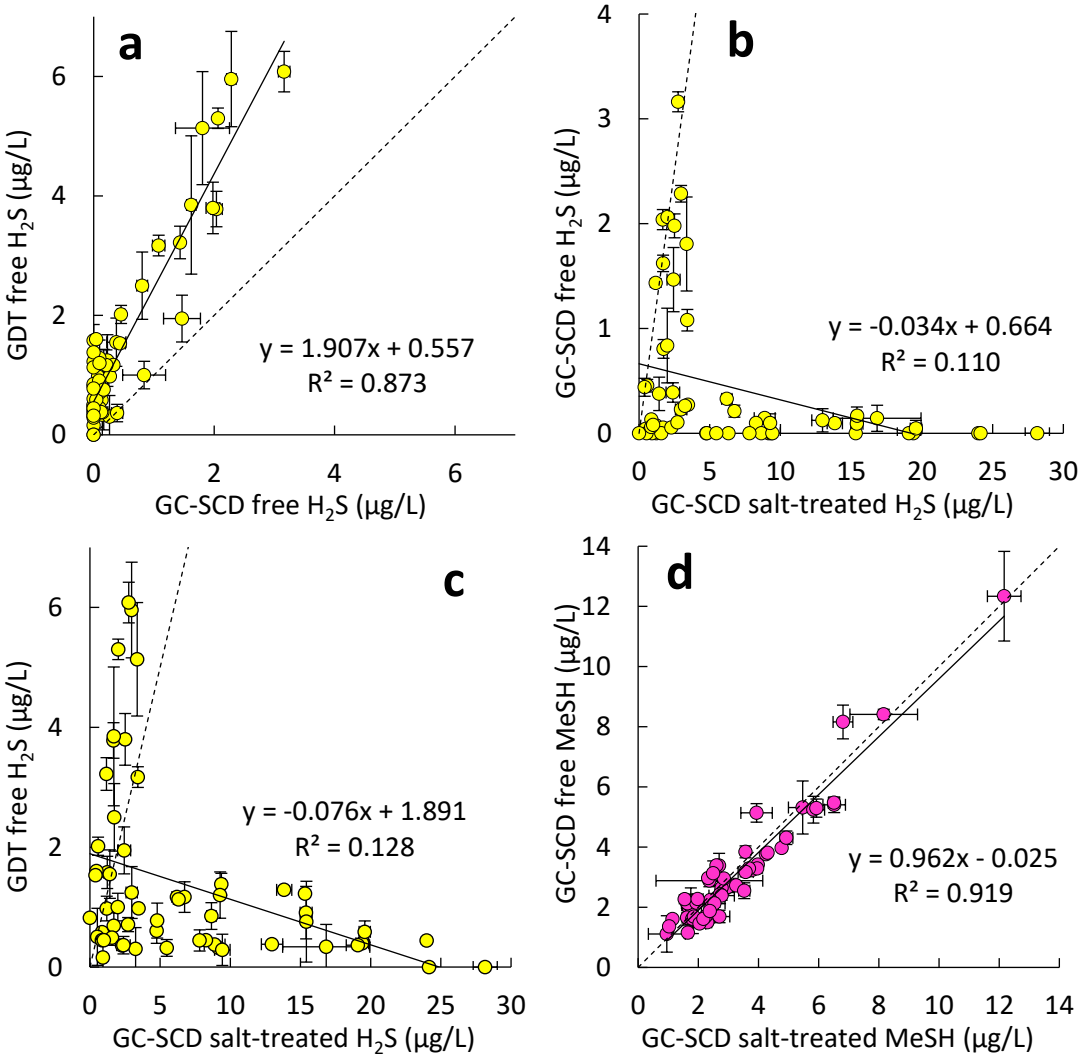


Figure 6.36. Measurement of 49 white wines for the comparison between (a) GDT and GC-SCD for free H_2S, (b) GC-SCD free and salt-treated H_2S, (c) GDT free H_2S and GC-SCD salt-treated H_2S, and (d) GC-SCD free and salt-treated MeSH. Wine with different bottling codes were split into separate data points. Error bars indicate the standard deviation. The solid line is the trend-line for the data, and the dashed line is the ideal case (with gradient = 1.00, intercept = 0). The data from one white wine (wine 21, Appendix 15) was omitted as it had extreme concentrations of H_2S. Appendix 17 shows the above graphs with the data from wine 21 included.

6.3.1.1 Comparison of analysis techniques for reductive aroma compound measurement in white wine.

A preliminary tasting trial of wines was conducted to identify around 20 white wines which displayed 'reductive' off-odours. In addition, another 30 commercial white wines were randomly selected to provide a total of 50 white wines (Appendix 15A and B).

The analysis of the white wines with two GC-SCD techniques (methods 2 and 5, Table 6.6) was conducted independently at two different laboratories to provide insight into the reproducibility of such measurements. In summary, the measures of free and salt-treated H_2S compared well between the laboratories (Appendix 16). However, there was a systematic bias for free and salt-treated MeSH whereby approximately 10 % higher concentrations were measured for MeSH at one laboratory compared to the other (Appendix 16). It was found that the slopes for the MeSH internal standard calibration graphs were consistently lower at one laboratory compared to the other, and this was consistent with the observed bias between the laboratories for MeSH quantitation. This data shows that although there is reasonable correlation between the data collected at different laboratories, there is some systematic offset between the absolute values quantified, which is not uncommon for such interlaboratory comparisons (Favell et al. 2022).

The comparison data for H_2S and MeSH by the different analysis techniques (methods 2, 4 and 5, Table 6.6) is shown in Figure 6.36. The free H_2S concentrations obtained by the different analysis techniques (i.e., GC-SCD versus GDT, Figure 6.36) had good correlation (R^2 = 0.873) with each other but with significant positive bias for the GDT free H_2S concentrations. The average positive bias for the GDT compared to GC-SCD for free H_2S was 1.0 ± 0.9 µg/L (n=58), but it increased to 2 ± 1 µg/L (n=10) for samples with H_2S concentrations higher than 1 µg/L as determined by GC-SCD. The likely explanation is that the $HgCl_2$-type GDTs utilised in the analysis can respond to some volatile thiol compounds in addition to free H_2S (Allison et al. 2022). Although $Pb(CH_3COO)_2$-type GDTs can be more selective for H_2S, the increased selectivity comes at the cost of sensitivity (Allison et al. 2022) and only the $HgCl_2$-type GDTs were sensitive enough to quantitate the vast majority of free H_2S concentrations in Figure 6.36. When the magnitude of the positive bias was plotted against free MeSH concentration the R_2 was 0.6877 (with the exclusion of one outlier, Appendix 18). This level of correlation suggested that MeSH could contribute to the positive bias in the measurement of H_2S by GDT compared to GC-SCD.

Both free methods for H_2S provided low correlations (R^2 < 0.13) with the concentrations obtained for the salt-treated H_2S method (Figure 6.36 b,c), whereby most of the salt-treated H_2S concentrations were much higher than the free H_2S concentrations. This implied a significant amount of copper sulfide in many of the wines, and that the chloride-pretreatment of the wine successfully enabled the extraction of H_2S from the copper sulfide.

The GC-SCD free and salt-treated measurement of MeSH (Figure 6.36d) show high correlation with each other (R^2 > 0.91), a gradient of 0.96 (i.e., close to 1.0) and an intercept of 0.025 (close to 0). This implied that the salt-treatment is not releasing any further MeSH than measured by the free method, and consequently that metal (particularly Cu) binding to MeSH in these samples is relatively negligible. This result for MeSH contrasts that observed for H_2S (compare Figure 6.36b and d). Previous studies have shown that after bottling the free MeSH concentration in wine typically increases and the metal-bound form (or brine-releasable) decreases (Franco-Luesma and Ferreira 2016a). Given that all the wines in Figure 6.36 had been in bottle for more than 1-year

when the measurements were undertaken (Appendix 15A), the lack of Cu binding by MeSH was as expected.

The results show that the GC analysis techniques for measuring 'reductive' aroma compounds provide comparable results across different laboratories albeit with some bias in the case of MeSH. Free H_2S quantitation by GDTs was consistently 2-fold higher than by GC-SCD but the overall correlation between results was encouraging. The comparison of free and salt-treated GC-SCD measures showed large discrepancies in most wines implying that majority of H_2S was bound to Cu in these wines, but this was not the case for MeSH.

6.3.1.2 Comparison of instrumental analysis techniques to wine sensory data.

PLS-R was conducted on a sub-selection of measurements to identify associations and understand the contribution of each measure in predicting aromas that described the presence of sulfidic off-odours in the samples (Figure 6.37). A four-factor model was considered optimal with chemical measures (x) explaining 55% of the variance in sensory scores (y). From this model, aroma attributes boiled and rotten egg (calibration R^2 = 0.69, R^2 validation = 0.60) and natural gas/sewerage/durian (calibration R^2 = 0.69, validation R^2 = 0.60), commonly noted to describe 'reductive' wine faults, were well calibrated and validated. The GC-SCD measures of free H_2S (H2S_F) and free and salt-treated MeSH (MeSH_F, MeSH_ST) as well as the GDT measure of free H_2S (GDT_H2S) were found to be most positive and significant for boiled and rotten egg aroma as well as natural gas/sewage/durian aroma.

MeSH_T, MeSH_F, GDT_H2S and phenylmethanethiol (PMT) were significantly negatively associated with overall fruit, aligning with the established literature supporting masking effects exerted by these low molecular weight sulfur compounds (Nandorfy et al. 2023). Canned corn/asparagus (calibration R^2 = 0.58, validation R^2 = 0.29) was significantly related to dimethylsulfide (DMS) while rubber aroma (calibration R^2 = 0.46, validation R^2 = 0.34), was associated significantly with MeSH_ST, MeSH_F and PMT, however these attributes were less well modelled. The determined concentrations of both salt-releasable MeSH (MeSH_SR) and salt-releasable H_2S (H2S_SR) did not correspond closely with the sensory ratings that identify reductive wine faults (Figure 6.37b). Overall PLS-R models illustrate that H_2S and MeSH are primarily responsible for the positioning of off-odours associated with 'reductive' wine aromas and highlight the importance of free concentrations to sensory response.

6.3.1.3 Links between sensory attributes and Cu fractions.

Figure 6.38 shows a three-way comparison between the H_2S concentrations, Cu concentrations and the intensity in the key reduction sensory attributes for the white wines. For this comparison, the wines in the top 15 % for ratings of boiled/rotten egg aroma or natural gas/sewage/durian aroma intensity were designated 'high' and highlighted with coloured data points in Figure 6.38. Six wines had high intensity for both sensory attributes (pink data points in Figure 6.38), one wine was high in boiled/rotten egg aroma only (i.e., yellow data point), and one wine was high in natural gas/sewage/durian aroma only (i.e., blue data point).

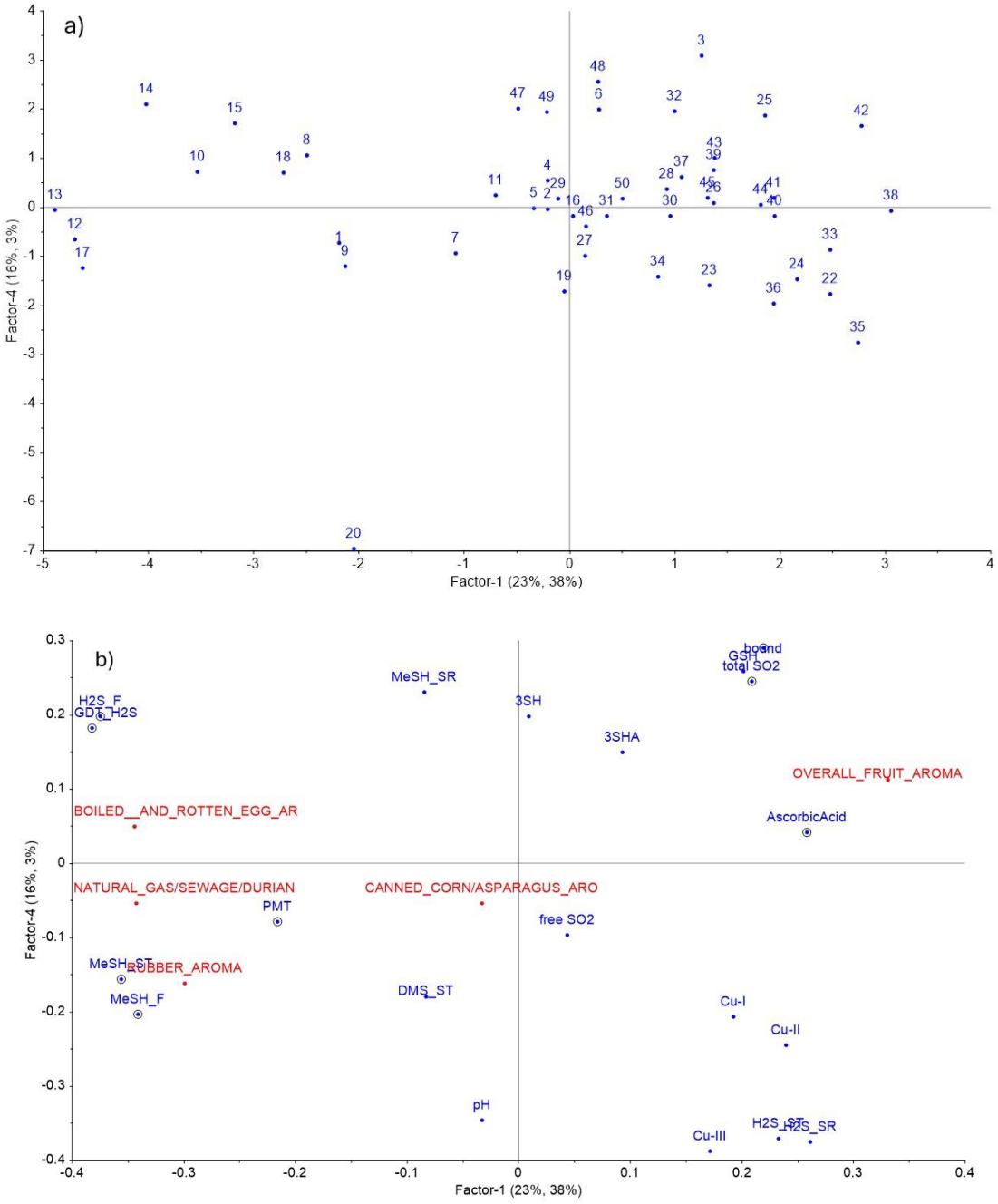


Figure 6.37. PLS-R regression models of the first four factors of (a) scores and (b) correlation loading plots relating the compositional compounds (x variables in blue (🔵)) to sensory attributes (y variables in red (🔴)) of the 49 wines analysed.

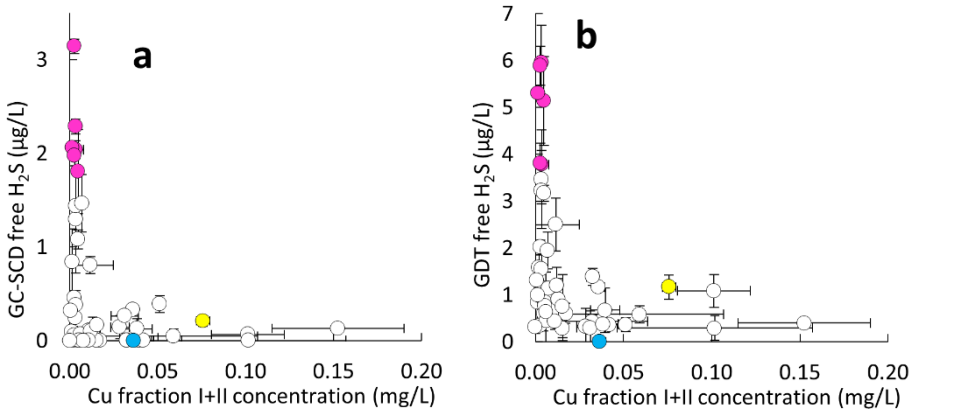


Figure 6.38. The comparison of (a) GC-SCD free H_2S, (b) GDT free H_2S, and (c) GC-SCD salt-treated H_2S with the combined Cu fraction 1 and 2 concentration. Coloured data points are for wines ranked within the top 15 % for *boiled and rotten egg* aroma intensity or *natural gas/sewage/durian* aroma intensity. Pink data points are for wines in the top 15 % of rankings (i.e., high) for both attributes, while blue is for a wine high in *natural gas/sewage/durian* aroma only, and yellow is for a wine high in *boiled and rotten egg* aroma only.

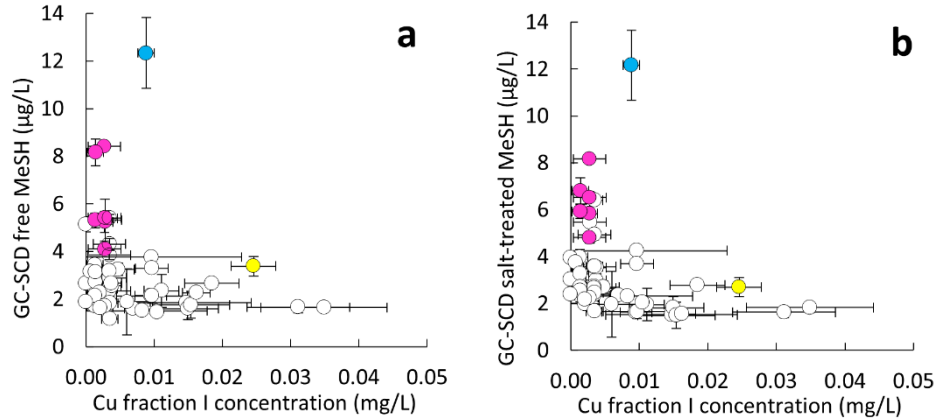


Figure 6.39. The comparison of (a) GC-SCD free MeSH, and (b) GC-SCD salt-treated MeSH with Cu fraction 1 concentration. Coloured data points are for wines ranked within the top 15 % for *boiled and rotten egg* aroma intensity or *natural gas/sewage/durian* aroma intensity. Pink data points are for wines in the top 15 % for rankings (i.e., high) of both attributes, while blue is for a wine high in *natural gas/sewage/durian* aroma only, and yellow is for a wine high in *boiled and rotten egg* aroma only.

Once the combined Cu fractions 1 and 2 fell below 0.02 mg/L, a link to potential GC-SCD free H_2S and GDT free H_2S accumulation (i.e., > 1.0 µg/L) was evident (Figure 6.38a,b). In addition, the accumulating free H_2S could be related to the intensity of boiled/rotten egg aroma and natural gas/sewage/durian aroma (Figure 6.38a,b). The wines with elevated intensities for both aroma attributes (pink data points) were grouped to higher free H_2S concentrations and low Cu fraction 1 and 2 concentrations. Interestingly, the wines with high intensity of boiled and rotten egg aroma (yellow data point) or natural gas/sewage/durian aroma (blue data point), but not both, did not follow this trend. For the latter (blue data point) this was a consequence of high MeSH concentration (see Figure 6.39) contributing to the natural gas/sewage/durian aroma in this wine rather than free H_2S. Alternatively, the wine corresponding to the yellow data point was low in free H_2S concentration and mid-range in MeSH despite being rated high in boiled/rotten egg aroma. However, of all 49 wines analysed, it had the second highest concentration of phenylmethanethiol (i.e., 0.14 ± 0.07 µg/L, wine 19, Appendix 15a), which is an aroma compound often associated with empyreumatic flint or struck match aroma (Tominaga et al. 2003, Nandorfy et al. 2023). At such high PMT concentration, and in combination with some free MeSH, it is possible that this combination was perceived similarly as boiled and rotten egg aroma. Another wine with high concentrations of phenylmethanethiol concentration (i.e., 0.2 ± 0.1 µg/L, wine 7, Appendix 15a) but low free H_2S and MeSH concentrations, was rated relatively high for the natural gas/sewage/durian aroma attribute (i.e., 8th highest rating out of the 49 wines).

The relationship between salt-treated H_2S measures with Cu fractions and sensory attributes (Figure 6.38c) is very different to that of the free H_2S (Figure 6.38a,b). All the wines with high ratings of both of the 'reductive' aroma attributes (pink data points) had low concentrations of salt-treated H_2S. This suggests that most of salt-releasable H_2S in wine is involatile.

Figure 6.39 shows the equivalent graphs to Figure 6.38 but using the GC-SCD measures of MeSH rather than H_2S. Cu fraction 1 linked better to free MeSH accumulation than other Cu fractions or total Cu (data not shown). The wines rated highest for the 'reduction' aroma attributes were grouped to wines of higher free MeSH concentrations and had less than 0.015 mg/L Cu fraction 1 (Figure 6.39a). The yellow data point corresponded to the wine with elevated phenylmethanethiol concentrations that has already been discussed. Given that the concentrations of free MeSH and salt-treated MeSH were strongly correlated with each other in these wines (Figure 6.36d), Cu fraction 1 had a similar link to salt-treated MeSH (Figure 6.39b) as for free MeSH (Figure 6.39a). The similarity in Figures 6.39a and 6.39b suggests that the inhibition of free MeSH accumulation by Cu fraction 1 is less likely to be due to the formation of Cu-complexes but more likely to be due to oxidation mechanisms (i.e., disulfide and/or polysulfide production). The oxidation of MeSH to dimethyldisulfide is a focus of the next section of this report (Section 6.3.3).

This study provided several key outcomes:

- For the first time, the link between 'reductive' aroma attributes (i.e., boiled and rotten egg and natural gas/sewerage/durian) was shown to relate to all free measures (i.e., GDT and GC-SCD) of H_2S and MeSH.
- Despite the bias to higher concentrations of free H_2S by GDTs compared to GC-SCD, the GDT method could still be readily used to quantitate compounds related to the sensory

perception of 'reductive' aroma in wine in situations when traditional analytical GC-SCD measurements are not possible.

- The salt-treated measure of H_2S did not provide any link to the sensory perception of reductive aroma attributes in the wines under study.
- The relationship of Cu fractions on the sensory perception of reductive aroma in a diverse range of white wines was established for the first time.

6.3.2 How universal is the link between Cu fractions and free sulfhydryl accumulation?

Given the link observed between Cu fractions and H_2S and MeSH (Figure 6.38-39), a large-scale survey of wines was conducted to establish whether the link was universal across wine type, age and storage conditions. Analysis was conducted on over 1300 bottles of wine, including periodically on wines stored at different temperature (14 °C or 40 °C) or conditions that are known to induce reductive aroma accumulation (e.g., exposed to light or in contact with metallic aluminium). Cu fractions were measured by colorimetry and/or electrochemistry, and free H_2S and free MeSH by GC-SCD (method 2, Table 6.6).

The aroma thresholds for H_2S and MeSH in white wine were used as upper limits for their designated acceptable accumulation in the surveyed wines. The thresholds were 1 and 3 µg/L, respectively (Siebert et al. 2009, Solomon et al. 2010). It must be noted that the exact concentrations for free H_2S or free MeSH to 'spoil' wine would be influenced by a variety of parameters, including wine matrix effects and the sensitivity of individual consumers, but it would typically be expected to be at concentrations above the given aroma thresholds. For example, the wines in Figure 6.38 and 6.39 were not designated as having the reductive characters of rotten egg or sewage until reaching concentrations around 2 µg/L for H_2S and 4 µg/L for MeSH, which were around 100 % and 33 %, higher than the respective aroma thresholds (i.e., 1 and 3 µg/L). Hence using the aroma thresholds as the maximum accumulation limit for H_2S and MeSH should be viewed as a conservative limit in terms of the compounds causing a noticeable spoilage of the wine.

Figures 6.40 and 6.41 compare Cu concentrations with free H_2S and free MeSH in approximately 900 bottles of wine. Of note is the particularly high concentration free H_2S and free MeSH in certain wines, reaching over 200 and 18 times the aroma thresholds, respectively. The wines highest in free H_2S and free MeSH were those stored in light or in contact with metallic aluminium, and they will be discussed in later sections of this report (Sections 6.5.5 and 6.6). The combined Cu fractions 1 and 2 provided the best link with free H_2S accumulation (Figure 6.40a,b), with the latter only reaching concentrations in excess of the aroma threshold when the Cu fraction 1 and 2 concentration was below 0.015 mg/L. There was only one exception in the 957 bottles analysed (Figure 6.40b), but even in this case the Cu fraction 1 and 2 concentration was low (0.018 mg/L) and the H_2S concentration was only marginally above 1 µg/L (i.e., 2.5 µg/L). These results were consistent with the free H_2S and Cu fraction 1 and 2 link discussed in the previous section (6.1.1.3),

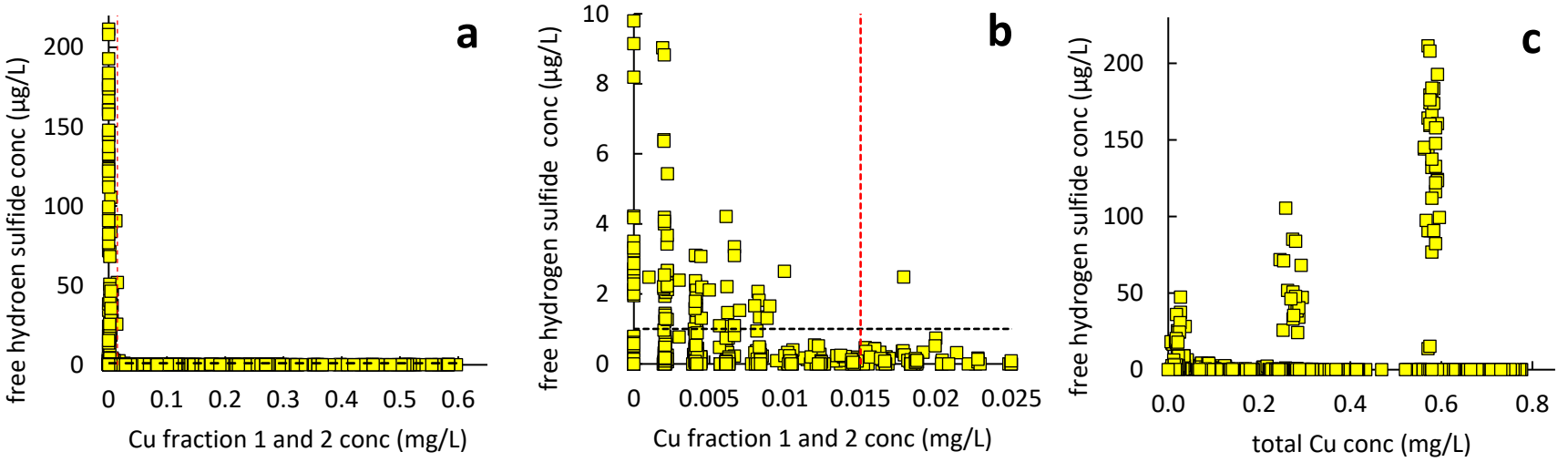


Figure 6.40. The concentration of free H_2S versus Cu fractions (a,b) and total Cu (c) in 957 bottles of white wine. Cu was measured by BCA colorimetry. Panel b is a zoomed in version of panel a. Black dashed: aroma threshold for H_2S. Red dashed lines: Cu fraction concentration to ensure limited free H_2S accumulation.

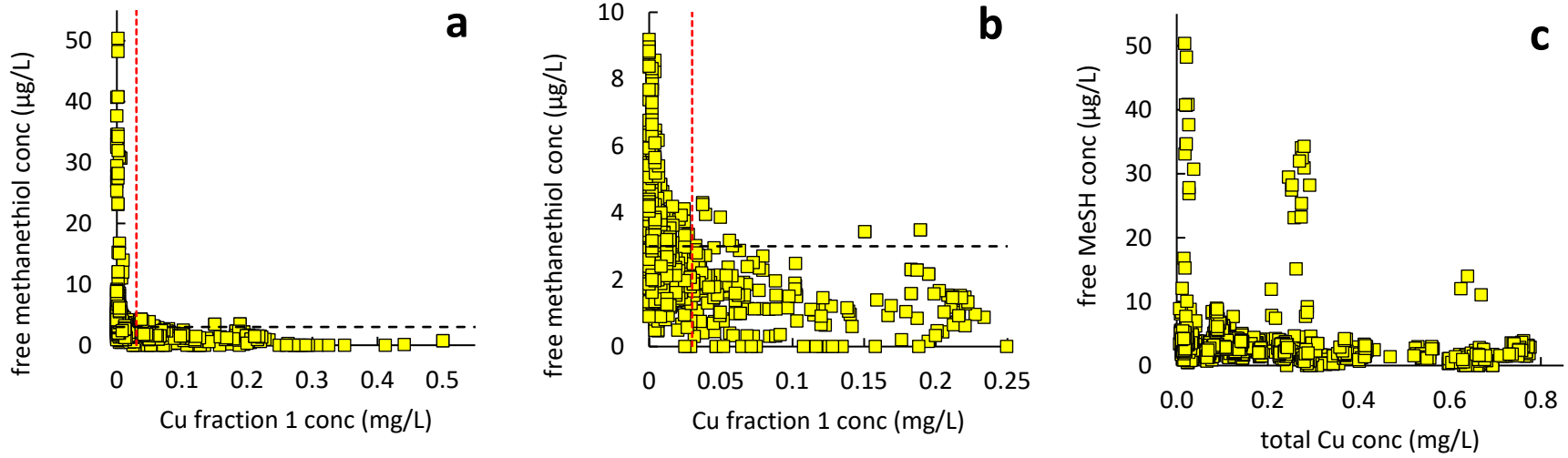


Figure 6.41. The concentration of free MeSH versus Cu fraction 1 (a,b) and total Cu (c) in 893 bottles of white wine. Cu was measured by BCA colorimetry. Panel b is a zoomed in version of panel a. Black dashed: aroma threshold for MeSH. Red dashed lines: Cu fraction concentration to ensure limited free MeSH accumulation.

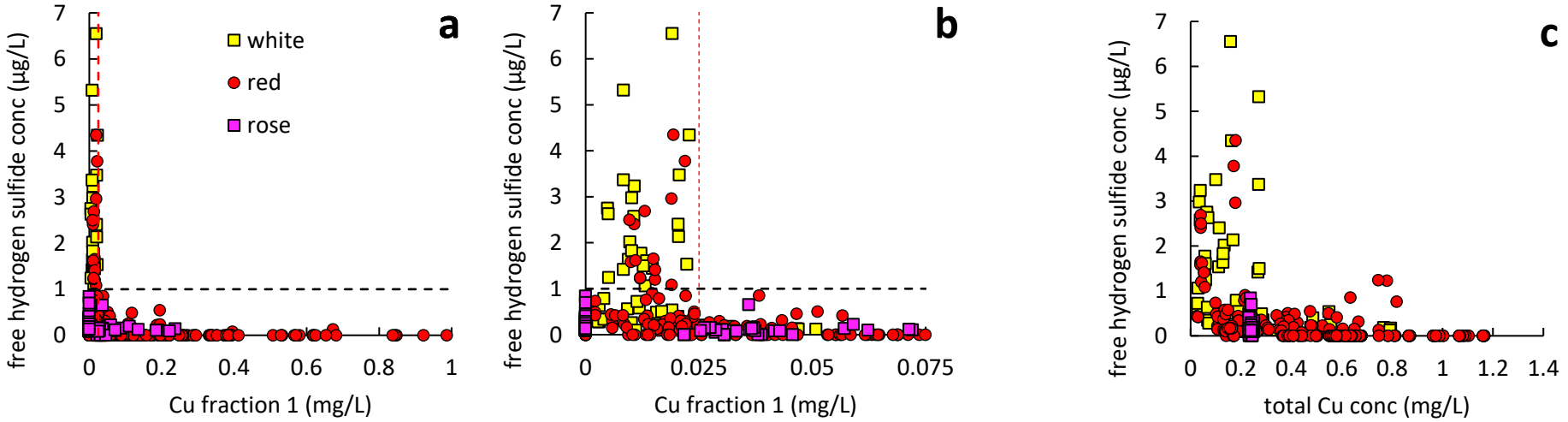


Figure 6.42. The concentration of free H_2S versus Cu fraction 1 (a,b) and total Cu (c) in 413 bottles of wine. Cu fraction 1 was measured by electrochemistry (total Cu by ICPOES). Panel b is a zoomed in version of panel a. Black dashed: aroma threshold for H_2S. Red dashed lines: Cu fraction concentration to ensure limited free H_2S accumulation.

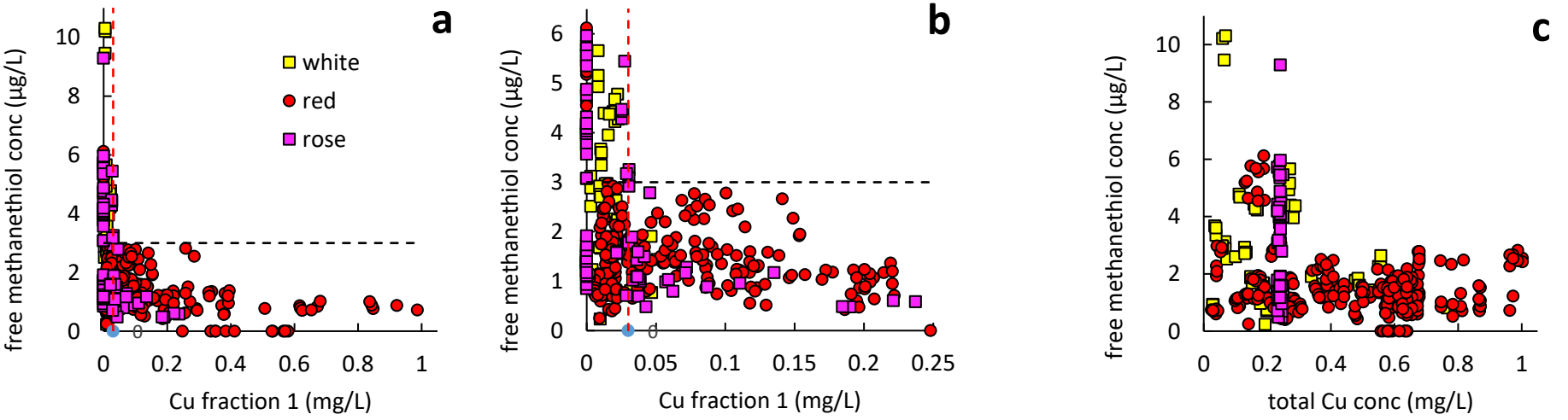


Figure 6.43. The concentration of free H_2S versus Cu fraction 1 (a,b) and total Cu (c) in 413 bottles of wine. Cu fraction 1 was measured by electrochemistry (total Cu by ICPOES). Panel b is a zoomed in version of panel a. Black dashed: aroma threshold for H_2S. Red dashed lines: Cu fraction concentration to ensure limited free H_2S accumulation.

including a similar result for the critical Cu fraction 1 and 2 concentration (i.e., 0.015 vs 0.020 mg/L). It must be noted that these results do not imply that wines with less than 0.015 mg/L Cu fraction 1 and 2 will always have accumulation of free H_2S. Instead, it shows that if a wine has a propensity for free H_2S to accumulate, it can only accumulate if the combined Cu fraction 1 and 2 concentration is below 0.015 mg/L. The relationship shown in Figure 6.40a did not eventuate if salt-treated measures of H_2S were used instead of free H_2S (Appendix 19b), or if total Cu was used instead of Cu fraction 1 and 2 (Figure 6.40c).

In Figure 6.40c, there appears to be some interesting clusters of samples with elevated free H_2S concentrations particularly at total Cu concentrations around 0.3 and 0.6 mg/L. These samples were predominantly those exposed to either light or stored in contact with metallic aluminium. For the remaining samples, it was generally the wines with lower total Cu (i.e., < 0.12 mg/L) that were able to accumulate high concentrations of free H_2S, but the link between free H_2S and total Cu was less defined than when using Cu fraction 1 and 2 (Figure 6.40a).

Cu fraction 1 was found to relate best to free MeSH (Figure 6.41a,b), rather than the combined Cu fraction 1 and 2 concentrations (data not shown). Free MeSH was only found to accumulate to appreciable concentrations when the Cu fraction 1 concentration was below 0.03 mg/L, which was around double the threshold found for the smaller scale study (Figure 6.39a). In contrast to the result for H_2S, the relationship shown in Figure 6.41b did still eventuate if salt-treated measures of MeSH were used instead of free MeSH (Appendix 19d). This was consistent with the results in 6.36d that showed in most wines the free and salt-treated MeSH concentrations were similar and that most wines had minimal binding of MeSH to Cu. This implied that the critical Cu fraction 1 concentration (i.e., the level required to prevent accumulation of free MeSH) of 0.03 mg/L, was most likely the lower limit that could afford efficient oxidation of MeSH rather than binding of MeSH. In terms of oxidation products, these could include disulfides and polysulfanes (Kreitman et al. 2017). As for free H_2S, the samples that accumulated high concentrations of free MeSH tended to have lower total Cu concentrations (i.e., < 0.05 mg/L). The exceptions were a cluster of samples around 0.25 mg/L that had been exposed to light during storage, and another set of wines with 0.6 mg/L Cu that were commercially sourced wines and had unknown, but most probably not ideal, storage conditions.

To confirm the same pattern also existed for red and rose wine styles, and for an alternate Cu fraction measurement technique, electrochemistry was used to measure the Cu fraction 1 concentration in 413 bottles of red, white and rosé wines. In this case, the electrochemical technique could not determine Cu fraction 2 and hence only Cu fraction 1 was related to the free concentrations of H_2S. The results in Figures 6.42a and 6.43a show that the electrochemical measure of Cu fraction 1 could be related to free sulfhydryl accumulation in a similar manner to the colorimetric results in Figures 6.40a and 6.41a. Indeed, the threshold concentrations Cu fractions required to prevent accumulation of free H_2S or free MeSH were similar in Figures 6.40-6.43. It must be noted that the transition of free H_2S to concentrations above their aroma threshold was more distinct when plotted with the colorimetric measure of Cu fraction 1 and 2 than for the electrochemical measure of Cu fraction 1. As for the white wine results in Figure 6.40c and 6.41c, the red, rosé and white wines with lower total Cu concentration were also those with more likelihood of having accumulation of free H_2S or free MeSH (Figure 6.42c and 6.43c).

These results show that the link between Cu fractions and free H_2S and free MeSH is universal across different wine types, ages and storage conditions. The combined Cu fraction 1 and 2 concentration relates best to free H_2S accumulation in white wine, and the Cu fraction 1 concentration relates best for red and rosé wines. The critical concentration for the combined Cu fractions 1 and 2 for the prevention of free H_2S

accumulation in white wine seems to be around 0.015 mg/L, and 0.025 mg/L of Cu fraction 1 in red and rosé wines. The Cu fraction 1 concentration of 0.03 mg/L appears to be sufficient to prevent free MeSH in white, red and rosé wines.

6.3.3 The interaction between Cu(II) and MeSH.

Franco-Luesma and Ferreira (2014) were the first to provide evidence for the metal bound form of methanethiol in wine conditions. While the free form of MeSH is volatile and associated with unpleasant rotten egg/sewage aromas to wine (see Section 6.3.1.3), the metal-bound forms were proposed to be involatile (Franco-Luesma and Ferreira, 2014). Franco-Luesma and Ferreira (2014) measured the free forms of MeSH using elevated temperature for extraction from wine (i.e., 60 °C for 10 minutes, method 1, Table 6.6) prior to GC analysis, while the free and metal-bound forms were measured by 50-fold dilution of wine with brine in conjunction with extraction at 35 °C for 20 minutes. Franco-Luesma and Ferreira (2014) found that the bound form of MeSH generally decreased during the bottle aging of wine, and this was consistent with the results from the earlier section (i.e., 6.3.1.1) showing minimal Cu-bound MeSH in the wines with 1-2 years of bottle age.

Later sections of this report (e.g., Section 6.3.4) demonstrate that Cu fractions 1 and 2 can gradually sequester sulfide from precursors in wine during wine aging and can transition exclusively to Cu fraction 3. This equates to a transition from Cu(II)-organic acid and Cu(I)-thiol complexes to copper sulfides. One potential consequence of the conversion of Cu(I)-thiol complexes to copper sulfides could be the release of methanethiol into the free form if it is dissociated from Cu by the stronger binding sulfide. To investigate this possibility, experiments were first conducted to confirm evidence for the binding of MeSH to Cu in model wine, white wine and red wine by using GC-SCD measurements of free MeSH and BCA colorimetric measurements of Cu fractions. Then hydrogen sulfide was added to a wine with Cu-bound MeSH and changes in free MeSH were monitored. It should be noted that the GC-SCD methodology used for free MeSH measurement was different than used by Franco-Luesma and Ferreira (2014), with a milder extraction temperature of 5 minutes at 45 °C rather than 10 minutes at 60 °C (Table 6.6). In later work, the Ferriera research group also used milder extraction temperatures (15 minutes at 30 °C) for free methanethiol measurement as the higher extraction temperature were seen as excessive and had the potential to cause equilibrium shifts from metal-complexed forms to free forms (Ontañón et al. 2019).

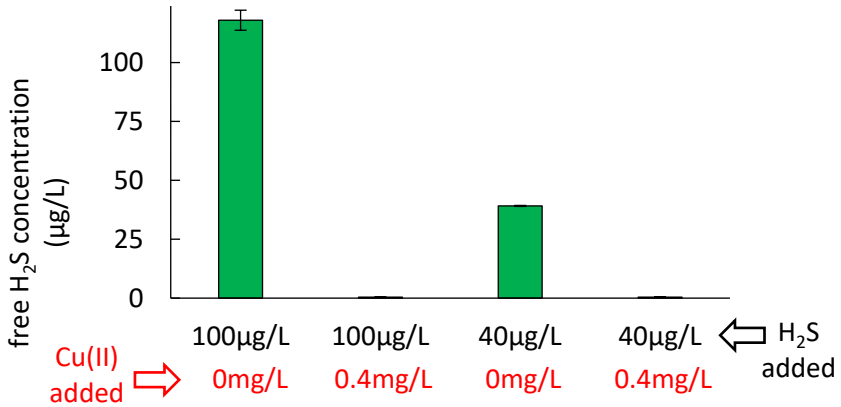


Figure 6.44. The concentration of free hydrogen sulfide in model wines before and after addition of 0.4 mg/L Cu(II).

Prior to assessing the interactions between Cu(II) and methanethiol, the binding between Cu(II) and hydrogen sulfide was reconfirmed. Figure 6.44 shows the measured free H_2S concentrations in model wines with 100 or 40 µg/L H_2S and either 0 mg/L or 0.4 mg/L Cu(II). The results show virtually no free hydrogen sulfide was present after Cu(II) addition to either sample. In both cases the Cu(II) added was in excess, with mole ratios for Cu(II) to H_2S being 5.3 : 1 and 2.1 : 1, respectively. These mole ratios would

generally be lower than those used in typical Cu fining during wine production but despite this they still show complete binding of hydrogen sulfide by Cu.

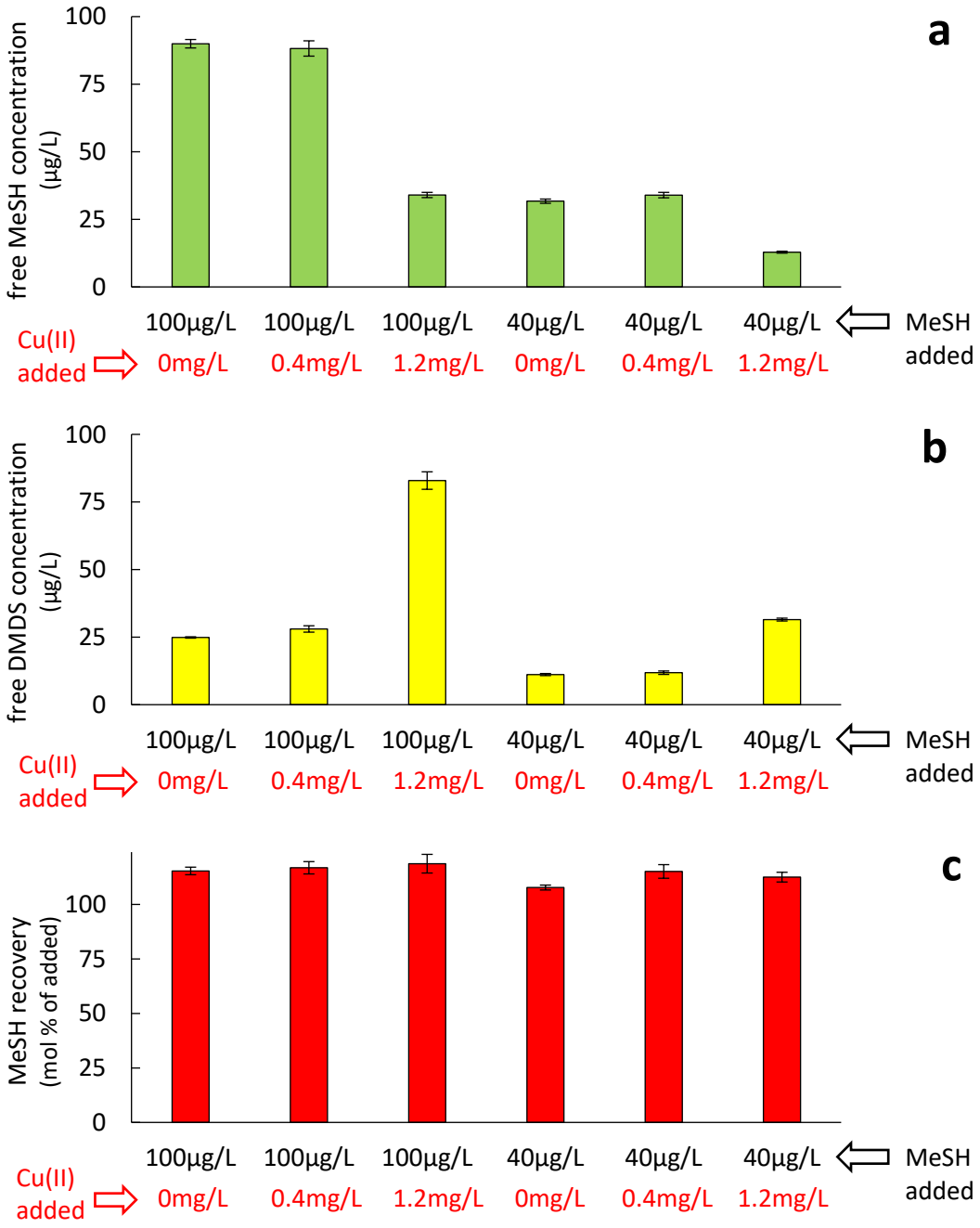


Figure 6.45. The concentration of free MeSH (a), free DMDS (b) and recovery of added MeSH (c) in model wine samples with variable MeSH and Cu(II) added. The recovery of added MeSH was calculated as: ([mol{freeMeSH} + (2 x mol{freeDMDS})]/mol{addedMeSH}) x100.

In contrast, when 0.4 mg/L Cu(II) was added to solutions of 100 µg/L or 40 µg/L methanethiol (Figure 6.45a), there was no decrease in free methanethiol concentration, implying no significant binding of methanethiol to Cu(II). This was despite Cu(II) being added in excess with mole ratios of Cu(II) to methanethiol of 3.1:1 and 7.6:1, respectively. Upon addition of a three-fold higher concentration of Cu(II)

(i.e., 1.2 mg/L), achieving Cu(II) to methanethiol mole ratios of 9.1:1 and 22.8:1, an appreciable loss in free methanethiol became evident (Figure 6.45a).

In conjunction with the loss of free MeSH was an increase in dimethyldisulfide (Figure 6.45b), which can be formed from the oxidative coupling of two MeSH molecules. This reaction is known to be catalysed by Cu(II) and can occur even in the absence of oxygen (Kreitman et al. 2017). When the recovery of the added methanethiol was calculated from the sum of the measured free methanethiol and the constituent methanethiol of dimethyldisulfide, the recovery values were around 100 % (Figure 6.45c). Based on this recovery, it suggests that any losses in free MeSH induced by Cu could be accounted for solely by dimethyldisulfide production and negligible binding of Cu(II) to MeSH.

Further experiments were repeated in the model wine system with variations of pH, incubation times, and phenolic compound/sulfur dioxide concentrations (see Appendix 20). In all cases, the recovery of added methanethiol could be accounted for by the measured free MeSH and the constituent methanethiol within dimethyldisulfide. Overall, these results suggested that if Cu(I) or Cu(II) were forming complexes with methanethiol, then they were not sufficiently strong to prevent the temperature-induced extraction (i.e., 5 minutes at 45 °C) of methanethiol during the GC-SCD analysis methodology. As an aside, it was found that dimethyldisulfide formation from methanethiol occurred more readily at a lower pH or in the presence of phenolic compounds provided sulfur dioxide was not present (data not shown).

Cu fractions were also measured in model wine systems to provide an alternative assessment for binding between Cu(II) and methanethiol. For this set of experiments, 0.4 mg/L Cu(II) was added to model wine solutions containing 10, 40 or 100 µg/L methanethiol and the resulting Cu fractions were measured immediately (i.e., 0 h) and after 24 hours. The results in Figure 6.46 show that the Cu(II) measured in all the model wines was exclusively in the form of Cu fraction 1, which was attributed to Cu(II)-tartrate or Cu(II)-hydrogen tartrate complexes. There was negligible Cu fraction 2 or 3 that would be indicative of soluble or aggregated Cu(I)-methanethiol complexes detected. This outcome was in contrast to that of

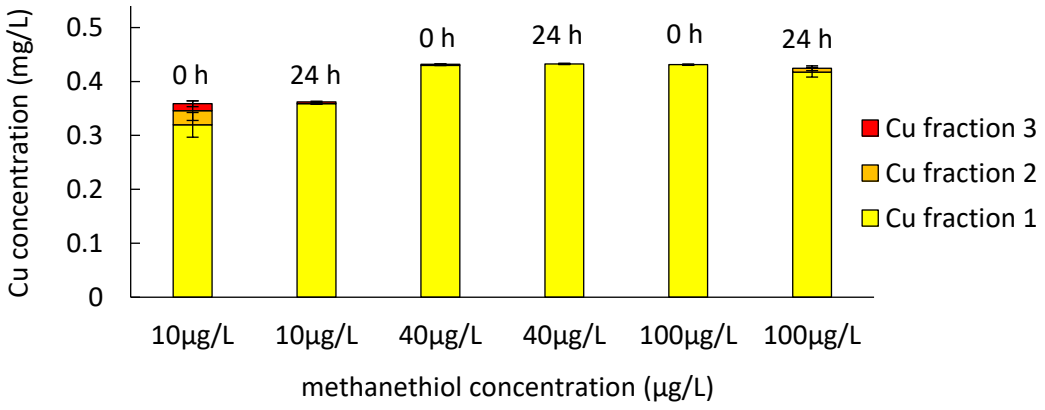


Figure 6.46. The Cu fractions in model wine systems containing 10, 40 and 100 µg/L methanethiol and measured after 0 and 24 hours.

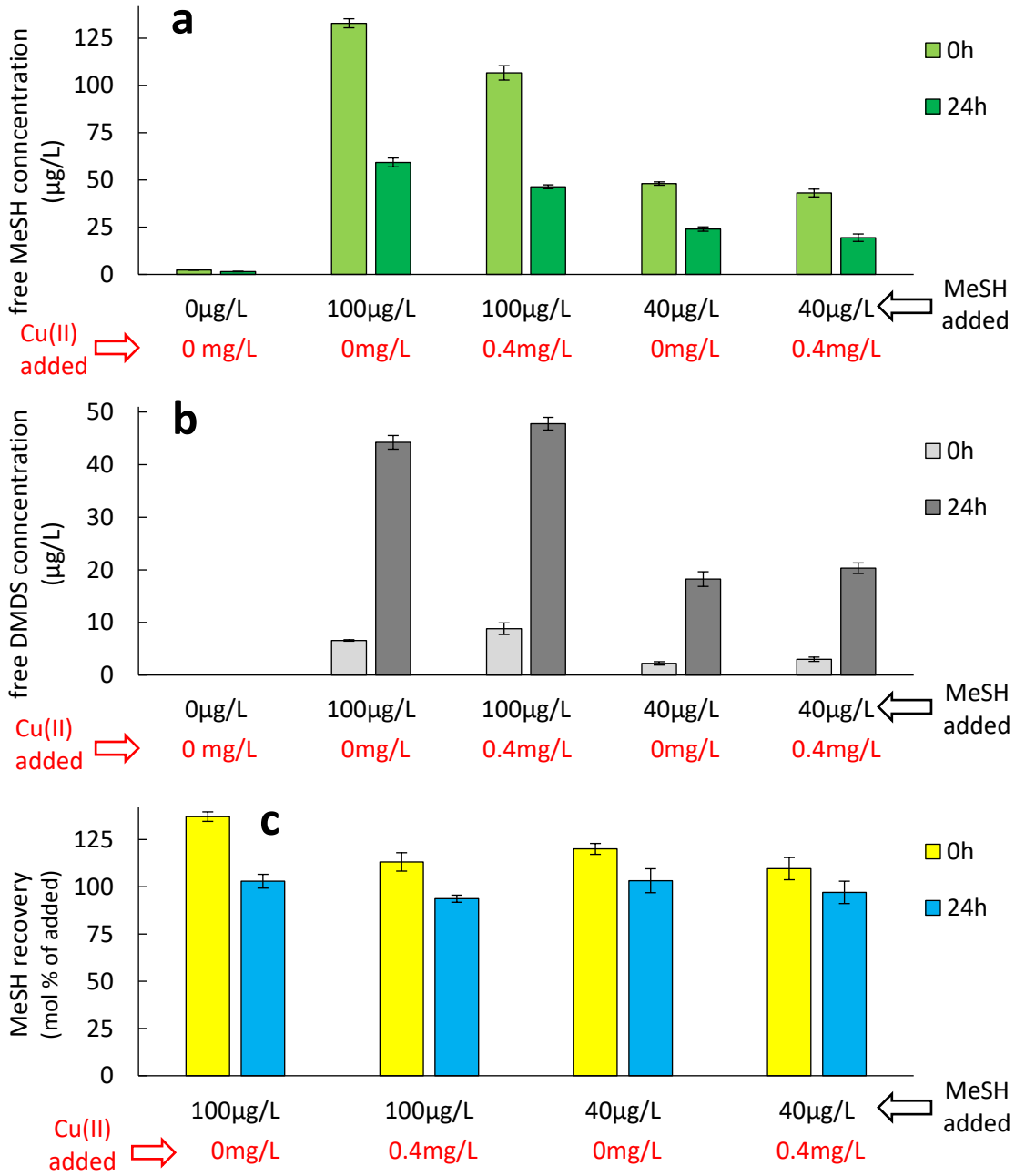


Figure 6.47. The free MeSH concentration (a), free DMDS (b) and recovery of added MeSH (c) in a white wine immediately after addition of MeSH and Cu(II) and then after 24 hours storage. The recovery of added MeSH was calculated as: ([mol{freeMeSH} + (2 x mol{freeDMDS})]/mol{addedMeSH}) x100.

the glutathione and Cu(II) interaction, which afforded significant amounts of Cu fraction 2 (Table 6.5). Glutathione contains acid and amino moieties, which are absent in methanethiol, and are likely to strengthen complexation to Cu(II). Overall, both the GC-SCD and BCA colorimetric results provided no evidence for complex formation between Cu(II) and methanethiol in the model wine system.

The interactions between Cu(II) and methanethiol were also investigated in a Chardonnay (Figure 6.47) and a Shiraz (Figure 6.48). The methanethiol levels in the wines were adjusted to either 100 or 40 µg/L and Cu(II) additions of 0 or 0.4 mg/L were made. The Cu present in the wines was low (i.e., < 0.05 mg/L) prior to any additions. The results in Figures 6.47 and 6.48 once again showed complete recovery of the added methanethiol based on the measured free methanethiol and the formed dimethyldisulfide. Like

the model wine system, this implied that the formation of any Cu(II) or Cu(I) complexes with methanethiol were not sufficiently strong to be detected by the GC-SCD methodology as originally proposed by Franco-Luesma and Ferriera (2014). A limitation of the Luesma and Ferriera (2014) study is that no results for dimethyldisulfide are described and hence much of the attributed metal-bound methanethiol may have been instead due to dimethyldisulfide formation. Also, the extractions conditions for free methanethiol determination by Luesma and Ferriera (2014) (i.e., 60 °C for 10 minutes) were harsher than utilised in recent methodologies (Table 6.6, Section 6.3.1). These higher extraction temperatures may have induced MeSH losses during free MeSH measurement, potentially through DMDS formation, rather than the decreased free MeSH being a consequence of bound MeSH. Upon addition of Cu(II) to methanethiol in a model wine system, Luesma and Ferriera (2014) could only account for 83 ± 9 % of the added methanethiol when using the measured free and 'bound' MeSH concentrations. From the results in this section, it is likely that the unrecovered methanethiol, and potentially the 'bound' methanethiol, in this model system was most likely due to dimethyldisulfide formation.

It is possible that in the more complex matrix of real wine, compared to the simplified model wine system, that the MeSH may be more inclined to bind to Cu when other specific ligands (e.g., specific residual proteins or oligopeptides), are also present. In a survey of wines with free and salt-treated MeSH measures by GC-SCD, only one wine was found to have significant amounts of 'bound MeSH' as determined by the difference between free and salt-treated concentrations. However, even in this case the concentration of bound MeSH was relatively low with a concentration of 1.2 µg/L relative to a free MeSH concentration of 5 µg/L. When H_2S was added to the wine at increasing concentrations H_2S of 0, 40, 80 and 160 µg/L, the concentration of free MeSH did not increase (Figure 6.49). This implied that the 1.2 µg/L of bound MeSH determined within this wine could not be dissociated to free MeSH based on H_2S addition.

In summary, these results show no evidence for any significant binding of between Cu(II) and methanethiol after their addition to model wines, as well as a white and red wine. This was based upon GC-SCD and BCA colorimetric studies and was consistent with the results observed in earlier sections of this report (i.e. Section 6.3.1 and 6.3.2). One white wine with a small concentration of 'bound' methanethiol was identified, but even after addition of high concentrations of hydrogen sulfide there was no release of further free methanethiol. Therefore, the increase in free MeSH due to the substitution of MeSH bound to Cu(I) by hydrogen sulfide is not likely to occur during the bottle aging of wine.

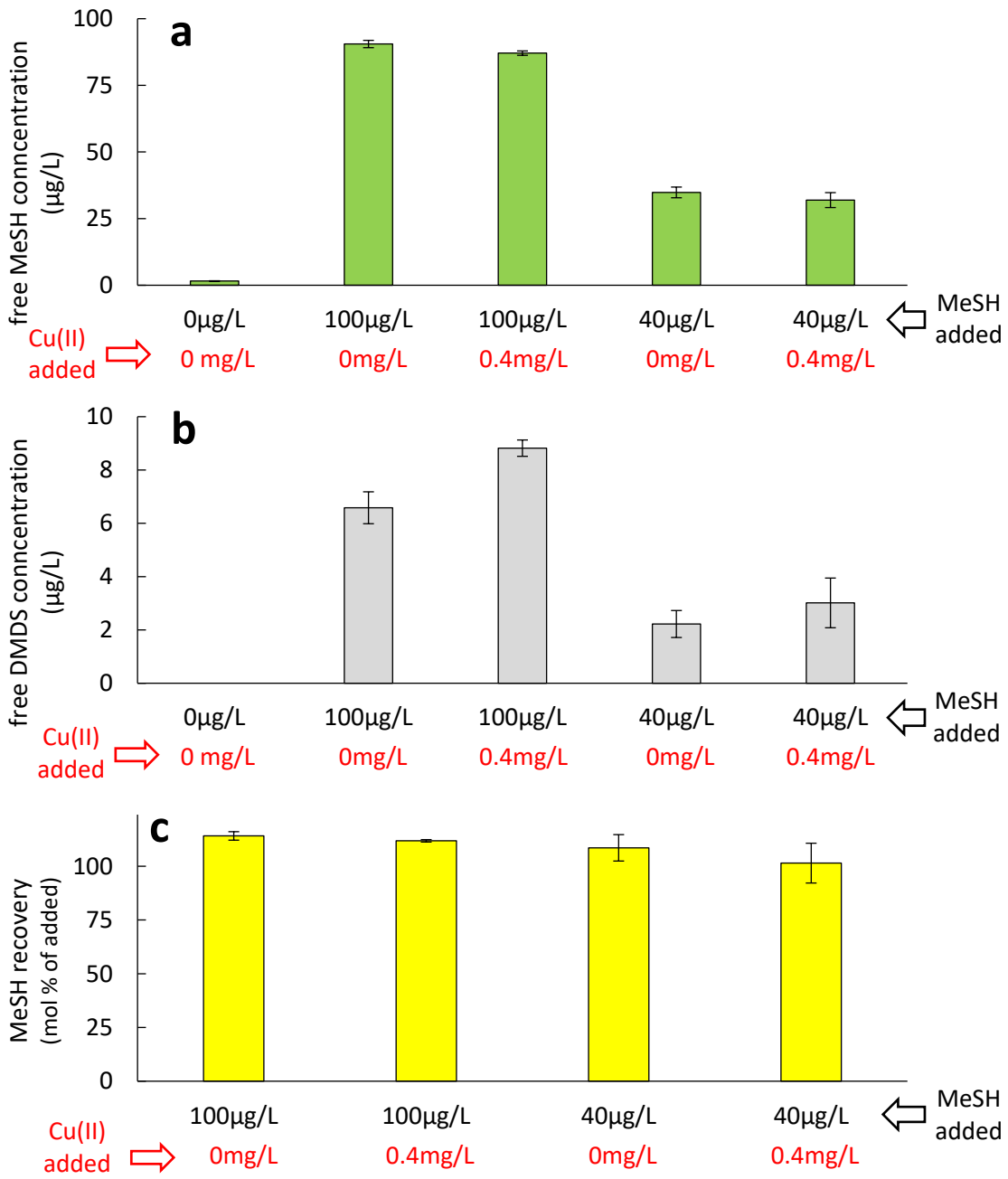


Figure 6.48. The free MeSH concentration (a), free DMDS (b) and recovery of added MeSH (c) in a red wine immediately after addition of MeSH and Cu(II). The recovery of added MeSH was calculated as: ([mol{freeMeSH} + (2 x mol{freeDMDS})]/mol{addedMeSH}) x100.

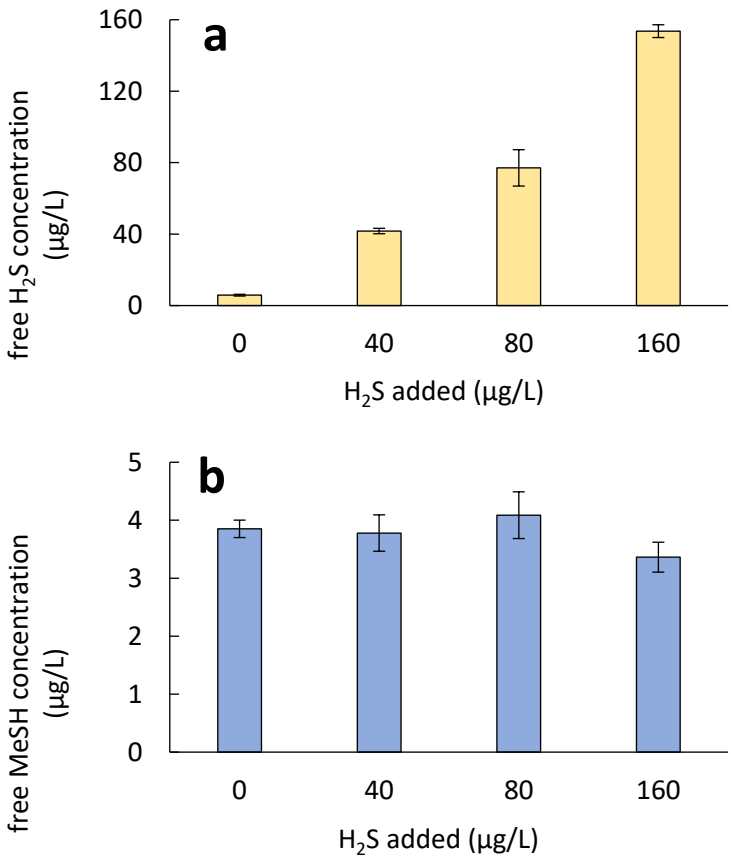


Figure 6.49. The free H_2S and free MeSH concentration in a white wine after the addition of increasing H_2S concentrations to the wine.

6.3.4 Cu fraction impacts on beneficial polyfunctional thiol aroma compounds.

The previous sections of this report (6.3.1 - 6.3.3) showed that specific Cu fractions in wine can inhibit the accumulation of detrimental sulfur-aroma compounds such as free H_2S and free MeSH. The mechanism of inhibition for H_2S involved the formation of copper sulfides, while for MeSH the more important mechanism was oxidation to disulfides. Although the removal of H_2S and MeSH is deemed favourable, wine also contains polyfunctional thiol compounds that contribute desirable varietal aromas (Table 6.7), and the loss of these thiols compounds is considered detrimental. This section of the work was conducted in collaboration with the AWRI (Ms Allie Kulcsar, Ms Lisa Pisaniello and Dr Marlize Bekker).

Studies have already shown that Cu can impact the varietal thiol compounds shown in Table 6.7. Cu applied to grapes as a fungicide led to decreased 3-sulfanylhexanol (3SH) and 4-methyl-4-sulfanylpentan-2-one (4MSP) concentrations in the final wines (Darriet et al. 2001). Later work showed similar effects of Cu(II) on 3SH concentration in wines prepared from Gros Manseng or Colombard grape varieties (Dournes et al. 2023), and it was shown that Cu(II) readily caused the oxidative conversion of 3SH to disulfides (Figure 6.50). Interestingly, if the reduced and oxidised (i.e., disulfide) forms of 3SH (Figure 6.50) were accounted for, then there were higher concentrations of these combined forms in the Gros Manseng wines with higher Cu(II) concentrations in the juice (Dournes et al. 2023). Cu(II) additions to a Sauvignon blanc at bottling induced lower concentrations of 3SH, and the magnitude of decrease remained similar after 6-months of bottle aging in low oxygen conditions (Ugliano et al. 2011).

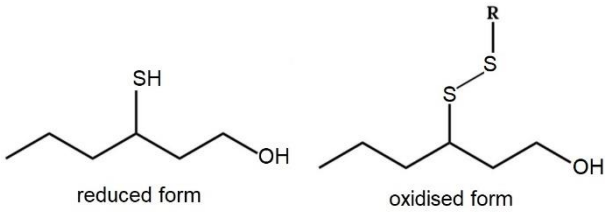
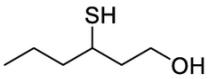
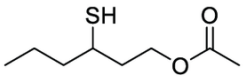
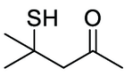


Figure 6.50. The reduced and oxidised form of 3-SH with R being an unidentified carbon chain. Modified from Dournes et al. (2023).

Table 6.7. Polyfunctional thiol aroma compounds in wine and their aroma thresholds.

Compound name (abbreviation)	Structure	Aroma	Aroma threshold^
3-Sulfanylhexanol (3SH)		grapefruit zest, passion fruit	60 ng/L
3-Sulfanylhexyl acetate (3SHA)		boxwood, passionfruit	0.8 ng/L
4-Methyl-4-sulfanylpentan-2-one (4MSP)		boxwood	4.2 ng/L

^ Ribéreau-Gayon et al. 2006.

This section of the report investigates the links between Cu fractions and the loss of varietal thiol aroma compounds induced by Cu(II) addition to wine. Cu(II) additions of 0, 0.15 and 0.30 mg/L were made during the bottling of two Sauvignon Blanc wines onsite at a commercial winery in South Australia. The bottling process was the standard process used by the commercial winery, with low oxygen conditions evidenced by minimal changes in the total sulfur dioxide concentration (i.e., < 10 mg/L) of the wines after 18-months of bottle aging. After bottling, the wines were dispatched to Charles Sturt University (Wagga Wagga) and they were stored in a cellar at 17 ± 1 °C in darkness prior to measurement. At each timepoint, three bottles of each wine were opened for measurement of Cu fractions by bicinchoninic acid (BCA) colorimetry, free

H_2S and MeSH by GC-SCD and the varietal thiol aroma compounds (Table 6.7) by LC-HRMS at AWRI as described within Zhang et al. (2024). The first measurement (designated time point 0 days) was conducted 2-4 weeks after the bottling of the wine.

Table 6.8 shows the Cu concentration in the wines at the first timepoint. Sauvignon blanc 1 had a lower concentration of Cu in the base wine compared to Sauvignon blanc 2, and all wines had concentrations of Cu fraction 1 + 2 above the critical level of 0.02 mg/L (i.e., identified in Section 6.3.2 to inhibit free H_2S accumulation). Only the Sauvignon blanc 1 base wine (i.e., 0 mg/L added Cu(II)) had a Cu fraction 1 concentration below 0.03 mg/L, identified in Section 6.3.2 as the critical concentration to inhibit free MeSH accumulation. The Cu additions at bottling led to increases in all Cu fractions but with Cu fraction 2 having the largest increase.

Table 6.8. Cu and varietal thiol concentrations in the two Sauvignon blanc wines at the 0-day timepoint measurement.

Cu added at bottling	Total Cu (mg/L)	Cu fraction 1 (mg/L)	Cu fraction 2 (mg/L)	Cu fraction 3 (mg/L)	3SH (ng/L)	3SHA (ng/L)	4MSP (ng/L)
Sauvignon blanc 1							
0 mg/L	0.081 ± 0.001^c	0.018 ± 0.001^c	0.050 ± 0.001^c	0.013 ± 0.001^c	520 ± 10^a	41 ± 2^a	89 ± 2^a
0.15 mg/L	0.228 ± 0.002^b	0.044 ± 0.002^b	0.153 ± 0.001^b	0.031 ± 0.002^b	426 ± 8^b	33 ± 3^b	72 ± 1^b
0.30 mg/L	0.371 ± 0.002^a	0.079 ± 0.005^a	0.240 ± 0.006^a	0.052 ± 0.002^a	320 ± 40^c	25 ± 2^c	54 ± 6^c
Sauvignon blanc 2							
0 mg/L	0.234 ±0.004^c	0.054 ±0.008^c	0.122 ±0.006^c	0.058 ±0.004^c	430 ± 22^a	20 ± 1^a	41 ± 1^a
0.15 mg/L	0.402 ±0.00 ^{b}	0.122 ±0.004^b	0.202 ±0.004^b	0.078 ±0.003^b	300 ± 20^b	14 ± 1^b	31 ± 1^b
0.30 mg/L	0.540 ±0.008^a	0.19 ±0.03^a	0.25 ±0.02^a	0.097 ±0.008^a	160 ± 20^c	7 ± 1^c	14 ± 1^c

^ 2-furylmethanethiol (aroma threshold 0.4 ng/L) and phenylmethanethiol (aroma threshold 0.3 ng/L) associated with struck flint were also measured but their concentrations were low and were only marginally above their aroma thresholds without any significant influence of Cu (data not shown). Values with different subscripts in the same column and for the same wine are significantly different using one way ANOVA with Tukey HSD / Tukey Kramer ($p < 0.05$).

At time point 0 (Table 6.8) there was a signficant impact of Cu(II) on all the varietal thiol aroma compounds. For example, for Sauvignon blanc 2 with 0.15 and 0.30 mg/L added Cu(II) the concentrations of 3SH were 36 and 69 % lower, respectively, than in the 0 mg/L sample. This outcome is consistent with a previous study (Ugliano et al. 2011) showing the impact of Cu(II) addition at bottling on 3SH concentrations measured 24 hours after bottling. Table 6.8 also shows a similar effect of Cu(II) addition on the concentrations of 3SHA and 4MSP as observed for 3SH. Despite added Cu(II) reducing the volatile thiol concentrations, all wines had concentrations of 3SH, 3SHA and 4MSP that were still in excess of their aroma thresholds (Table 6.7) by a factor of at least 2-3.

Figure 6.51 shows the changes in Cu fractions in the Sauvignon blanc 2 wine that had 0.3 mg/L Cu(II) added at bottling. Similar plots for all the other wines can be viewed in Appendix 21 and 22. During the bottle aging, all wines showed similar trends in the changes of the Cu fractions, that is, decreases in Cu fractions 1 and 2, and an increase in Cu fraction 3 during bottle aging (Figure 6.51). These changes will be explored further in a later section of this report (Section 6.5.3), but demonstrate that Cu can readily acquire sulfide during bottle aging, which results in the conversion of Cu(II) organic acid complexes (i.e., Cu fraction 1) and Cu(I)-thiol complexes (i.e., Cu fraction 2) to copper sulfides.

Given the importance of the Cu fraction 1 on inhibiting the accumulation of the detrimental thiol compound (i.e., free MeSH), its concentrations in the two Sauvignon blanc wines are shown in Figure 6.52. Also highlighted in Figure 6.52 (i.e., red dashed line) is the 0.03 mg/L concentration of Cu fraction 1 that was found to inhibit MeSH in white wines. The Sauvignon blanc 1 with 0 mg/L Cu(II) added at bottling was below 0.03 mg/L Cu fraction 1 throughout the entire 18-month bottle aging period, while the samples with added Cu required 8-months to fall below this threshold. In Sauvignon blanc 2, the '0 mg/L Cu(II)' sample was below the critical concentration after 5-months, while the samples with Cu(II) addition (i.e., both 0.15 and 0.30 mg/L) required 12-months to fall below the critical concentration.

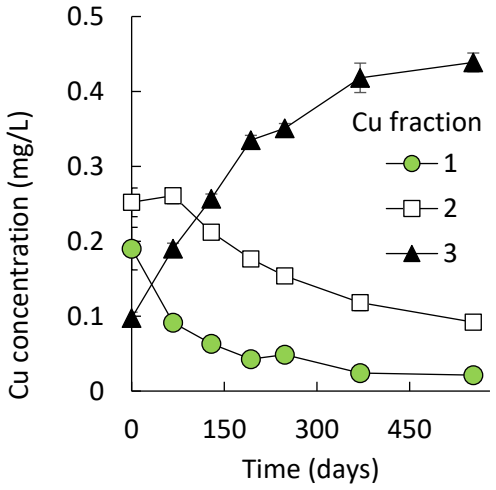


Figure 6.51. The concentration of Cu fractions during the bottle aging of Sauvignon blanc 2. This wine had 0.30 mg/L Cu(II) added at bottling. The error bars indicate the standard deviation of triplicate samples. See Appendix 21 and 22 for the individual graphs of each sample in Table 6.8.

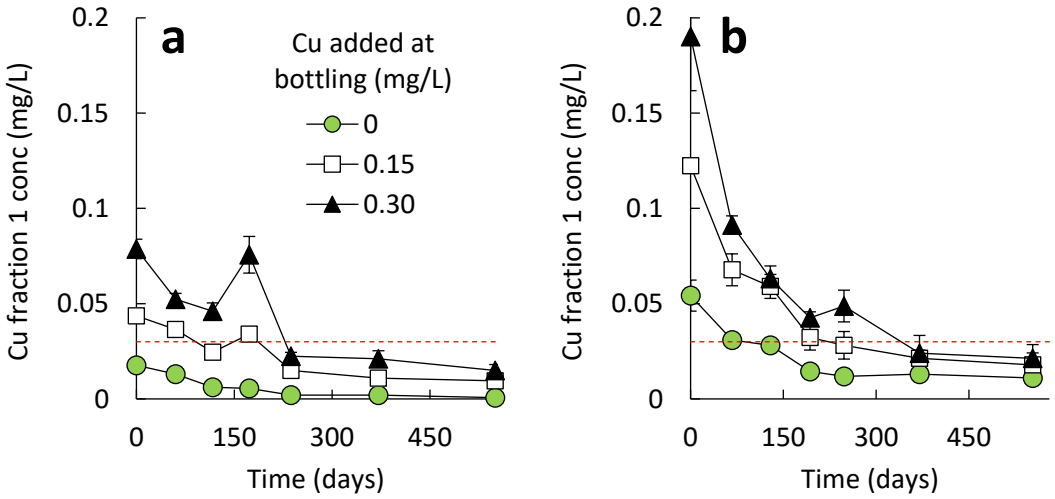


Figure 6.52. The concentrations of Cu fraction 1 in Sauvignon blanc 1 (a) and Sauvignon blanc 2 (b) during bottle aging over 18 months and with different Cu(II) addition rates at bottling. The red dashed wine highlights the critical concentration Cu fraction 1 of 0.03 mg/L.

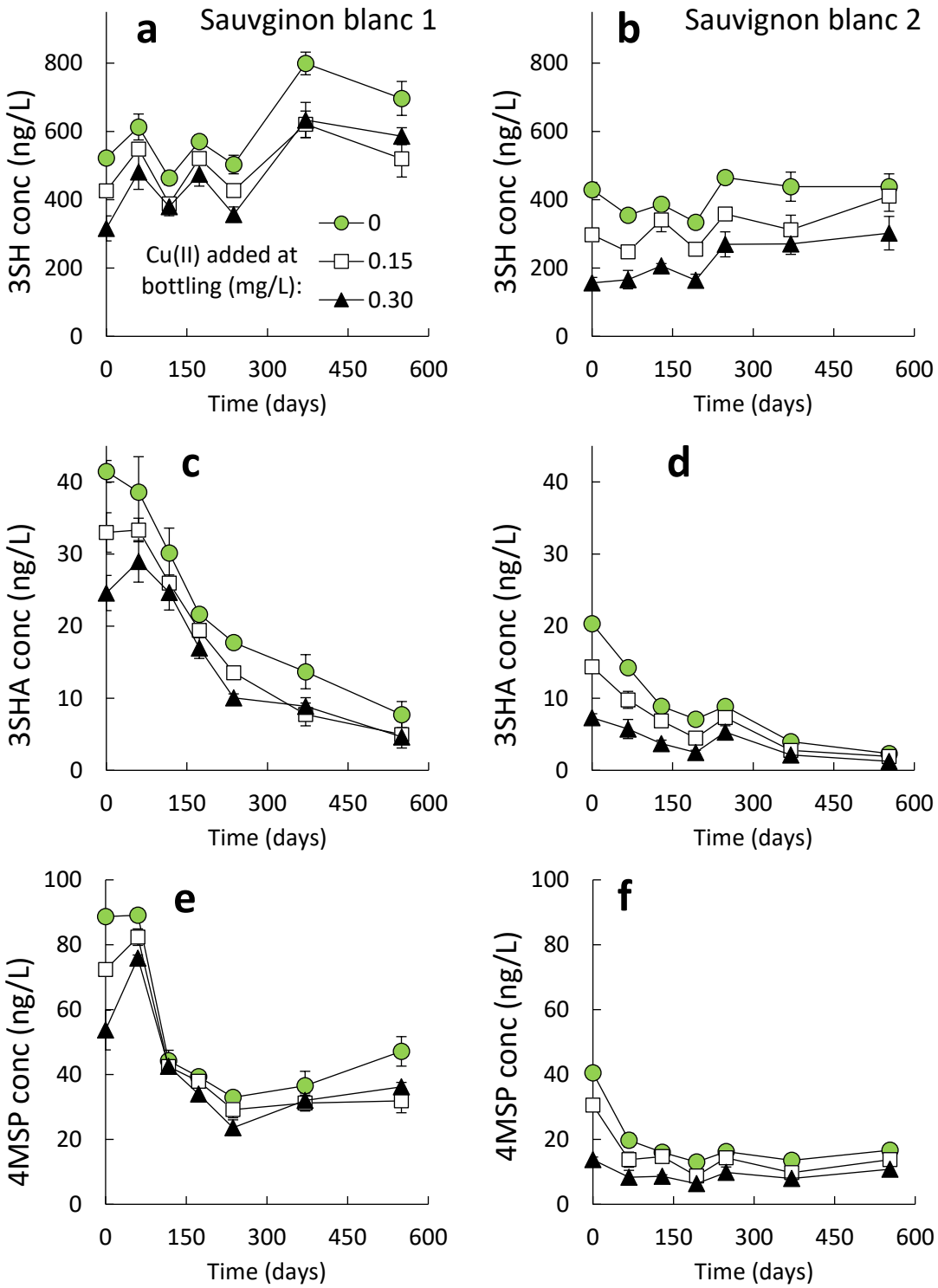


Figure 6.53. The concentrations of 3-sulfanylhexanol (a,b), 3-sulfanylhexylacetate (c,d) and 4-methyl-4-sulfanylpentan-2-one (e,f) in Sauvignon blanc 1 (a,c,e) and Sauvignon blanc 2 (b,d,f) during bottle aging with 0, 0.15 and 0.30 mg/L Cu(II) added at bottling.

During bottling aging all varietal thiol aroma compounds had lower concentrations in Sauvignon blanc 2 compared to Sauvignon blanc 1. For 3SH, the differences in concentration induced by Cu(II) addition remained reasonably constant throughout aging (Figure 6.53a,b). Although, there did seem to be a larger overall increase in the 3SH concentration over the 18-month period for the wines treated with 0.3 mg/L Cu(II) (i.e., black triangles, Figure 6.53a,b) compared to those with the 0 mg/L treatment (i.e., green circles). There was no obvious link between the changes in Cu fraction 1 (Figure 6.52) and the 3SH concentration losses induced by Cu(II) (Figure 6.53a,b).

3SHA and 4MSP had an overall decrease in concentration during the 18 month storage period (Figure 6.53c-f) which was in contrast to the concentration profile oberved for 3SH. For 3SHA and 4MSP the impact of Cu(II) on their concentration was largest at the first measurement time point and became progressively less apparent with bottle aging. The decreased influence of Cu on 3SHA and 4MSP during bottle aging does have parallels with the changes in Cu fraction 1 (Figure 6.52) but further evidence would be required for the the link to be proven causative.

In contrast to the varietal thiol compounds, MeSH had a clear increase in concentration during 12-months of bottle aging (Figure 6.54). There was no significant difference ($p<0.05$) on the concentration of MeSH with Cu treatments for any individual timepoint, although there was a trend to higher MeSH concentrations in the sample without added Cu(II). The concentration of free MeSH only exceeds the aroma threshold once the Cu fraction 1 concentration had fallen below 0.03 mg/L in any sample.

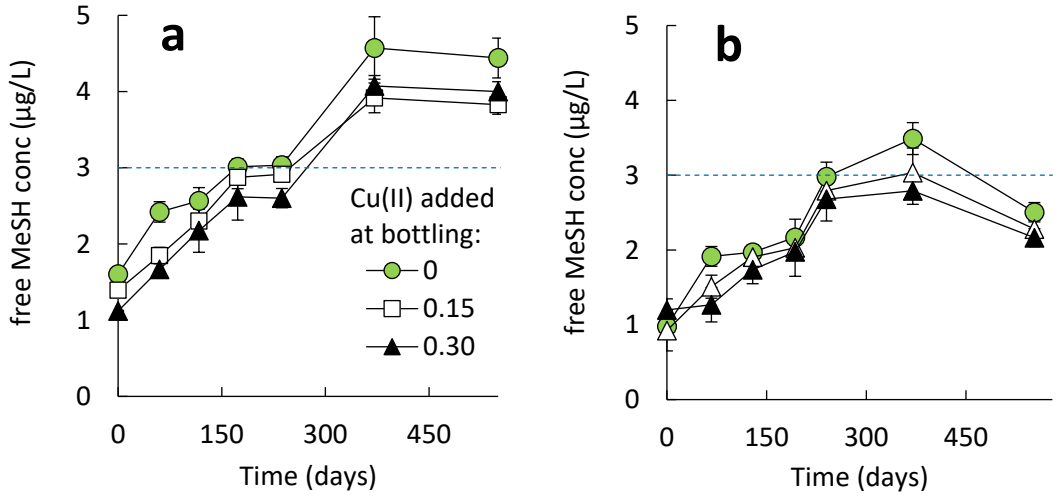


Figure 6.54. The concentration of free MeSH in Sauvignon blanc 1 (a) and Sauvignon blanc 2 (b). The blue dashed line represents the typical aroma threshold for MeSH in wine. The error bars represent the standard deviation of triplicate samples

The combined concentrations of Cu fraction 1 and 2, known for inhibiting hydrogen sulfide, throughout the bottle aging of the two Sauvignon blanc wines is shown in Figure 6.55. In this case, only the 0 mg/L sample of Sauvignon blanc 1 fell below the critical concentration of 0.02 mg/L and this was at 8-months after bottling. It was only in this same wine that the concentration of free H_2S exceeded the aroma threshold and this was after 18-months of bottle aging (Figure 6.56). Therefore, there was a lag period of around 10-months after the decrease of Cu fraction 1 and 2 below the concentration of 0.02 mg/L and the emergence of > 1 µg/L free H_2S. The concentration of free H_2S in Sauvignon blanc 2 remained at trace concentrations that are well below the aroma threshold (data not shown) which was consistent with its high Cu fraction 1 and 2 concentrations (Figure 6.55b).

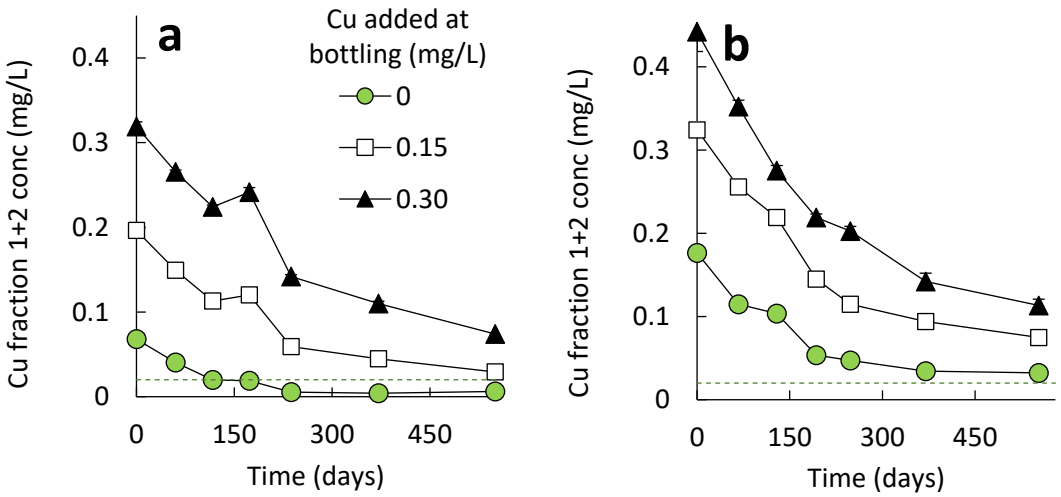


Figure 6.55. The combined concentration of Cu fraction 1 and 2 in Sauvignon blanc 1 (a) and Sauvignon blanc 2 (b) during bottle aging over 18 months and with different Cu(II) addition rates at bottling. The green dashed line highlights the critical combined concentration Cu fraction 1 and 2 of 0.02 mg/L.

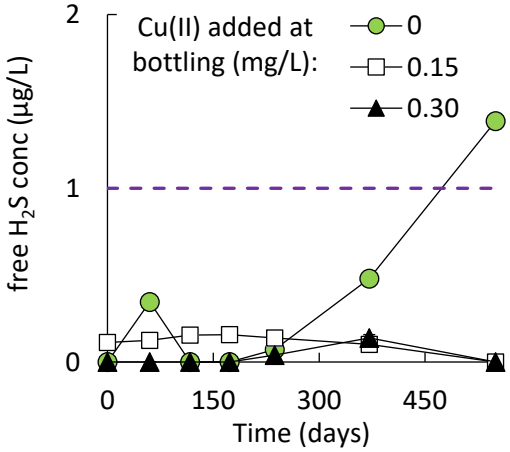


Figure 6.56. The concentration of free H_2S in Sauvignon blanc 1. The purple dashed line represents the typical aroma threshold quoted for H_2S in wine. The error bars represent the standard deviation of triplicate samples.

Sensory analysis was conducted on the wines after 18-months of storage using the Pivot® Profile sensory methodology (Pearson et al. 2020). In addition, sensory analysis was also conducted on freshly opened bottles of control wine (i.e., stored for 18 months without Cu(II) addition at bottling) immediately after the addition of 0.15 and 0.30 mg/L Cu(II). These samples were labelled as *Fresh_0.15Cu* and *Fresh_0.30Cu*, respectively. The sensory panellists characterised the wines using 12 descriptors for Sauvignon Blanc 1 and 13 descriptors for Sauvignon Blanc 2. Correspondence analysis (CA) was utilised to analyse these data sets and produce Figure 6.57a and 6.57b.

For Sauvignon Blanc 1, 63.3 % of variance is explained by F1 and 31.5 % by F2. F1 is predominantly driven by *tropical/floral* and *fruit/citrus* descriptors, and F2 by *grassy/capsicum*, *cabbage* and *H_2S/sewage* descriptors. Separation of the wine with and without Cu addition at bottling (i.e., 0.15_Cu and 0.30_Cu versus 0_Cu) was more driven by F2 with the 0_Cu sample associated with the *grassy/capsicum*, *cabbage* and *H_2S/sewage* descriptors. This is consistent with the higher concentrations of free H_2S and methanethiol measured in the wine without Cu added at bottling (Figure 6.56 and 6.54a). Interestingly, the 0_Cu sample was not more closely associated with the *tropical/floral* or *fruit/citrus* descriptors than the Cu-treated samples, despite having higher concentrations of 3SH, 3SHA and 4MSP (Figure 6.53a,c,e). This was perhaps a consequence of the elevated free H_2S and MeSH concentrations in the 0 Cu sample masking the aroma contributions from the varietal thiol compounds.

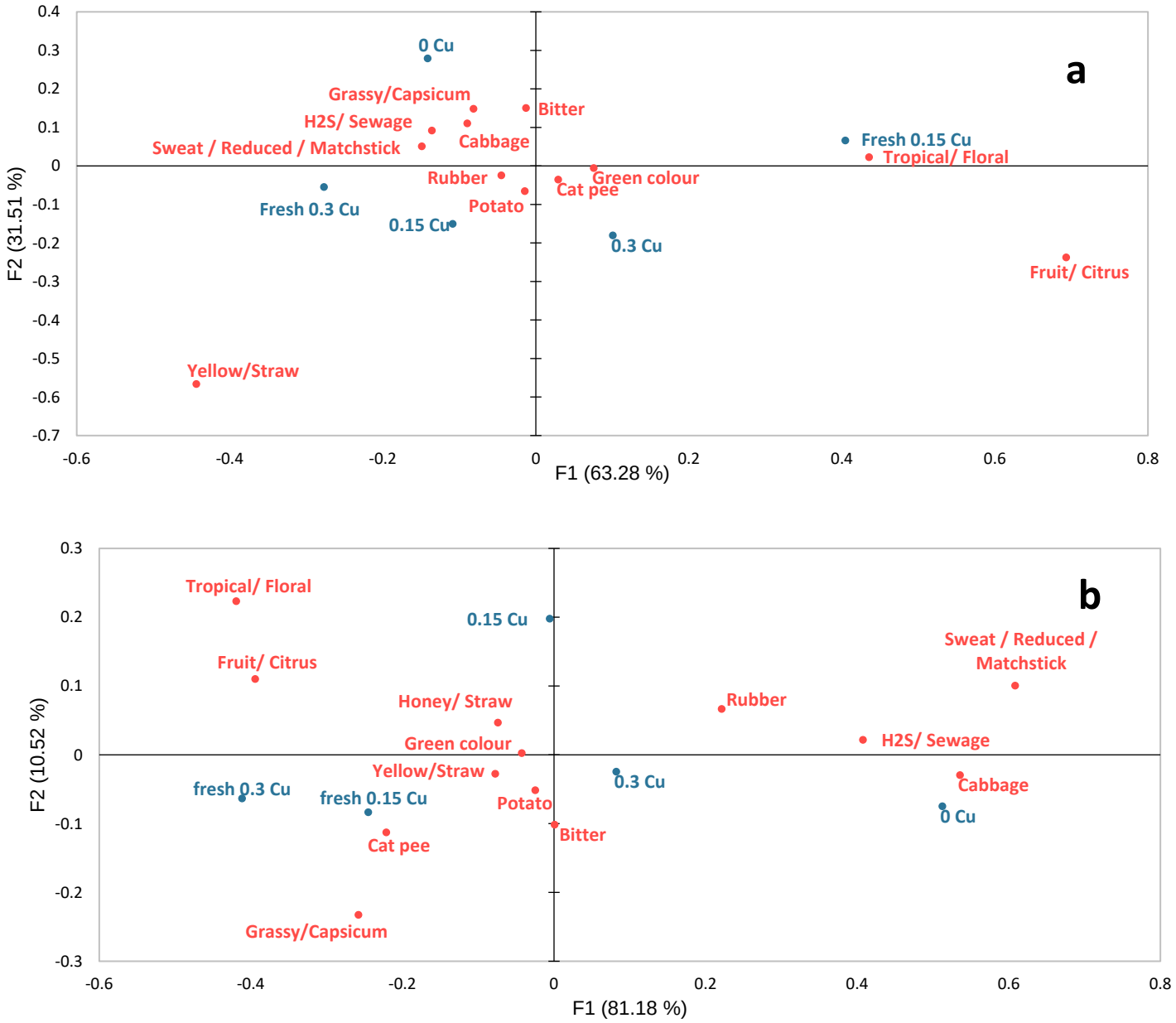


Figure 6.57. Correspondence analysis (CA) biplot of Sauvignon Blanc 1 (a) and Sauvignon Blanc 2 (b) after 18-months of storage. The CA was generated using Pivot® Profile.

Indeed, when sensory analysis was conducted on the 0_Cu sample immediately after addition of 0.15 mg/L Cu(II) (i.e., *Fresh_0.15Cu*) the wine position was driven by the *tropical/floral* descriptors and was distant from the *grassy/capsicum*, *cabbage* and *H_2S/sewage* descriptors. The absence of free H_2S in the Fresh_0.15Cu sample was confirmed by GC-SCD (data not shown). When the Cu(II) addition was made to another fresh bottle of the 0_Cu sample at a higher concentration (i.e., *Fresh_0.30Cu*), the alignment with the *tropical/floral* and *fruit/citrus* descriptors was no longer apparent and possibly due to greater oxidative loss of the varietal thiol compounds.

For Sauvignon Blanc 2, the correspondence analysis shows that 81.2 % of the variance is explained by F1 and 10.5 % by F2. In this case, F1 was driven by *H_2S/sewage*, *cabbage* and *sweat/reduced/matchstick* descriptors, and F2 by *tropical/floral* descriptors. Intriguingly, despite the 0_Cu sample having no measurable free H_2S (data not shown) and a free MeSH concentration similar to the Cu(II)-treated samples (Figure 6.53b,d,f), this wine was located further to the right-hand side of F1 than any other sample. Cu(II) treatment, either at bottling or immediately prior to the sensory analysis, caused the samples to be located in the opposite direction along F1. The sample associated most with the *tropical/floral* descriptor was the wine treated with 0.15 mg/L Cu(II) at bottling, despite it having similar 3SH, 3SHA and 4MSP concentrations as the 0_Cu sample (Figure 6.53b,d,f). It is hard to explain why the 0_Cu sample for this wine aligns so strongly with the *H_2S/sewage*, *cabbage* and *sweat/reduced/matchstick* descriptors despite having low concentrations of free H_2S and methanethiol, however wines with Cu(II) addition are clearly positioned away from these negative descriptors. Further research is required to ascertain whether the perceived reductive characters in a wine may be synergistically enhanced by a combination of different sulfhydryl aroma compounds.

In summary, the varietal thiol compounds are sensitive to Cu in wine. The impact of Cu(II) on the varietal thiol compounds was significant at the first timepoint measurement (i.e., 2-4 weeks after the addition of Cu(II)). The influence of Cu(II) on 3SH remained evident throughout the storage period of 18-months, while for 3SHA and 4MSP there was a diminishing influence of Cu(II) during storage. There was no obvious link between 3SH concentration and Cu fractions. The concentration decreases for 3SHA and 4MSP during aging mirrored the profiles for Cu fraction 1 but a causative effect of Cu fraction 1 on 3SHA and 4MSP was not demonstrated. The only wine to accumulate free H_2S above 1 µg/L at 18-months was the only wine (Sauvignon Blanc 1) with less than 0.02 mg/L combined Cu fraction 1 and 2 at this same timepoint. Interestingly, despite the observed impact of Cu(II) addition on the concentration of varietal thiol compounds, Cu(II) addition did not always lead to wines being less associated with the aroma descriptors associated with these compounds. In fact, in some cases, Cu(II) addition led to wines being perceived to have more *tropical* and *fruit* characters, possibly due to the ability of Cu(II) to the remove free H_2S, and other potential masking compounds. Preventative Cu(II) additions should be carefully considered when made to wines in which varietal thiol aromas are desirable (e.g.., Sauvignon Blanc), but some decrease in the concentration of varietal thiol aroma compounds may be worthwhile if excessive accumulation of masking compounds, such as fee H_2S and MeSH, is prevented.

6.4 The impact of Cu speciation on the amount of sulfur dioxide consumed by oxygen.

A critical antioxidant role of sulfur dioxide in wine is to remove products formed after oxygen reacts with wine phenolic compounds (Figure 6.58). According to theory, for each oxygen molecule that reacts, one molecule of sulfur dioxide is required to scavenge the hydrogen peroxide (pathway 1 in Figure 6.58) and another is required to scavenge an *ortho*-quinone (pathway 2 in Figure 6.58). Both reactions of sulfur dioxide are irreversible, meaning that they decrease the total sulfur dioxide concentration in the wine. Consequently, for each mmol/L of oxygen consumed there will be a loss of around 2 mmol/L of total sulfur dioxide, or in units more typical of winemaking, for each mg/L of oxygen consumed there will be a loss of around 4 mg/L total sulfur dioxide.

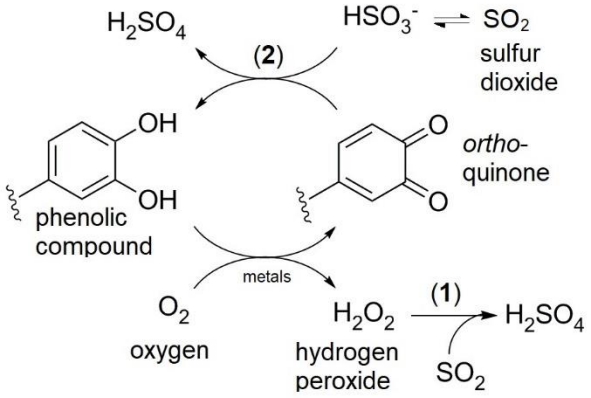


Figure 6.58. The metal catalysed reaction of molecular oxygen with a wine phenolic compound to afford hydrogen peroxide and an *ortho*-quinone. One molecule of sulfur dioxide reacts irreversibly with hydrogen peroxide (pathway **1**) and another, in the equilibrium form of hydrogen sulfite, reacts irreversibly with the *ortho*-quinone (pathway **2**).

An alternate mechanism presents itself in white wines containing ascorbic acid (Figure 6.59), whereby oxygen reacts with ascorbic acid to produce hydrogen peroxide and dehydroascorbic acid. In this case, sulfur dioxide will scavenge the hydrogen peroxide irreversibly (pathway 1 in Figure 6.59), but react with dehydroascorbic acid in a reversible addition reaction (pathway 2 in Figure 6.59). This means that the reaction with hydrogen peroxide will decrease total sulfur dioxide concentration, while the reaction with dehydroascorbic acid will not. The latter reaction will only decrease free sulfur dioxide concentration. Consequently, for each mmol/L of oxygen consumed in a white wine with ascorbic acid, there will be a loss of 1 mmol/L of total sulfur dioxide, which is equivalent to a 1 mg/L of oxygen inducing loss of 2 mg/L total sulfur dioxide. That is, for the same amount of oxygen, the loss of total sulfur dioxide from a white wine with ascorbic acid will be half as much as the same white wine without ascorbic acid.

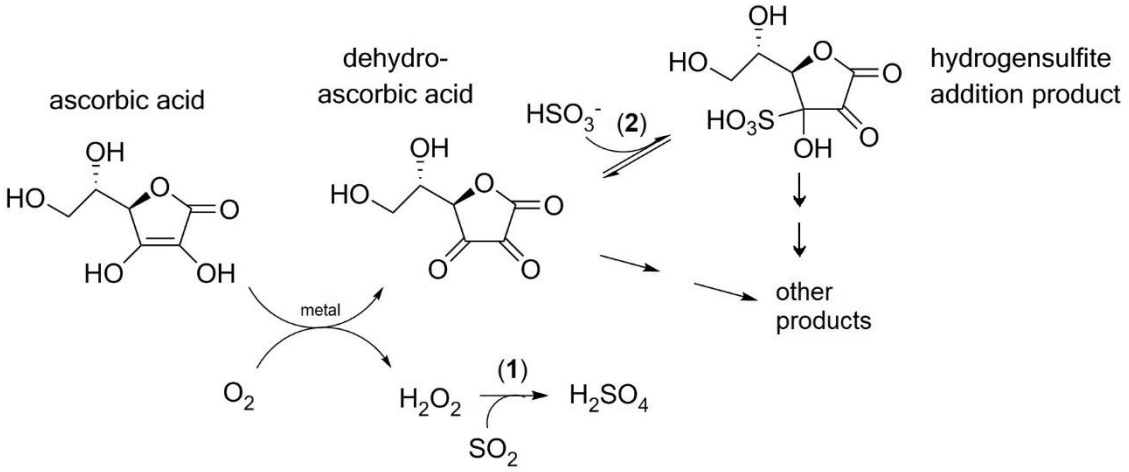


Figure 6.59. The metal catalysed reaction of molecular oxygen with ascorbic acid to afford hydrogen peroxide and dehydroascorbic acid. One molecule of sulfur dioxide reacts irreversibly with hydrogen peroxide (pathway **1**) and another reacts reversibly with dehydroascorbic acid (pathway **2**).

Both oxidation mechanisms (Figures 6.58 and 6.59) show a reliance on metal ions to catalyse the reaction of oxygen with phenolic compounds or ascorbic acid. When Cu is acting as a catalyst for the reaction between oxygen and ascorbic acid (Figure 6.59), its complexing ligands can impact the speed of the oxidation reaction. That is, Cu(II)-organic acid and Cu(I)-thiol complexes were efficient at catalysing the reaction between molecular oxygen and ascorbic acid, while copper sulfides were not. The ligands of Cu were less critical when Cu was catalysing the reactions between oxygen and phenolic compounds (Figure 6.58) rather than between oxygen and ascorbic acid (Figure 6.59).

Despite the theory described already, the amount of sulfur dioxide consumed in wine per mmol/L of oxygen can be quite variable (Waterhouse et al. 2016a). This variability in sulfur dioxide loss during wine oxidation means that certain wines may be more prone to oxidative spoilage than others. The lack of knowledge in this area limits the control a winemaker has on the ability to predict the aging of wine in bottle and to potentially lower the amounts of sulfur dioxide used in wine. The following section examines the influence of Cu fractions on the sulfur dioxide to oxygen consumption ratios.

6.4.1 White wine with ascorbic acid: Cu fractions versus SO_2 to O_2 consumption ratios.

The experiment was conducted on a current vintage Chardonnay wine, and the bulk wine was adjusted to a total Cu concentration of 0.65 mg/L. The wine was sparged with compressed air vigorously for 10 minutes to provide a dissolved oxygen concentration of 8 mg/L. Ascorbic acid was added at a concentration of 100 mg/L and potassium metabisulfite was added to afford a free sulfur dioxide concentration of around 50 mg/L. This high free sulfur dioxide concentration was necessary to match the saturated oxygen conditions to be used in the experiment. During bottling of the wine in 187 mL bottles, H_2S (added as Na_2S) was adjusted to three different concentrations. The first set of wines had no addition, the second had a 'medium' level addition

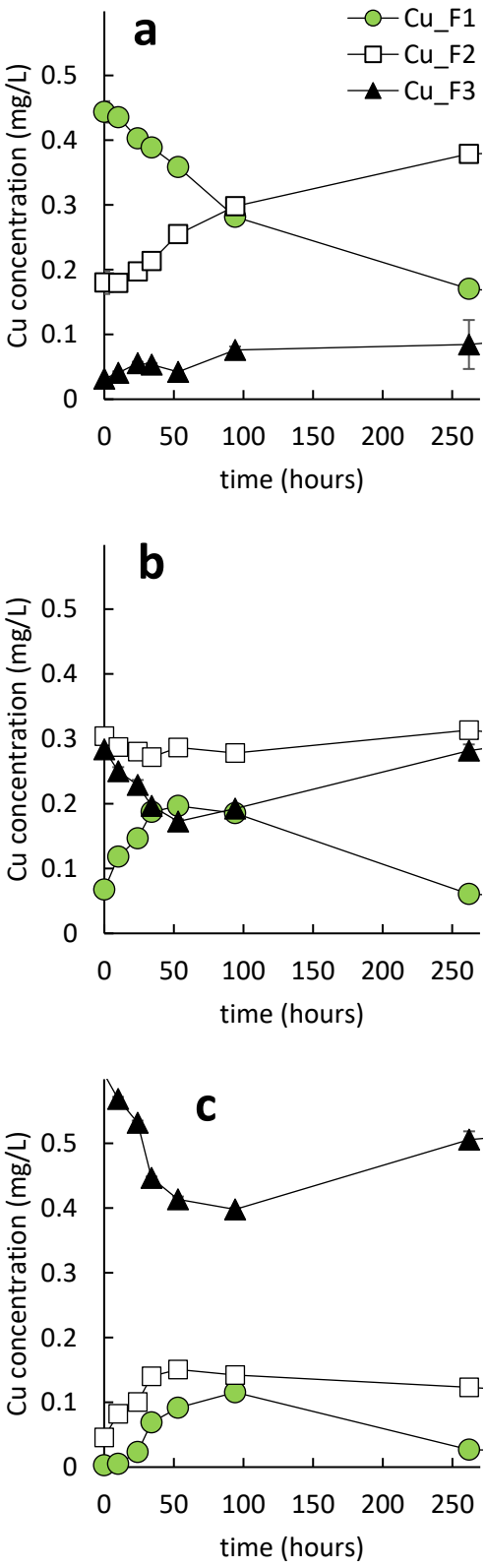


Figure 6.60. The concentration of Cu fractions in samples with different levels of added Na_2S. The H_2S was adjusted to three different mole ratios (i.e., H_2S to added Cu(II)): 0 : 1 (a), 0.8 to 1 (b), and 2 : 1 (c).

of H_2S (i.e., mole ratio of 0.8:1 for H_2S to added Cu(II)), and the third was 'high' level addition of H_2S (i.e., mole ratio of 2:1 for H_2S to added Cu(II)). This provided three distinct Cu fraction profiles for each of the white wine (see timepoint 0 hours in Figure 6.60). The samples were coded with respect to the ratio of Na_2S to added Cu(II) in the wines:

- **0 to 1** (dominated by Cu Fraction 1 at bottling),
- **0.8 to 1** (dominated by Cu Fraction 2 and 3 at bottling), and
- **2 to 1** (dominated by Cu fraction 3 at bottling).

The Cu fractions were measured by BCA colorimetry. The concentrations of Cu fractions in the different samples changed during the reaction of oxygen in the wine (Figure 6.60). Throughout the storage period, the **0 to 1** sample was dominated by either Cu fraction 1 or 2 depending on the timepoint of analysis. In contrast, the **0.8 to 1** sample had appreciable concentrations of all three Cu fractions, while the **2 to 1** sample was dominated by Cu fraction 3 throughout the experiment.

All measurements were conducted on triplicate sacrificial samples during 0 – 2037 hours of storage in darkness at 17 °C. Throughout the experiment the oxygen concentration was measured by luminescence sensors and ascorbic acid by DCPIP titration. The concentrations of free and total sulfur dioxide were measured by the traditional aspiration-oxidation method and also by colorimetry utilising an automated analyser.

The saturated oxygen concentrations fell to 0 mg/L in all samples after 250 hours (Figure 6.61a). Afterwards (i.e., 250 – 2038 hours) there was no increase in dissolved oxygen implying that the bottle closures were sufficient to prevent accumulation of dissolved oxygen (Appendix 23). As expected from previous work (Kontoudakis and Clark, 2020), the loss of oxygen was faster in the samples with higher Cu fraction 1 and 2 concentrations (i.e., treatment 0 to 1 and 0.8 to 1) and slowest for the sample dominated by Cu fraction 3 (i.e., treatment 2 to 1). This pattern also held for the relative decrease in ascorbic acid, free sulfur dioxide and total sulfur dioxide concentration (Figure 6.61b-d). The measurement of sulfur dioxide using the automated analyser (Appendix 24) and the oxidation-aspiration method (Figure 6.61c and 6.61d) showed similar results to each other. The mole consumption ratios were calculated after the amount of total sulfur dioxide lost in the samples became significant, which was after 34 to 53 hours depending on the specific samples.

Table 6.9 shows the overall mole consumption ratios calculated throughout the experiment, while Figure 6.62 and Appendix 25 show the ratios calculated at individual timepoints for the aspiration-oxidation and automated analyser measures of sulfur dioxide, respectively. The ratios matched the mechanistic theory for the reaction of oxygen with ascorbic acid and sulfur dioxide. The ratio between ascorbic acid to oxygen consumption was close to 1:1, as was the consumption ratio for total sulfur dioxide to oxygen. The ratio for free sulfur dioxide to oxygen was marginally higher than unity (i.e., 1.2:1), implying that only a small amount of sulfur dioxide became bound to dehydroascorbic acid or its degradation products. Importantly, there was no significant difference amongst the consumption ratios for the samples of different Cu fraction profiles (Figure 6.62, Table 6.9). Therefore, despite the rate of oxygen reaction being highly sensitive to the Cu profile in the wine, the latter did not influence the amount of sulfur dioxide consumed for a given amount of oxygen and ascorbic acid.

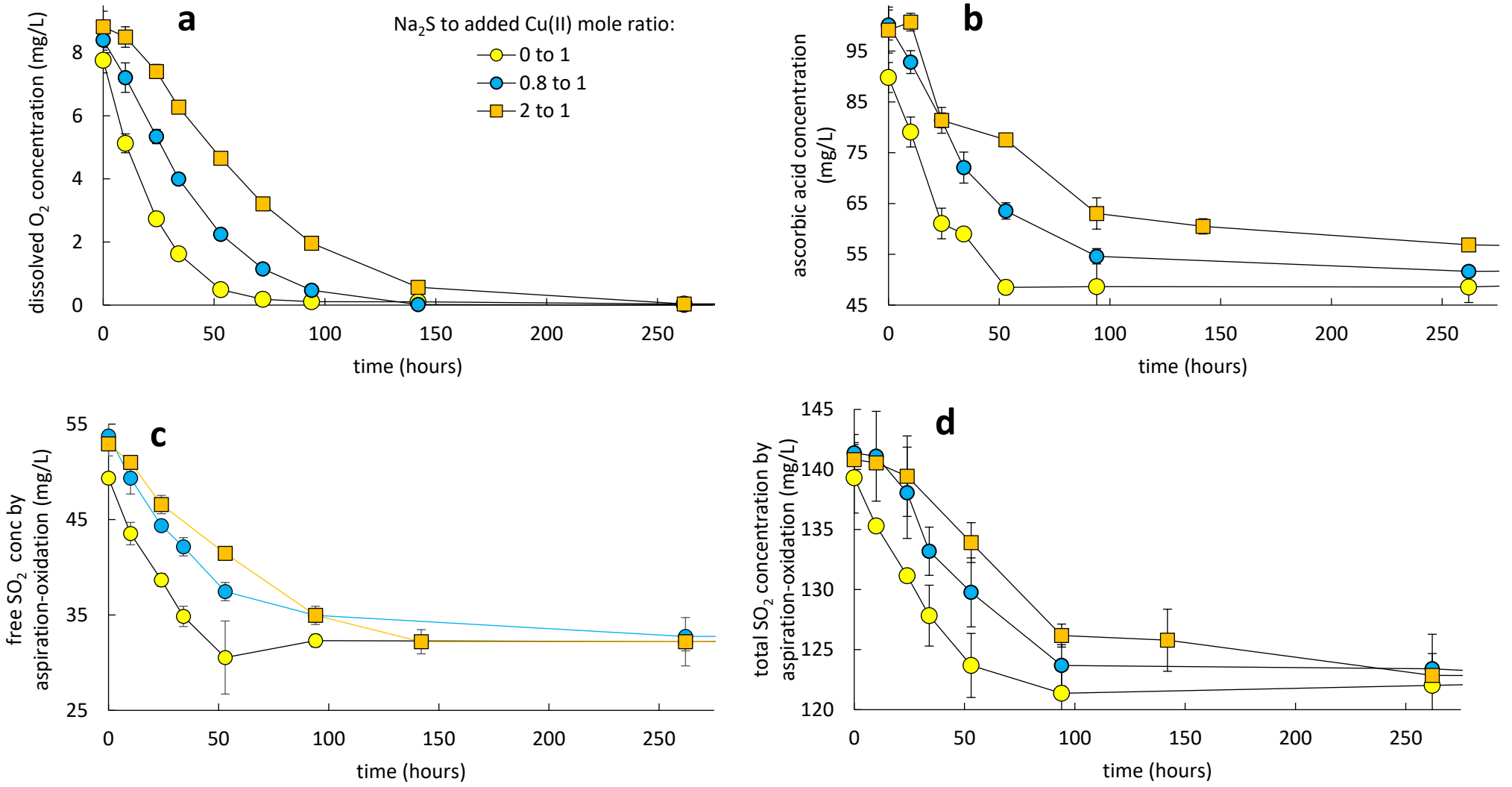


Figure 6.61. The concentrations of oxygen (a), ascorbic acid (b), free sulfur dioxide (c) and total sulfur dioxide (d) in the Chardonnay samples throughout the first 262 hours of the storage period.

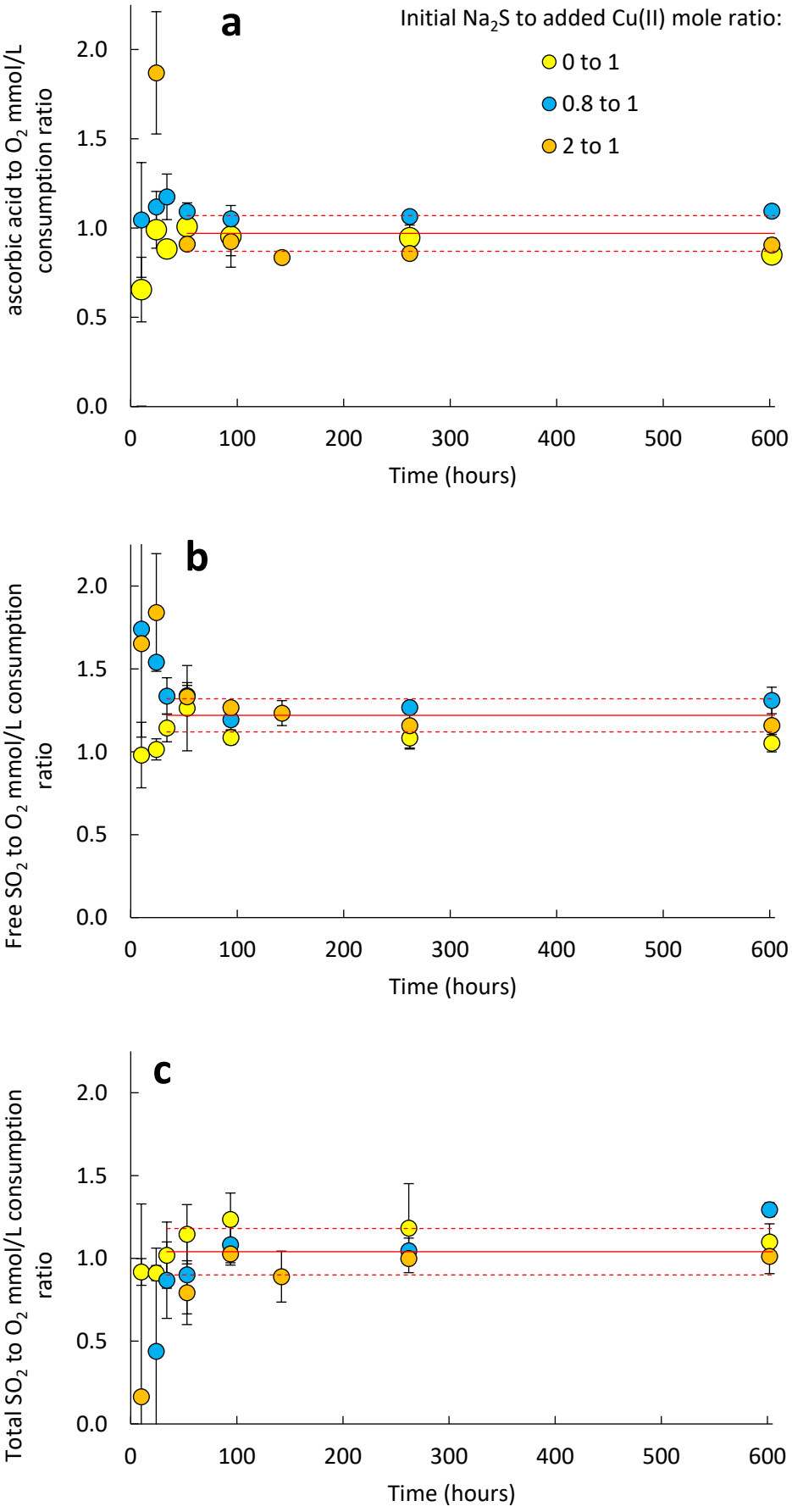


Figure 6.62. The mole consumption ratios for ascorbic acid to oxygen (a), free sulfur dioxide to oxygen (b) and total sulfur dioxide to oxygen (c). The solid red lines indicate the average consumption ratios from 34 to 600 hours, and the dashed lines indicate the standard deviation. The sulfur dioxide concentrations were measured by the aspiration-oxidation method.

Table 6.9. The mole consumption ratios for ascorbic acid, free sulfur dioxide or total sulfur dioxide to oxygen in white wine containing ascorbic acid.

mole consumption ratio*	SO_2 method	Sample (Initial Na_2S to added Cu(II) mole ratio)			Average
		0 to 1	0.8 to 1	2 to 1	
ascorbic acid : oxygen	n/a	0.93 ± 0.06	1.10 ± 0.05	0.89 ± 0.04	1.0 ± 0.1
free SO_2 : oxygen	asp.-ox.	1.13 ± 0.08	1.29 ± 0.06	1.23 ± 0.07	1.2 ± 0.1
	auto anal.	1.11 ± 0.09	1.28 ± 0.06	1.3 ± 0.2	1.3 ± 0.2
total SO_2 : oxygen	asp.-ox.	1.14 ± 0.08	1.0 ± 0.2	0.9 ± 0.1	1.0 ± 0.1
	auto anal.	1.0 ± 0.2	1.2 ± 0.1	1.0 ± 0.2	1.1 ± 0.2

* The mole consumption ratios were calculated from 34 to 602 hours apart from the SO_2 ratios for the automated analyser. Due to a delayed loss of SO_2 when measured by the automated analyser, the ratios were measured over the period of 53-602 hours and 94-602 hours for the free SO_2 and total SO_2 ratios, respectively.

6.4.2 Wines without added ascorbic acid: Cu speciation impacts on SO_2 to O_2 consumption.

The experiment outlined in the previous section was repeated on the Chardonnay wine but this time without ascorbic acid in the wine. The Cu concentration of the wine was adjusted to 0.65 mg/L and Na_2S added to afford the three mole ratios for Na_2S to added Cu(II), that is, ratios of 0 to 1, 0.8 to 1 and 2 to 1. The initial distribution of Cu fraction in each of the wines can be viewed at time point 0 in Figure 6.63. The dominating fractions at the start of the experiment was Cu fraction 1 in the 0 to 1 sample (Figure 6.63a), Cu fractions 1 and 2 in the 0.8 to 1 sample (Figure 6.63b) and Cu fraction 3 in the 2 to 1 sample (Figure 6.63c).

As the oxidation reactions commenced there was a rapid change in the Cu fractions (Figure 6.63) that was not evident when ascorbic acid had been in the wine (Figure 6.60). Cu fraction 1 rapidly increased at the cost of Cu fraction 3. By the first measurement timepoint after bottling (i.e., 18 hours), the Cu profiles were much the same across all samples with concentrations decreasing in the order Cu fraction 1 > Cu fraction 2 >> Cu fraction 3.

In contrast to the rapid change in Cu fractions, oxygen reacted slower in the wine without ascorbic acid (Figure 6.64a), whereby 671 hours was required for it be depleted rather than 250 hours (Figure 6.61a). Therefore, although oxygen reacted slower in the wine without ascorbic acid, it induced a much faster change in the Cu fractions. The rapid loss of Cu fraction 3 in the ascorbic acid-free wine could potentially be caused by *ortho*-quinones inducing the dissociation of copper sulfides. *Ortho*-quinones are known to react with the acidified form of sulfide (i.e., hydrogen sulfide) and if this occurs before the *ortho*-quinone is scavenged by sulfur dioxide the irreversible loss of sulfide would cause dissociation of copper sulfide. In contrast, when ascorbic acid is present the *ortho*-quinones are rapidly reduced back to phenolic compounds (Figure 6.59) and therefore are too short lived to cause significant dissociation of copper sulfides.

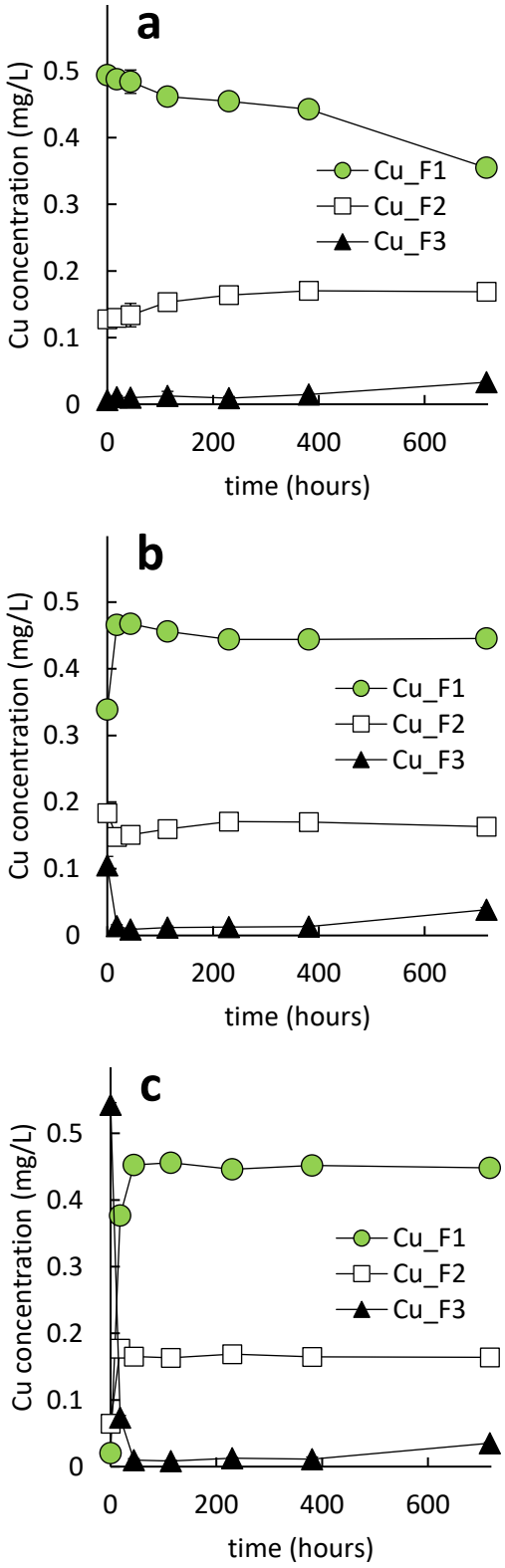


Figure 6.63. The concentration of Cu fractions in Chardonnay wines (no ascorbic acid) with different levels of added Na_2S. The Na_2S was adjusted to three different mole ratios (i.e., Na_2S to added Cu(II)): 0 : 1 (a), 0.8 to 1 (b), and 2 : 1 (c).

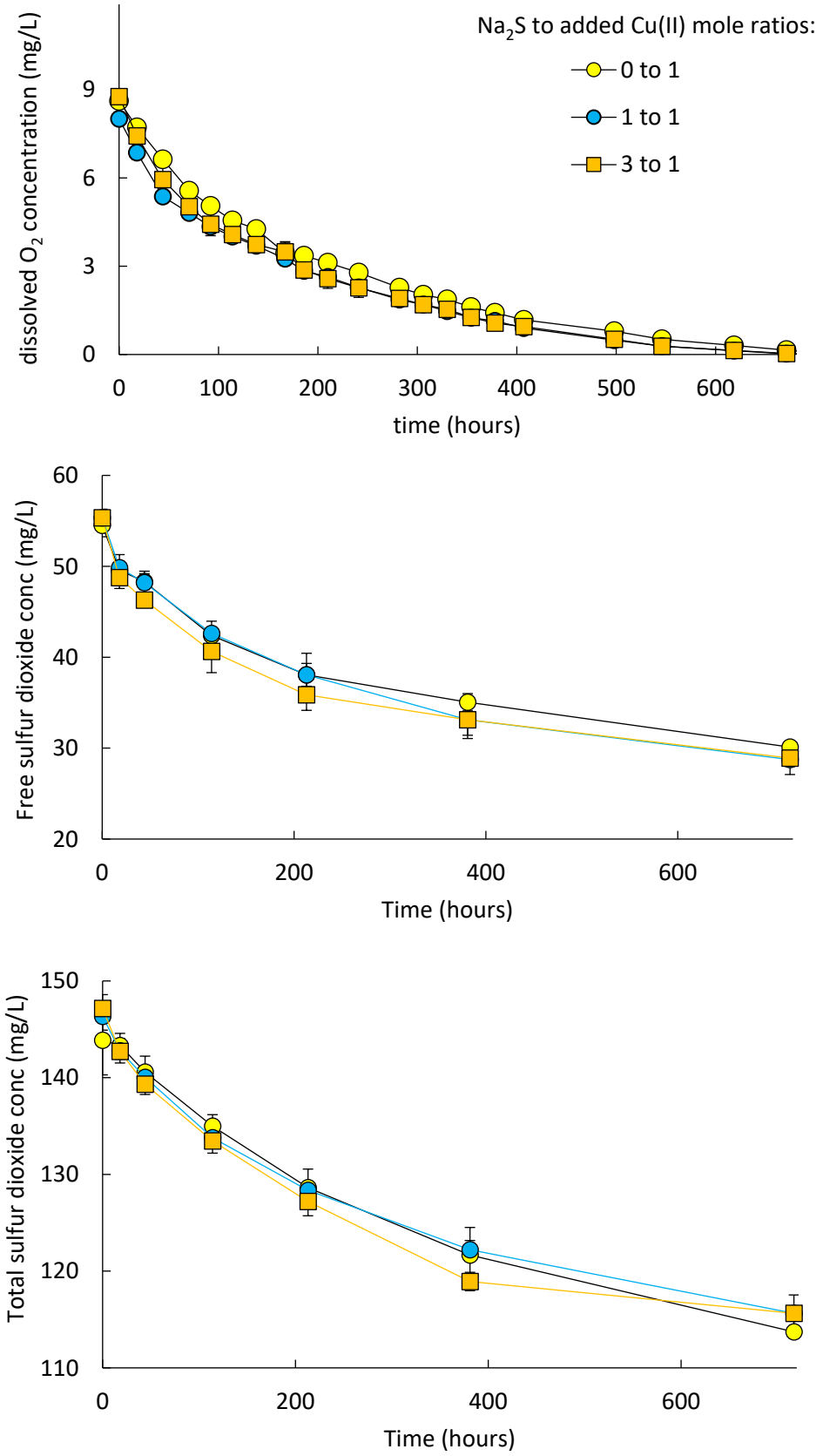


Figure 6.64. The concentrations of oxygen (a), free sulfur dioxide (b) and total sulfur dioxide (c) in the Chardonnay wines (no ascorbic acid) throughout the first 717 hours of the storage period. The sulfur dioxide concentrations were determined by the aspiration-oxidation method.

Given the similarities in Cu fractions across all the samples from the 18 to 671-hour timepoints (Figure 6.63), it is unsurprising that there were no significant differences in the oxygen or sulfur dioxide concentrations throughout the experiment (Figure 6.63a-c). This was also reflected in the lack of significant differences in the determined total sulfur dioxide to oxygen consumption ratios amongst the different samples (Table 6.10). The ratio averaged across all samples was 1.8 ± 0.3 : 1 when sulfur dioxide was measured by the aspiration-oxidation method, which was not significantly different to the 2: 1 ratio predicted by theory (Figure 6.58). A similar ratio was determined when an automated analyser was used for determination of total sulfur dioxide (Table 6.10). The consumption ratio for free sulfur dioxide to oxygen was not dissimilar to the total sulfur dioxide ratio, implying minimal changes in bound sulfur dioxide throughout the experiment. In comparing these ratios (Table 6.10) to that of the white wine with ascorbic acid (Table 6.9), it is clear that the total sulfur dioxide consumed by oxygen was around 2-fold higher when ascorbic acid was not present. Given the rapid change in Cu fractions provided similar profiles across all samples within 18-hours, it is not surprising that the different Cu fraction profiles at time 0 did not influence oxidation rates or sulfur dioxide to oxygen mole consumption ratios.

The experiment was repeated in a Shiraz and Cabernet Sauvignon blend red wine that had Cu(II) added to achieve a total Cu concentration of 0.6 mg/L. Based on previous studies (Kontoudakis and Clark, 2020), it was known that a larger amount of Na_2S needs to be added to red wine than for white wine to achieve appreciable Cu fraction 3 concentration. Consequently, the Na_2S to added Cu(II) ratios used for the red wine were higher than those used for white wine (i.e., 0 to 1, 1 to 1 and 3 to 1). Figure 6.65 showed that the Cu fraction 1 concentration was progressively lower with increased Na_2S addition to the red wine. However, after commencement of oxygen reaction the Cu fractions changed rapidly, similar to the case for white wine without ascorbic acid, such that there was no significant difference in Cu profiles in the different samples after the first 23 hours of the experiment (Figure 6.65).

Table 6.10. The mole consumption ratios for free sulfur dioxide and total sulfur dioxide to oxygen in white wine and red wine. There is no ascorbic acid in the white or red wines.

mole consumption ratio*	SO_2 method	Sample (Initial Na_2S to added Cu(II) mole ratio)			
			white wine		
		0 : 1	0.8 : 1	2 : 1	average
free SO_2 : oxygen	asp.-ox.	1.5 ± 0.1	1.6 ± 0.1	1.57 ± 0.07	1.6 ± 0.1
	auto anal.	1.9 ± 0.4	1.7 ± 0.4	2.0 ± 0.4	1.9 ± 0.4
total SO_2 : oxygen	asp.-ox.	1.8 ± 0.2	2.0 ± 0.3	1.6 ± 0.2	1.8 ± 0.3
	auto anal.	1.9 ± 0.4	1.8 ± 0.3	2.0 ± 0.4	1.9 ± 0.3
			red wine		
		0 : 1	1 : 1	3 : 1	average
free SO_2 : oxygen	asp.-ox.	1.0 ± 0.1	1.09 ± 0.09	1.16 ± 0.08	1.1 ± 0.1
	auto anal.	0.4 ± 0.2	0.4 ± 0.3	0.4 ± 0.4	0.4 ± 0.3
total SO_2 : oxygen	asp.-ox.	1.5 ± 0.2	1.5 ± 0.2	1.3 ± 0.2	1.4 ± 0.2
	auto anal.	1.6 ± 0.2	1.4 ± 0.2	1.5 ± 0.1	1.5 ± 0.2

The oxygen concentration throughout the experiment is shown in Figure 6.66a, and its decay was faster than in the white wine (Figure 6.64a) but slower than in the white wine with ascorbic acid (Figure 6.61a). As for the white wine without added ascorbic acid, there was no significant impact caused by the different Cu fractions on the rate of oxygen decay or the sulfur dioxide loss (Figure 6.66a-c). Again, this result was expected due to the rapid change in the Cu fractions that resulted in them being so similar after just 23

hours. Consequently, the total sulfur dioxide to oxygen consumption ratios were similar across all samples (Table 6.10) providing an average of 1.5 ± 0.2 : 1 for the aspiration-oxidation measurement of total sulfur dioxide. A similar ratio was also obtained if the automated analyser was used (Table 6.10). This result was lower than suggested by theory (i.e., 2:1, Figure 6.58), but has been observed in previous studies on red wines (Danilewicz and Standing 2018). It is proposed that the high concentration of phenolic compounds in red wine means that some *ortho*-quinones escape reaction with sulfur dioxide leading to lower sulfur dioxide consumption ratios than predicted by theory. The free sulfur dioxide consumption ratios were lower than the total sulfur dioxide values but the free sulfur dioxide activity in red wine is complicated by both its binding to anthocyanins and its ability to be replenished from bound sulfur dioxide reserviors. The results confirm no impact of the Cu fraction profiles on the sulfur dioxide to oxygen consumption ratios.

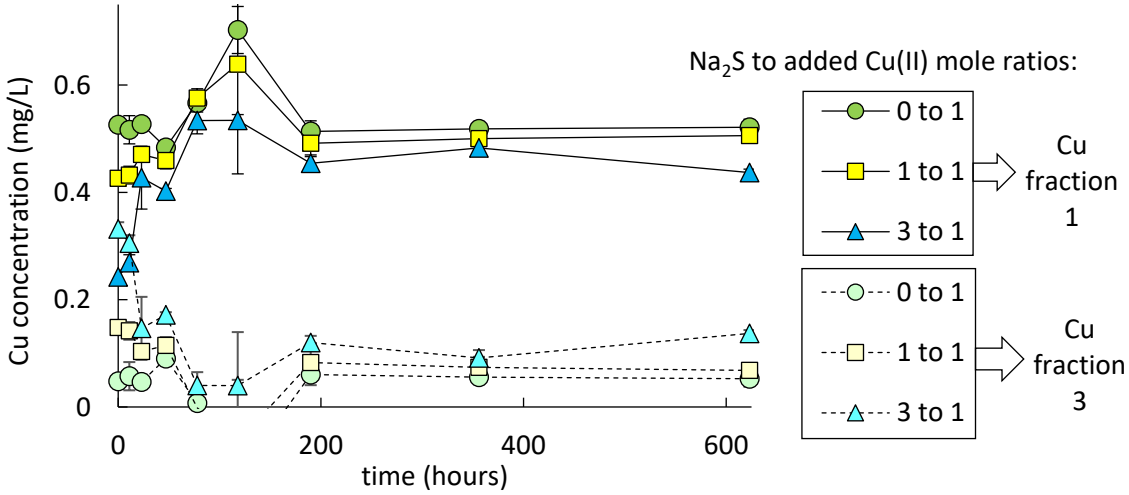


Figure 6.65. The concentration of Cu fractions in Shiraz / Cabernet Sauvignon wines with different levels of added Na_2S.

In summary, it was confirmed that different Cu fractions impacted the rate of decrease of oxygen, free sulfur dioxide and total sulfur dioxide in white wine containing ascorbic acid. However, they did not impact any of these parameters in white wine (without ascorbic acid) or red wines. This impact of ascorbic acid was most likely a consequence of it delaying the oxidative dissociation of Cu fraction 3 in the presence of high oxygen concentrations. In all cases, the wines containing different Cu fractions at bottling did not impact the relative rates of sulfur dioxide to oxygen consumption ratios.

As an aside, the free and total sulfur dioxide measurements for all samples were conducted by two different techniques: aspiration-oxidation and an automated analyser. In comparing all results, the total sulfur dioxide concentrations compared rather well across both red and white wines (Appendix 26). The free sulfur dioxide concentrations had improved agreement in white wine compared to red wine.

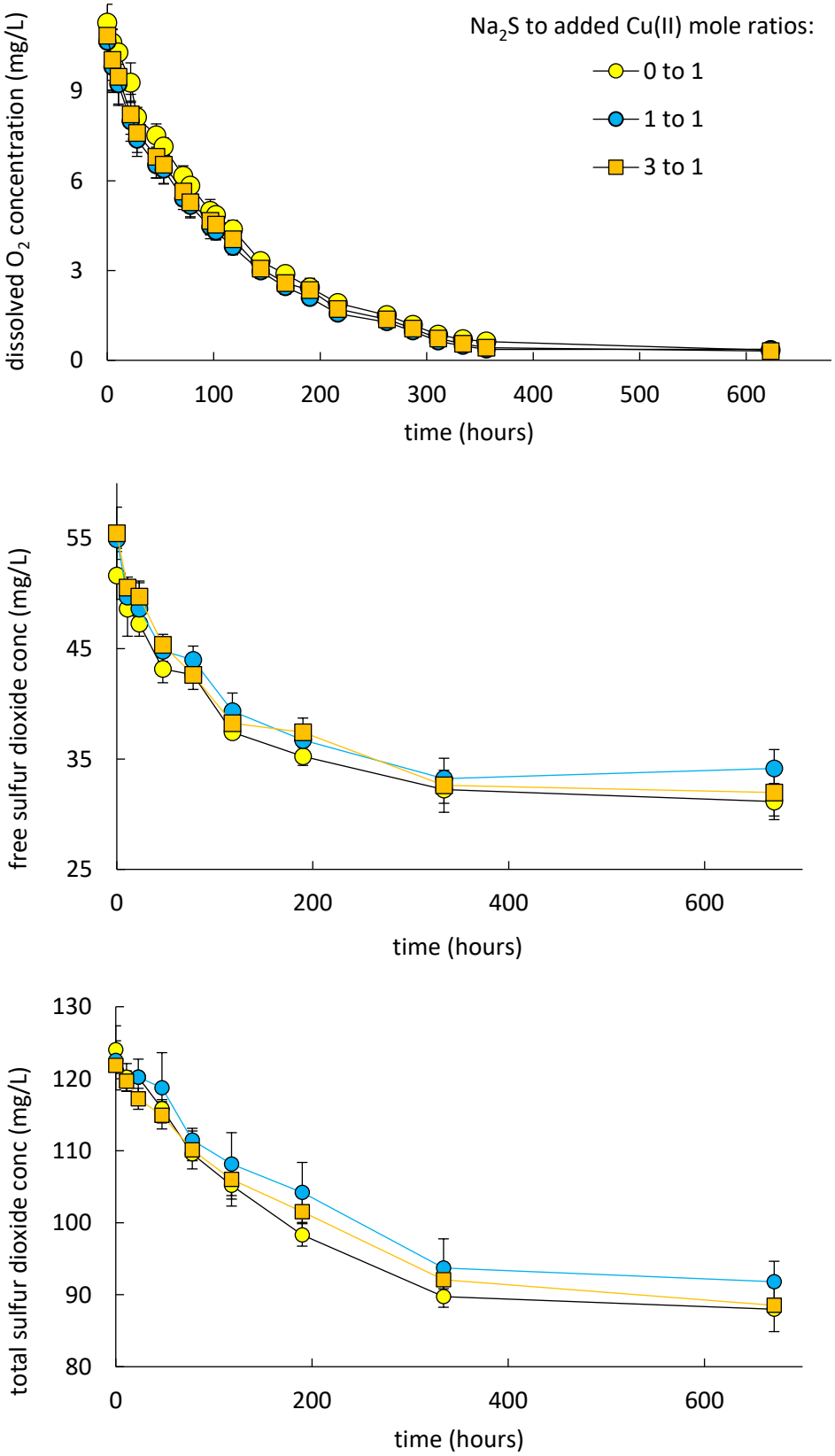


Figure 6.66. The concentrations of oxygen (a), free sulfur dioxide (b) and total sulfur dioxide (c) in the Shiraz / Cabernet Sauvignon wines throughout the first 671 hours of the storage period.

6.5 The transition between different Cu fractions in wine

Section 6.5 identifies the fractions of Cu that are dominant in wine after Cu(II) addition, and how the fractions change when wine is stored in both ideal and non-ideal conditions. The non-ideal storage conditions include the wine being exposed to excessive oxygen, being stored at elevated temperatures, exposed to light or in contact with metallic aluminium (Al(0)). The concentrations of free H_2S and free MeSH were also performed for aging experiments in low oxygen conditions.

6.5.1 The fractions of Cu in wine after Cu(II) addition.

Cu addition to wine can be for reactive or preventative purposes. The reactive addition is made during wine production when a wine has obvious sulfidic-off aromas due to elevated concentrations of free hydrogen sulfide and/or methanethiol. In contrast, preventative additions are made to a finished wine (i.e., without any faults) to limit the incidence of hydrogen sulfide or methanethiol accumulation during bottle aging. Prior to this project, the concentrations used for preventative additions by winemakers were more based on anecdotal and experience-based guidelines rather than being supported by published scientific data.

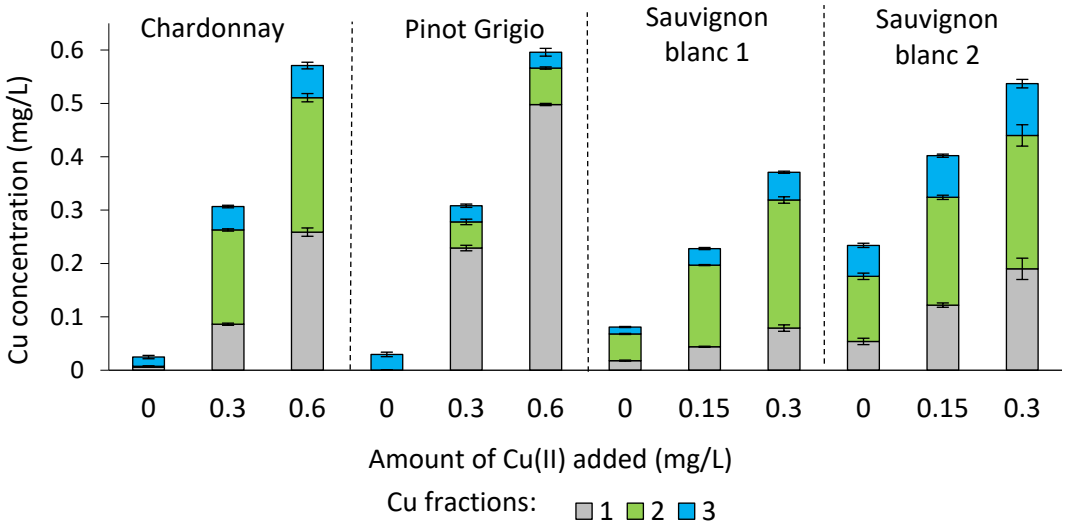


Figure 6.67. The Cu fractions in four white wines after the protective addition of the specified Cu(II) concentrations. The Chardonnay and Pinot Grigio were measured 2-days after the Cu(II) addition, the Sauvignon blanc 1 after 2-weeks and the Sauvignon blanc 2 after 4-weeks. The error bars represent the standard deviation (n = 3).

The first set of experiments established the dominant Cu fractions in wine when a preventative Cu(II) addition was made to wines. The Cu fractions were measured by BCA colorimetry when two rates of Cu(II) were added to four white wines. Figure 6.67 shows that the added Cu(II) generally distributed itself amongst Cu fractions 1 and 2. This meant that the added Cu(II) was generally binding to organic acids (Cu fraction 1), such as tartaric acid, or thiol compounds (Cu fraction 2), such as glutathione. For each wine, the partitioning of added Cu amongst Cu fractions 1 and 2 was wine dependent. For example, the Sauvignon blanc 1 had a large proportion of the added Cu apportion to Cu fraction 2 while in the Pinot

Grigio most of the added Cu become Cu fraction 1. A white wine with a high concentration of efficient Cu(II)-binding compounds, such as glutathione, would be likely to favour Cu fraction 2 over Cu fraction 1.

Figure 6.68 shows the distribution of Cu fractions in two red wines after preventative Cu(II) additions. The Cu fraction 1 concentrations were measured by stripping potentiometry and Cu fraction 3 was calculated from the difference between total Cu and Cu fraction 1. After the addition of 0.3 and 0.6 mg/L, it is evident that the added Cu distributed itself predominantly as Cu fraction 1, with only a slight increase in Cu fraction 3. Despite no overt H_2S aroma in the wines prior to the addition of Cu(II), it is possible for some of the added Cu(II) to sequester sulfide from other weaker binding metals, such as Zn(II) or Fe(II) (Ontañón et al. 2019).

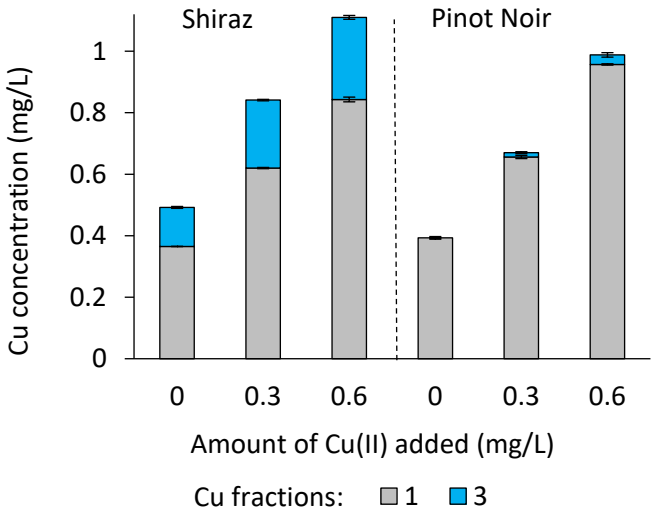


Figure 6.68. The Cu fractions in two red wines after the protective addition of 0, 0.3 and 0.6 mg/L Cu(II). The Shiraz was measured 6 days after the Cu(II) addition, and the Pinot Noir after 5 days. The error bars represent the standard deviation (n = 3).

A red and white wine were used to assess the change in Cu fractions when Cu(II) was added to wines as a reactive measure. That is, addition of Cu(II) to wines with overt sulfidic-off odour, which in this case would be due to the presence of H_2S added to the wine (as Na_2S). Cu(II) (0.6 mg/L) was added to a white and red wine containing progressively higher concentrations of hydrogen sulfide, whereby the wine had no hydrogen sulfide present, corresponding to a mole ratio of H_2S to Cu of 0 : 1, or had hydrogen sulfide present at a mole ratio of 0.8 or greater (Figure 6.69). It is evident from Figure 6.69 that Cu fraction 3 concentrations increased when Cu(II) was added to the wines of higher H_2S concentration. The yield of Cu fraction 3 from hydrogen sulfide appeared higher in white wine compared to red wine. This may have been a consequence of quinones, which are more easily generated in red than white wines, scavenging H_2S before its interaction with Cu(II).

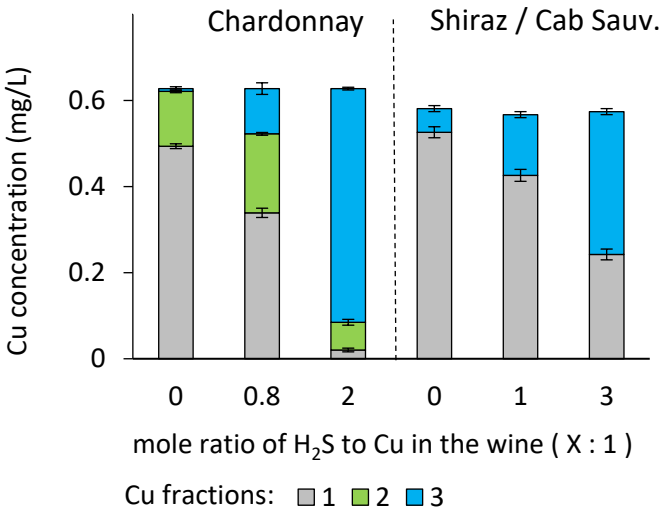


Figure 6.69. The Cu fractions in a white and red wine, which had been adjusted to three different free hydrogen sulfide concentrations, after the addition of 0.6 mg/L Cu(II). The Cu fractions were measured ~2 hours after the addition of the Cu. The error bars represent the standard deviation (n = 3).

Overall, this work shows that preventative Cu(II) additions to white wine favour Cu fractions 1 and 2, while addition to red wine favours Cu fraction 1. Only minor increases of Cu fraction 3 were evident for the preventative additions. Alternatively, the reactive addition can result in significant increases in Cu fraction 3 depending on the concentration of hydrogen sulfide present in the wine.

6.5.2 The change of Cu fractions during the oxidation of wine.

Section 6.4 has already shown the impact of the high oxygen concentrations in wine on Cu fractions. To briefly summarise the results, once red or white wines were saturated with oxygen, Cu fraction 3 was depleted within 1-day and replaced by Cu fraction 1 and 2 in white wine (Figure 6.63) or Cu fraction 1 in red wine (Figure 6.65). The exception was white wine containing ascorbic acid, whereby the ascorbic acid protected Cu fraction 3 from depletion and allowed its concentration to remain higher throughout storage (Figure 6.60). Upon depletion of oxygen, the presence of ascorbic acid also allowed the Cu fraction 3 concentration to increase rapidly, at the expense of the other Cu fractions.

The following sections of the report show the impact of oxygen saturation and ascorbic acid on the Cu fractions in a Rosé wine, and the impact of a closure with a high oxygen ingress rate (i.e., 27 µg/(L·day)) on the Cu fractions in red and white wines.

6.5.2.1 The impact of oxygen saturation and ascorbic acid on Cu fractions in rosé.

Unlike white wine, ascorbic acid is rarely used in red wine as the higher concentrations of phenolic compounds in red wines negate the need for further antioxidant addition (Waterhouse et al, 2016b). Rosé wines contain significantly lower concentrations of phenolic compounds than found in typical red wines (Paixão et al. 2007; Wirth et al., 2012), and consequently rosé would be expected to benefit more from ascorbic acid usage than red wine. However, the impact of ascorbic acid on the development of rosé in is uncertain. Full details of this work can be viewed within Zhang et al. (2023), but the focus reported in this section was the impact of oxygen and ascorbic acid on the Cu fractions in rosé during its bottle aging.

Rosé wine was made from Tempranillo grapes (vintage 2019, NSW, Australia) with 1500 L undergoing a standardised winemaking protocol as per Šuklje et al. (2019). Prior to bottling, 0.25 mg/L of Cu(II) was added to the base wine with 500 mg/L $CuSO_4 \cdot 5H_2O$ stock solution. The composition of the final wine is shown in Appendix 27.

The wine was divided into two halves, with one half saturated with oxygen by sparging air through the wine for 10 minutes via hosing connected to an air compressor, while the other half was not sparged with oxygen. During bottling, additions of ascorbic acid were made to individual 750 mL bottles to achieve final concentrations of 0, 50 or 500 mg/L ascorbic acid in the bottled wine. This provided the treatments of Control, 50Asc, and 500Asc, respectively, for the half of wines without the sparging of air prior to bottling (i.e., lower oxygen concentration), and Ox, 50AscOx, and 500AscOx for the air-saturated treatments. These concentrations of ascorbic acid were utilised as they were consistent with both the routine addition of ascorbic acid to white wines (i.e., 50 mg/L) and also would provide insight into more extreme additions (i.e., 500 mg/L). A total of 24 bottles were prepared for each treatment (i.e., 144 bottles in total) and triplicate bottles were open at each analysis time point. Bottles were sealed with screw caps with tin liners. For each treatment, triplicate bottles were prepared with Pst3 oxygen sensor spots attached to both the bottom and neck of each bottle to enable measurement of dissolved and headspace oxygen concentrations. All bottled wines were then stored at 14 ◦C in darkness.

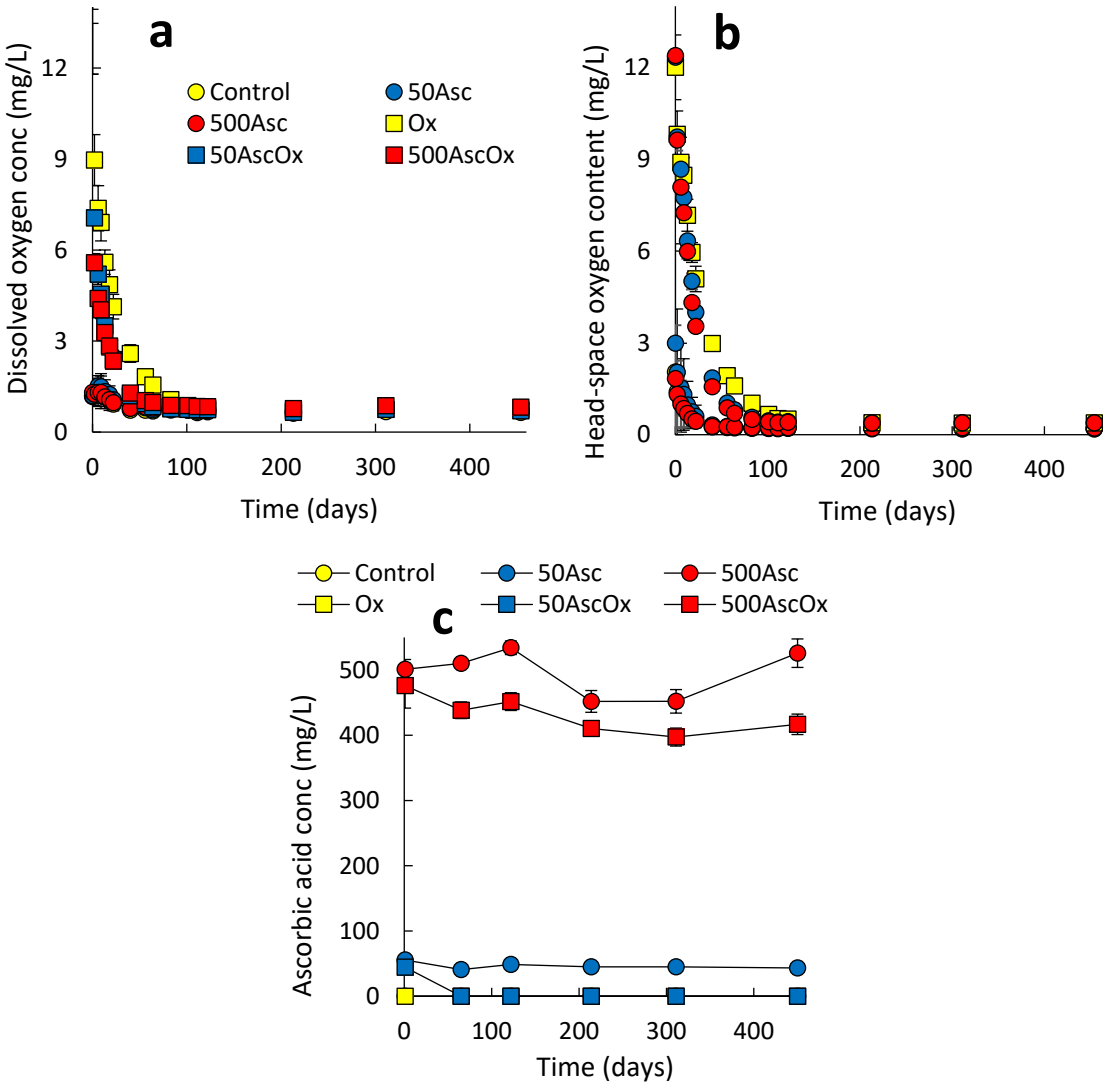


Figure 6.70. The concentration of dissolved oxygen (a), head-space oxygen (b) and ascorbic acid (c) in the rosé wines. In plate (a), the symbols for the Control samples are concealed by the 500Asc symbols. In plate (c) the symbols fo Ox are concealed after 0 days by the symbols for 50AscOx.

Figure 6.70a and b show the dissolved and head-space oxygen concentrations of wines during the storage period. The head-space oxygen corresponds to oxygen residing between the screw cap closure and the surface of the wine. The wines with low oxygen had near depletion of oxygen after 1-month of storage, while the high oxygen wines required approximately two to four months for depletion of the majority of oxygen. For the high oxygen samples, the oxygen was depleted faster in samples containing ascorbic acid, with first order oxygen decay rates that were 0.030, 0.039 and 0.040 day^{-1} (all ± 0.001 day^{-1}) for the Ox, 50AscOx and 500AscOx samples, respectively.

The sulfur dioxide (Appendix 28) and ascorbic acid (Figure 6.70c) concentrations matched the changes in oxygen concentrations. That is, minimal changes in the low oxygen samples, and the largest concentration decreases for the first 2 to 4-months of aging in the samples with high oxygen.

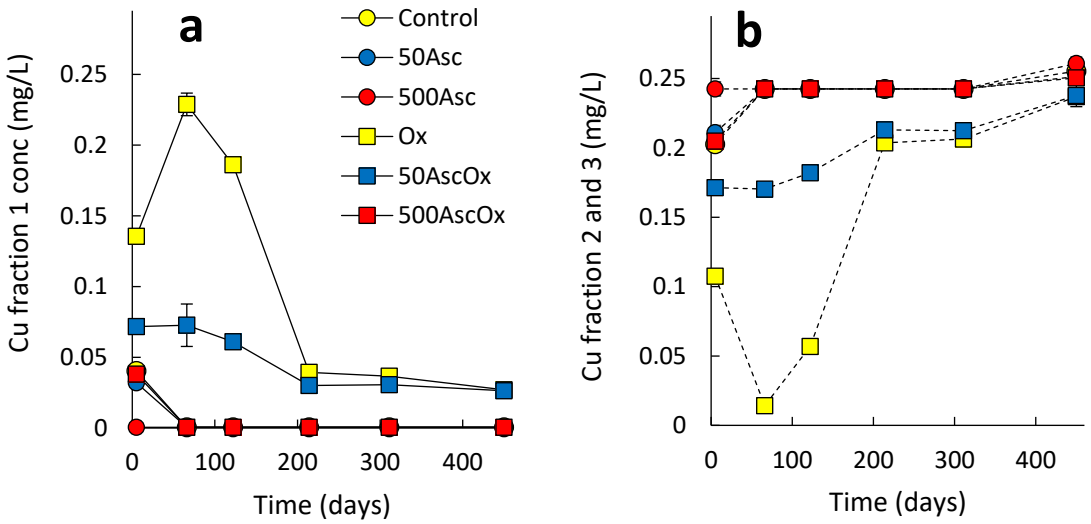


Figure 6.71. The concentrations of Cu fraction 1 (a) and Cu fractions 2 and 3 combined (b) in the rosé samples. Error bars indicate the standard deviation of triplicate samples, and where not evident are smaller than the size of the symbol. In (a) and (b) the Control symbols (yellow circles) are concealed by the 500AscOx symbols (red squares).

At the first analysis timepoint (i.e., 5-days after bottling), the samples treated with high oxygen had higher concentrations of Cu fraction 1 than those with the low oxygen treatments for a given ascorbic acid treatment (Figure 6.71a). Furthermore, the ascorbic acid treatments significantly decreased the Cu fraction 1 concentration, irrespective of oxygen bottling concentrations, although the magnitude of Cu fraction 1 decrease was more pronounced for the wines bottled with higher oxygen concentrations (Figure 6.71a).

During storage, the wines bottled with low oxygen had complete loss of Cu fraction 1 by 2-months (Figure 6.71a). This was also the case for the sample with both high ascorbic acid concentration and high oxygen (500AscOx). The other high oxygen samples (i.e., Ox and 50AscOx) had significantly higher Cu fraction 1 concentrations throughout wine aging.

Overall, these results show for the first time that lower oxygen conditions favour the conversion of Cu fraction 1 to fractions 2 and/or 3 in rosé, while higher oxygen conditions favour the opposite transition. Also, only extremely high ascorbic acid concentrations can afford the complete conversion of Cu fraction 1 to Cu fraction 2 and/or 3 by 2-months in high oxygen conditions. This influence of ascorbic acid was only observed at 500 mg/L despite the lower ascorbic acid sample (i.e., with 50 mg/L) having a similar oxygen decay rate as the 500Asc sample.

This study shows that the total packaged oxygen at bottling increased the concentration of Cu fraction 1 in rosé at the cost of sulfide- and/or thiol-bound forms of Cu (i.e., Cu fractions 2 and 3). This influence of oxygen on Cu fraction 1 was lessened if ascorbic acid was present. Based on these results, the implication is that ascorbic acid can facilitate the reaction of Cu fraction 1 with either thiol compounds or hydrogen sulfide to form Cu fraction 2 or 3, and it may also have a role in protecting the Cu fraction 2 and 3 species from dissociating in the presence of oxygen. The ramifications for the accelerated loss of Cu fraction 1, a fraction of Cu that offers protection against sulfidic off-aroma, will be explored further in Section 6.5.3.

6.5.2.2 The impact of SO_2 with a high oxygen ingress closure on Cu fractions.

This section investigates the change in Cu fractions during the aging of the wine that have bottle closures that allow the ingress of oxygen at a high rate. The wines were bottled at two different sulfur dioxide concentrations to assess its impact on Cu fraction changes.

Table 6.11. The four white and red wines used for the high oxygen ingress study.

white wine	red wine
W1 = Chardonnay	R1 = Pinot Noir
W2 = Chardonnay	R2 = Shiraz
W3 = Sauv. blanc	R3 = Shiraz
W4 = Riesling	R4 = Cabernet Sauvignon

Four commercial white and red wines (Table 6.11) were purchased and prepared in two batches, whereby one had 40 mg/L of sulfur dioxide added and the other was left unadjusted. The wines were re-bottled in 40 mL vials in an anaerobic hood with care taken not to have any ullage in the vials. The dissolved oxygen concentration in the vials was measured to be 0.1-0.2 mg/L. Based on previous trials, the vial closures had an average oxygen ingress rate of 1.6 ± 0.3 µg/day that enabled an increase of 39.1 µg/L of dissolved oxygen per day (i.e., µg/L·day) into the 40 mL of wine. To place this in perspective, the equivalent rates for the following closures on a 750 mL volume of wine would be (Lopes et al. 2005, 2006, 2007):

- 0.2 – 11 µg/(L·day) for cork,
- 1.5 µg/(L·day) for technical cork,
- 1.2 µg/(L·day) for screw cap with tin liner,
- 3.7 µg/(L·day) for screw cap with saranex liner, and
- 12.3-23 µg/(L·day) for synthetic closures.

Consequently, the closures being used equated to oxygen ingress levels that were double that of synthetic closures. The oxygen ingress rate of 39.1 µg/(L·day) allowed 14.6 mg/L ingress of dissolved oxygen over a period of 1-year, with an estimated total sulfur dioxide consumption of around 58 mg/L, assuming a total sulfur dioxide to oxygen mole consumption ratio of 2 to 1. The Cu fraction 1 concentration was measured by stripping potentiometry and total Cu concentrations by ICP-OES. No BCA colorimetric measures of Cu fractions 1, 2 and 3 were conducted in white wine, as this study pre-dated the development of the technique.

During aging, the dissolved oxygen concentration of the wines was measured in sacrificial samples and it remained low (<0.2-0.3 mg/L). The low steady state concentration meant that the oxygen entering the wine was largely reacting before it could accumulate. Figure 6.72 shows an example of the decrease in free and total sulfur dioxide concentrations in a red wine (R1 and R1+SO_2) and white wine (W2 and W2+SO_2) throughout the aging period. The decrease in total sulfur dioxide (Figure 6.72b) is relatively linear, consistent with the expected ingress of oxygen into the containers. Similar profiles for all the white and red wines can be viewed in Appendix 29 and 30, respectively. The free sulfur dioxide concentration dropped below 10 mg/L in all wines except for white wines 3 and 4 with extra sulfur dioxide added at bottling (i.e., W3+SO_2, W4+SO_2).

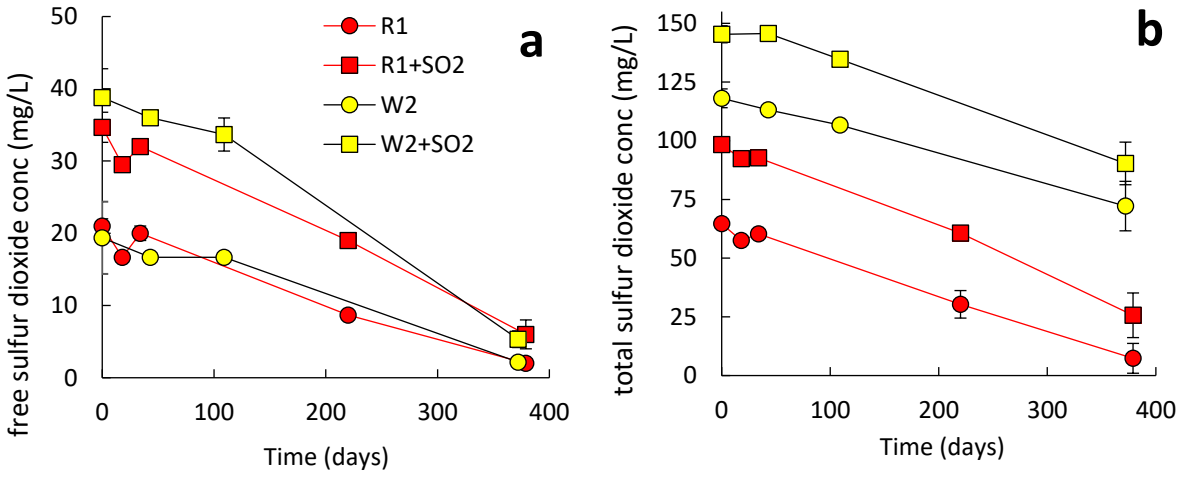


Figure 6.72. An example of the free sulfur dioxide (a) and total sulfur dioxide (b) decay in red wines (R1 and R1+SO_2) and white wines (W2 and W2+SO_2) bottled at two different sulfur dioxide concentrations. The sulfur dioxide concentrations for all samples during the aging period can be viewed in Appendix 29.

Figure 6.73 and 6.74 show the Cu fraction 1 concentrations throughout the storage period. For the first 100 days, the Cu fraction 1 concentration has either a slight decrease or remains relatively stable. After the first 100 days, the Cu fraction 1 concentration then increased towards the total Cu concentration. In all but two wines, the Cu fraction 1 concentration reached the total Cu concentration by 1-year. This was not the case in two wines, white wines 3 and 4, in which the free sulfur dioxide concentration remained above 20 mg/L. At the timepoints around 100 and 250 days there were several wines with significantly ($P < 0.05$) lower Cu fraction 1 concentrations in the treatments with higher sulfur dioxide concentrations. This included W2+SO2, W3+SO2, W4+SO2, R1+SO2 and R4+SO2 (Figure 6.73 and 6.74). This is potentially a consequence of sulfur dioxide scavenging oxidised phenolic compounds (i.e., *ortho*-quinones) in these higher sulfur dioxide treatment samples before the quinones reaction with thiol compounds or hydrogen sulfide. Such reaction of quinones with thiol compounds or hydrogen sulfide could cause dissociation of Cu(I)-thiol complexes and copper sulfides, respectively, and thereby increase the concentration of Cu(II)-organic acids (i.e., Cu fraction 1). There was one wine (R2) that had more Cu fraction 1 with added sulfur dioxide at one timepoint of analysis (i.e., 100 days) but this may have been a consequence of having the highest Cu fraction 1 concentration of any other wine at bottling.

These results show that closures with high oxygen ingress rates will favour Cu fraction 1 over Cu fraction 2 and 3. A higher sulfur dioxide concentration at bottling (i.e., by 40 mg/L) was generally able to slow the transition of Cu to Cu fraction 1.

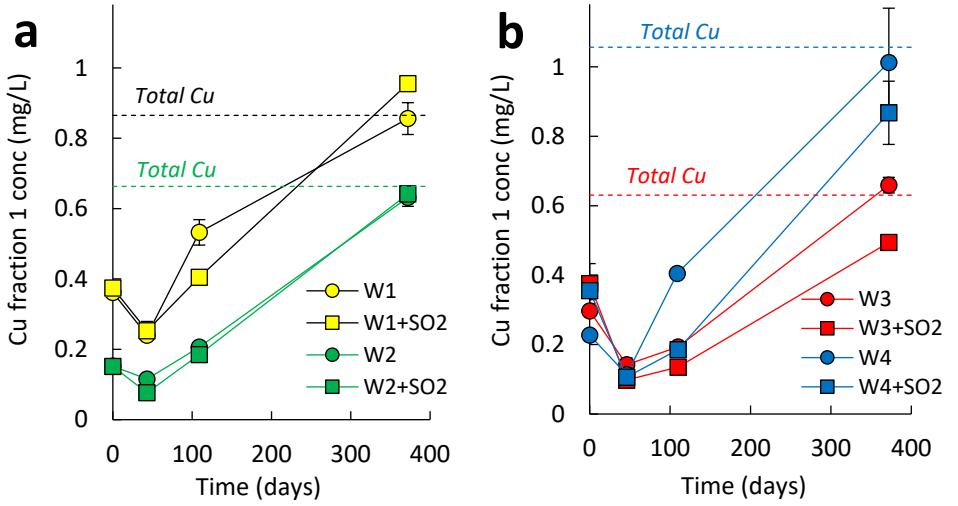


Figure 6.73.
The Cu fraction 1 concentration in white wines 1-4. The wines with '+SO2' had an extra 40 mg/L SO_2 added at bottling.

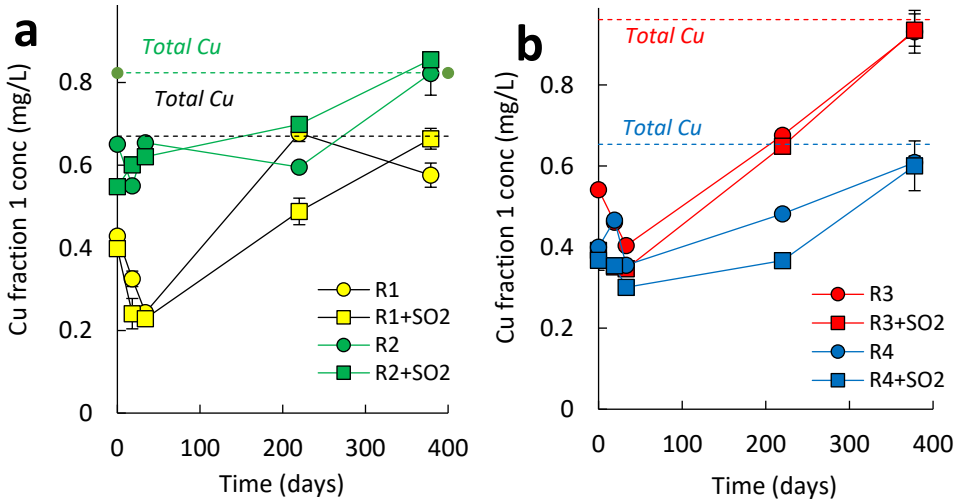


Figure 6.74.
The Cu fraction 1 concentration in red wines 1-4. The wines with '+SO2' had an extra 40 mg/L SO_2 added at bottling.

6.5.3 The change in Cu fractions during the aging of wine in ideal storage conditions.

Cu fractions 1 and 2 can inhibit the accumulation of reductive aroma compounds in wine (Section 6.3.2), however, these same Cu fractions were also shown to transition to Cu fraction 3 during the bottle aging of two Sauvignon blanc wines in low oxygen and ideal conditions (Section 6.3.5). The transition of Cu fraction 1 to 3 was also shown during the bottle aging of rosé in low oxygen conditions. This section attempts to address several key questions regarding possible optimisation of Cu fraction 1 and 2 concentrations prior to the bottling of wine:

- Do higher Cu fraction 1 and 2 concentrations at bottling offer increased protection against the reductive aroma development of wine?
- During wine aging, how long does it take free H_2S and free MeSH to accumulate to detrimental levels if Cu fractions 1 and 2 become depleted?
- Is there a limit to the amount of Cu fractions 1 and 2 that can be depleted by a wine?
- How variable are the rates of decay for Cu fraction 1 and 2 across different wines?
- At the bottling of wine, can we predict how long before Cu fractions 1 and 2 become depleted?

To address these questions, the following sections (6.5.3.1-6.5.3.3) show the changes in Cu fractions, free H_2S and free MeSH in two white wines and two red wines bottled with variable Cu concentrations and aged in low oxygen conditions. To determine the variability in Cu fraction 1 and 2 transition to Cu fraction 3 across different wines, the decay rates for combined Cu fraction 1 and 2 concentrations were determined in 8 white wines, and the decay rates for Cu fraction 1 determined in 6 red wines and 8 white wines. An overall scheme for the transition between Cu fractions in low and high oxygen conditions is also proposed.

6.5.3.1 The change in Cu fractions during the aging of two white wines in ideal storage conditions.

Additions of 0, 0.3 or 0.6 mg/L Cu(II) were made to Chardonnay and Pinot Grigio at bottling. Throughout a 12 to 14-month storage period, Cu fractions were determined by colorimetry, and sulfhydryl compounds by gas chromatography with sulfur chemiluminescence detection. The full experimental details of this study can be found within Zhang et al. (2022).

Total sulfur dioxide measurements (data not shown) showed a 15–20 mg/L decrease across wines during the first two months of storage, and relatively stable concentrations thereafter. These results implied that oxygen was consumed by wines within the first two months of bottle aging, and after this period low oxygen concentrations prevailed.

After bottling, low concentrations of total Cu (≤0.03 mg/L) were measured in the Control Chardonnay and Pinot Grigio wines (first time point, Figure 6.75a,d) and the majority was in the form of Cu fraction 3. For the Cu-treated wines, Cu fraction 1 dominated in the Pinot Grigio, with minor amounts of Cu fraction 2 present (Figure 6.75b,c), while for the Chardonnay, a significant proportion of Cu existed as both Cu fractions 1 and 2 (Figure 6.75e,f). The elevated concentrations of Cu fraction 2 in the Chardonnay compared to Pinot Grigio wines suggested a higher concentration of thiol compounds capable of complexing Cu in the base Chardonnay wine.

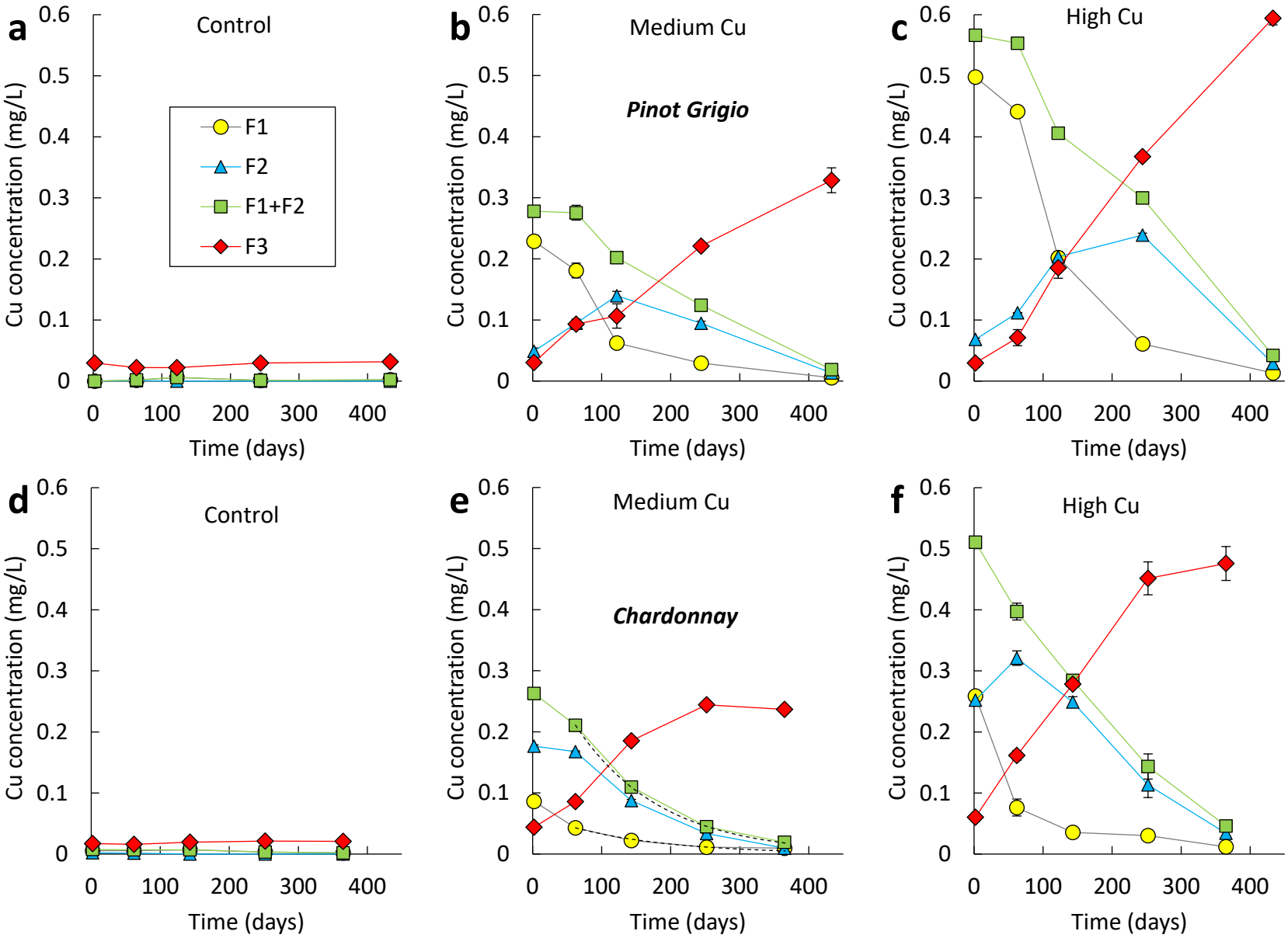


Figure 6.75. The concentrations of Cu fractions 1, 2 and 3 in Pinot Grigio (a-c) and Chardonnay wines (d-f), including the combined Cu fraction 1 and 2 concentrations. Error bars represent the standard deviation from triplicate samples.

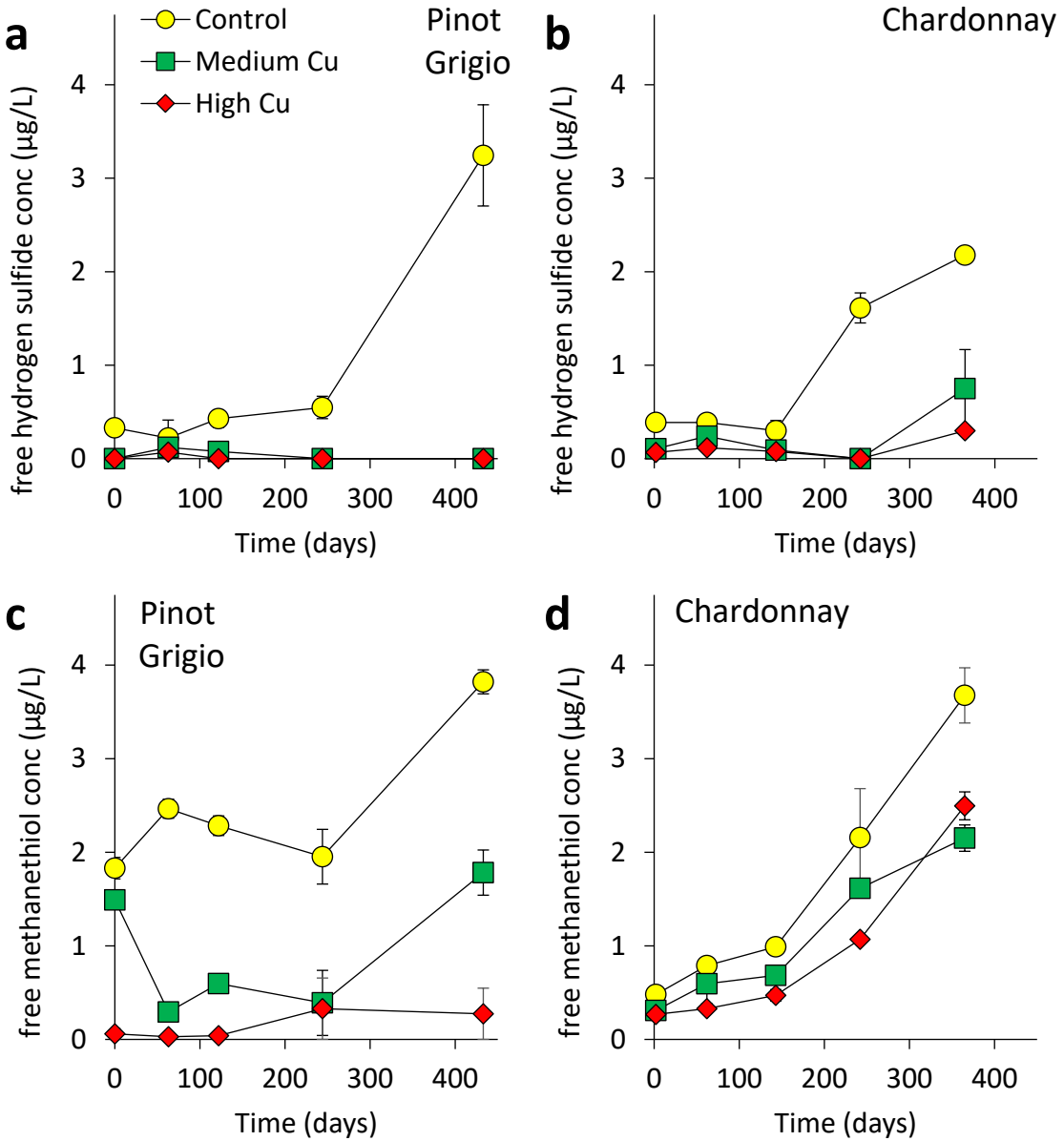


Figure 6.76. The concentrations of free H_2S (a, b) and methanethiol (c, d) in Pinot Grigio (a, c) and Chardonnay (b, d) wines. Error bars represent the standard deviation from triplicate samples.

During the aging of wines in bottle for 12 or 14 months at 14 °C, the concentration of Cu fractions changed in a similar manner as observed for the bottle aged Sauvignon blanc wines as discussed in Section 6.3.5 (Figure 6.51). The concentration of Cu fraction 1 in the Control wines remained low (<0.015 mg/L, Figure 6.75a,d). In all the Cu-treated wines (Figure 6.75b,c,e,f) the concentration of Cu fraction 1 continuously decreased throughout the wine storage period. However, the concentration of Cu fraction 1 concentration was always higher for the wines with increased Cu addition rates at bottling. These results also show that Cu fraction 1 can remain in a wine for around 5 – 14 months.

Cu fraction 2 concentrations were negligible in all Control samples throughout storage (Figure 6.75a,d). The Cu-treated Pinot Grigio samples initially had increasing Cu fraction 2 concentrations (Figure 6.75b,c) up to 4–8 months, before a decrease to negligible concentrations by 14 months. For the Cu-treated Chardonnay samples, Cu fraction 2 was either stable (Medium) or increased (High) up to 2-months, before near total depletion by 12-months (Figure 6.75e,f). These results suggest contrasting behaviours for the Cu-thiol compound interactions in the two wines. In Chardonnay, the thiols are largely of a type to immediately bind to Cu and form Cu fraction 2. Alternatively, the Pinot Grigio is more dominated by a pool of thiol precursors, which over time, release thiols that then react to form Cu fraction 2.

The changes described for Cu fraction 1 and 2 in the Cu-treated wines coincided with an increase in Cu fraction 3 (Figure 6.75b,c,e,f). In fact, Cu fraction 3 was the dominant form of Cu in Chardonnay wines by 4-months and in Pinot Grigio wines by 8-months.

After bottling, there were negligible amounts of free H_2S and free MeSH present in the Chardonnay wines (Figure 6.76b,d). The Pinot Grigio also had negligible free H_2S concentrations (Figure 6.76a) but had free MeSH above the aroma threshold in the control wine (Figure 6.76c). The free methanethiol concentrations in the Pinot Grigio became progressively lower with Cu-treatment at bottling.

During the aging period, free H_2S concentrations did not exceed 1 µg/L (i.e., the quoted aroma threshold (Waterhouse, Sacks, & Jeffery, 2016b)) in any of the Cu-treated wines (Figure 6.76a,b). However, for the Control wines, the free H_2S concentration exceeded 1 µg/L at the 7-months measurement for the Chardonnay (Figure 6.76b) and at 14-months for the Pinot Grigio (Figure 6.76a).

The concentration of free methanethiol remained below 2.5 µg/L in all the Cu-treated samples (Figure 6.76c,d). Alternatively, in the Control samples the free methanethiol concentrations increased during storage. In the Pinot Grigio Control, the free methanethiol concentration exceeded the 2 µg/L odour threshold (Kreitman et al., 2019) for the entire duration of the bottle aging trial (Figure 6.76c), while for the Chardonnay Control the odour threshold was exceeded after 4–8 months (Figure 6.76d).

Sensory analysis was conducted on the wines after 12-14 months of bottle aging utilising the Pivot® Profile sensory methodology (Pearson et al. 2020). Sensory analysis was also conducted on freshly opened Control bottles of Chardonnay and Pinot Grigio, at the 12 to 14-month timepoint, after the addition of 0.3 and 0.6 mg/L Cu(II). These samples were labelled as 'Fining_0.3Cu' and 'Fining_0.6Cu', respectively.

Panellists characterised the wines using 19 descriptors for Pinot Grigio and 25 for Chardonnay. Correspondence analysis (CA) was utilised to analyse these data sets and produce Figure 6.77a and b. For the Pinot Grigio (Figure 6.77a), 82.7 % of variance was explained by F1 and F2, while F3 explained 10.1 %

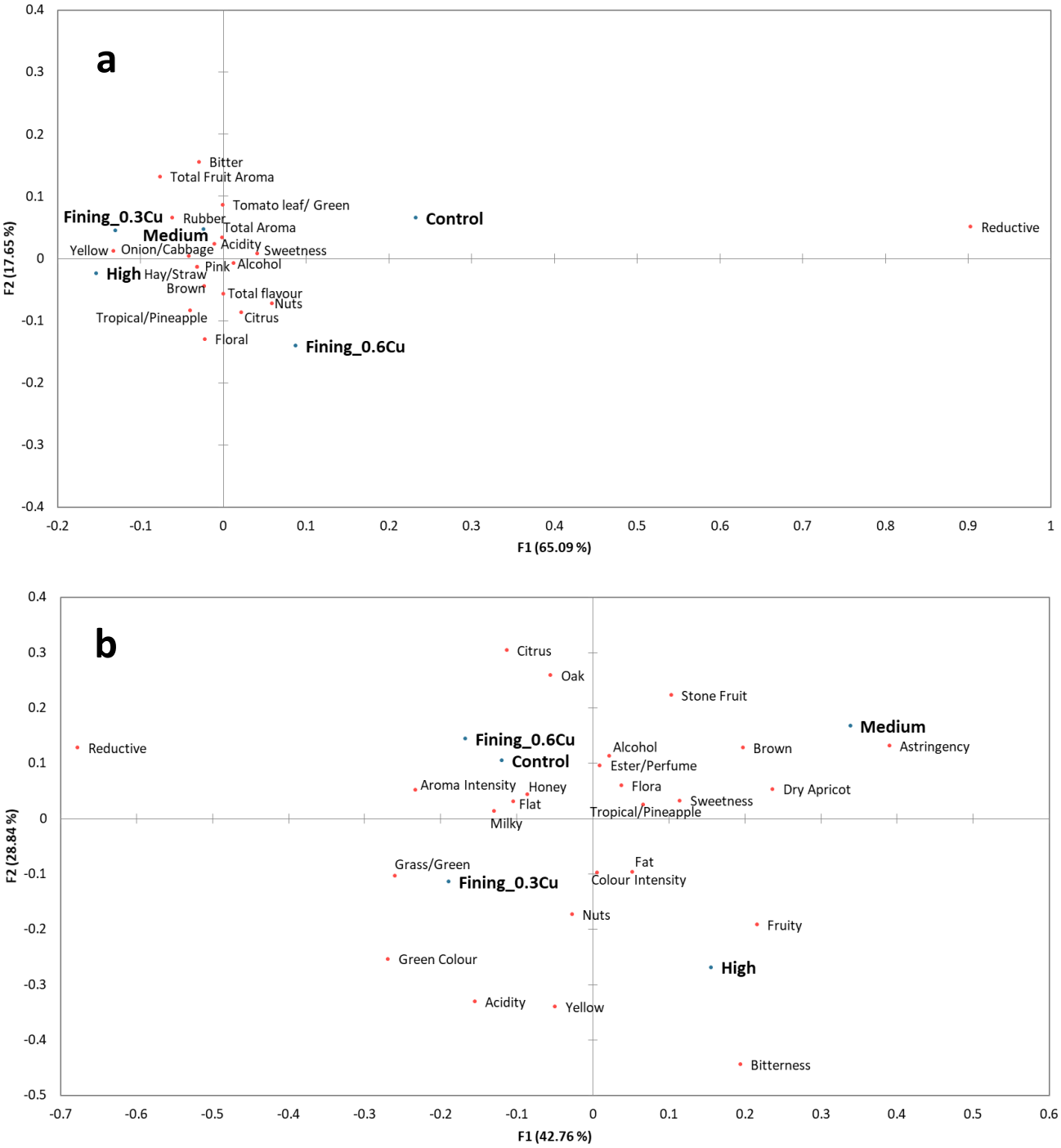


Figure 6.77. Correspondence analysis biplot of the Pinot Grigio (a) and Chardonnay (b) wines after 12 or 14 months of bottle ageing using Pivot® Profile.

and F4 explained 7.2 % (data not shown). F1 is predominantly driven by differences in the 'reductive' descriptor, with the Control sample further to the right-hand side of the figure indicating a high association with the reductive attribute. The Cu-treated (Medium and High) samples were described as having lower reductive character. This result was in line with the concentrations of free H_2S and free methanethiol being above their aroma thresholds in the Control but not in the Cu-treated Pinot Grigio wines (Figure 6.76a,c). The Cu additions immediately prior to sensory analysis (i.e., treatment Fining_0.3Cu and Fining_0.6Cu) also demonstrated a suppression of the reductive character, however, interestingly the sensory influence was more pronounced with the lower addition rate (i.e., Fining_0.3Cu, Figure 6.77a). The removal of free methanethiol and free H_2S in these samples was confirmed (data not shown), as was elevation of Cu fractions 1 and 2 above 0.015 mg/L.

For the Chardonnay sensory data (Figure 6.77b) 71.6 % of variance was accounted for by F1 and F2, while F3 and F4 explained 16.0 % and 12.4 % (data not shown), respectively. Separation of the Control treatment from the Medium and High Cu addition treatments was evident along F1, which again was primarily influenced by the reductive attribute. Differences in astringency, fruity and dry apricot also influenced the location of the Cu addition wines. Fresh Cu fining treatment did not appear to reduce the reductive character in the Control Chardonnay samples, with all Control, Fining_0.3Cu and Fining_0.6Cu samples still associated with the reductive attribute to a similar extent.

The decay rates were determined for Cu fraction 1 and 2 during the low oxygen aging-phase of the wines, which was from 2-months after bottling. These will be discussed in more detail in Section 6.5.3.3, but the decay rate for Cu fraction 1 was significantly faster in the Pinot Grigio compared to Chardonnay, with the time required to halve the Cu fraction 1 concentration (i.e., half-life) being 110 ± 20 days for the Chardonnay and 70 ± 4 days for the Pinot Grigio (the quoted uncertainty is the standard deviation with n=3). In contrast, the decay rate for the combined Cu fraction 1 and 2 concentrations were similar for both wines, with half-lives of 91 ± 8 and 98 ± 3 days (n=3) for the Chardonnay and Pinot Grigio, respectively. Therefore, across these two white wines the decay rates for Cu fraction 1 were different while those for the combined Cu fraction 1 and 2 concentrations were similar.

These results show that with Cu(II) addition at bottling, Cu fractions 1 and 2 can remain in a wine for 5-14 months during bottle aging. The overall decrease in the Cu-organic acids (Cu fraction 1) and labile Cu(I)-thiol complexes (Cu fraction 2) is concomitant with an increase in the Cu fraction related to sulfide-bound Cu and more inert Cu(I)-thiol species (Cu fraction 3). Only the Control wines, with no Cu addition at bottling, showed elevated concentrations of free H_2S and free methanethiol, and this was consistent with wines being able to be separated during sensory analysis, largely based on the presence of reductive character, particularly in the case of the Control Pinot Grigio. The results suggest that Cu(II) addition at bottling can offer protection to wine against reductive aroma development even after Cu fraction 1 and 2 have been depleted.

6.5.3.2 Cu fraction 1 concentration during red wine aging in ideal storage conditions.

This section investigates the change of Cu fraction 1 during the bottle aging of red wine in low-oxygen conditions, and investigates how much Cu fraction 1 can transition to Cu fraction 3 during bottle aging. To this end, and to gain insight into varietal effects, Shiraz and Pinot noir were bottled with increasing Cu concentrations and stored for 12 months in cellar conditions (i.e., 14 °C in darkness) and the periodic monitoring of Cu fraction 1 was carried out using stripping potentiometry. The full details of this study can be viewed within Zhang and Clark (2023).

The Cu concentrations in Pinot noir and Shiraz wines on the day of bottling were 0.43 ± 0.01 and 0.56 ± 0.02 mg/L, respectively. Immediately prior to bottling, an additional 0.3 mg/L or 0.6 mg/L of Cu(II) was added to various 750 mL wine bottles, while no Cu addition was made to '0 mg/L added' samples (i.e., the Control). After filling the bottles with wine, the headspace was sparged with CO_2 and the bottles sealed with screw cap closures and stored in cellar conditions (14 °C in darkness). After bottling, the total packaged oxygen in samples was largely depleted within the first 2 weeks of storage (Appendix 31a,b). During this time, the free and total SO_2 concentrations also decreased and then remained relatively stable for the remaining storage period (Appendix 31c,d).

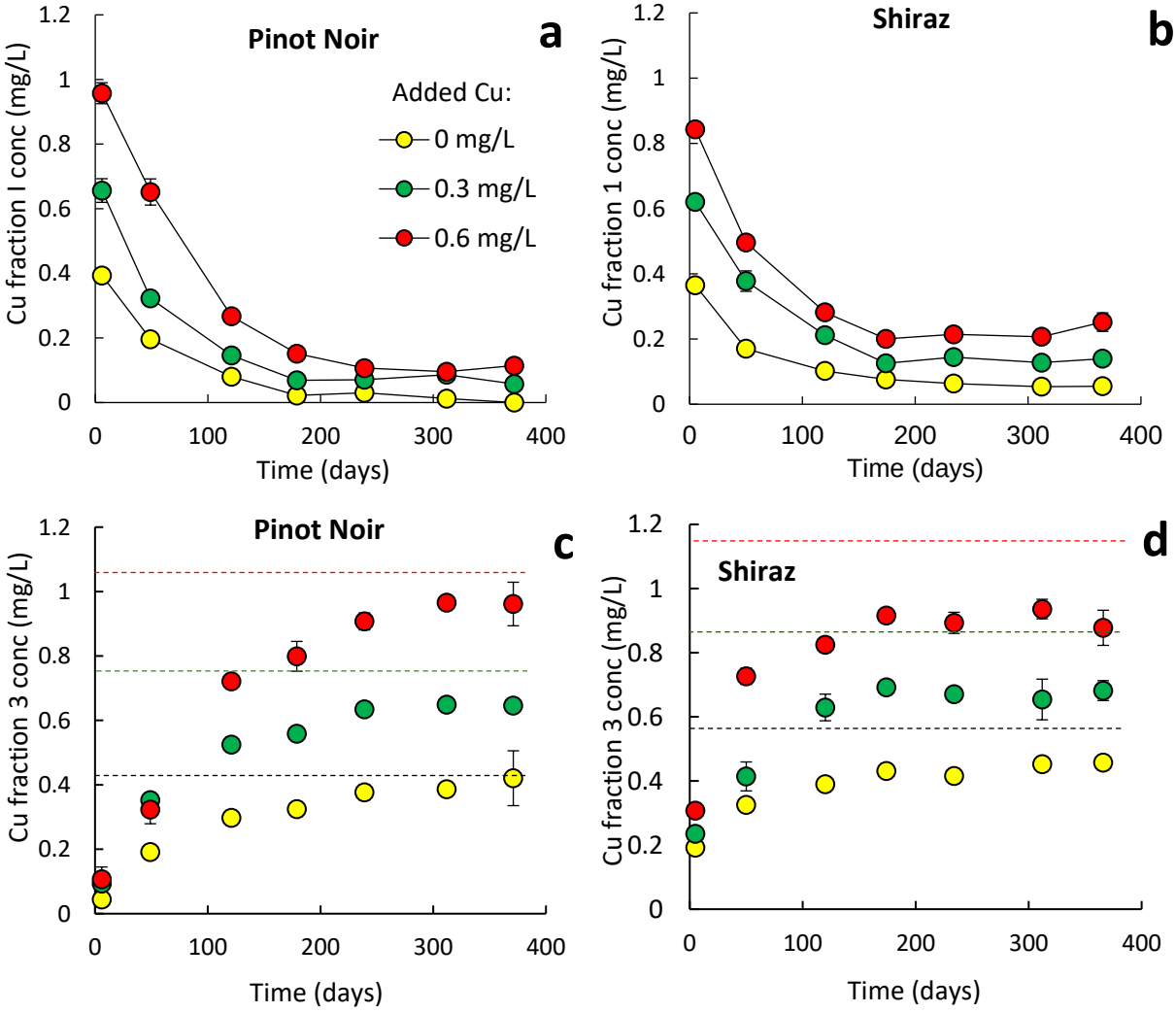


Figure 6.78. Cu fraction 1 (a, b) and Cu fraction 3 (c, d) concentrations, as determined by stripping potentiometry, in the Pinot noir and Shiraz wines during the storage experiment. Error bars represent the standard deviation of triplicate sample measures. The horizontal dashed lines in panels c and d represent the total Cu concentrations for the respective wines.

Figure 6.78 shows the Cu fraction 1 and 3 concentrations in the Pinot noir and Shiraz throughout the storage experiment. In both wines, the Cu fraction 1 concentration decreased (Figure 6.78a,b), and the Cu fraction 3 concentration increased during storage (Figure 6.78c,d). The decrease in Cu fraction 1 occurred over 0-6 months for samples with 0.3 and 0.6 mg/L Cu(II) addition at bottling, and over 0-4 months for samples with 0 mg/L Cu addition (i.e., the control) at bottling (Figure 6.78a,b). After these periods of time the Cu fraction 1 was depleted, reached a plateau or slightly increased (Figure 6.78a,b). The decay of Cu fraction 1 was found to be a first-order process, and the rates of decay remained relatively similar across the different Cu(II) treatments and the different wines (see the following Section [6.5.3.3]). The half-life associated with the first-order decay rates suggested that the Cu fraction 1 concentration would halve approximately every 2 months in these red wines.

Figure 6.78 also demonstrated that both red wines could convert the majority of Cu fraction 1 present at bottling to Cu fraction 3 during the storage period. In fact, the amount of Cu fraction 1 that underwent transition was high when compared to the typical concentrations of Cu found in wine. For example, the average total Cu concentration in 29 commercial red wines has been reported to be 0.3 mg/L (Kontoudakis et al., 2019b), and all but one of the red wine treatments in Figure 6.78 had > 0.3 mg/L of Cu fraction 1 transition to Cu fraction 3. Additionally, it is evident that increased Cu(II) additions at bottling led to higher Cu fraction 1 concentrations both at bottling and throughout aging (Figure 6.78a,b). This was particularly evident in the Shiraz wines, in which appreciable levels of Cu fraction 1 remained in the 0.3 and 0.6 mg/L Cu-treated wines, but not in the 0 mg/L treatment (Figure 6.78b). This implied that the unknown precursors for sulfide in the Shiraz wines, utilised for the conversion of Cu fraction 1 to 3, were depleted in the wines treated with 0.3 and 0.6 mg/L Cu(II) at bottling.

Based on the high capacity for red wine to transform Cu fraction 1 to 3, it is difficult to envisage Cu treatment during wine production that would comply with good practice (i.e., Cu legal limits, avoiding Cu-induced haze) and permanently maintain Cu fractions 1 concentrations above 0.02 mg/L. In the present study, plateau concentrations of > 0.02 mg/L for Cu fraction 1 were observed in the red wines (Figures 6.78a and b), but this was only achieved with relatively high total Cu concentrations in the wines (i.e., > 0.39 mg/L). Instead, it would appear more feasible that Cu additions be made to wine at bottling to maintain Cu fraction 1 concentrations at > 0.02 mg/L for a certain length of time (e.g., 4 to 6-months). However, further studies are required to determine what the optimum length of time might be. Also, further studies may elucidate whether a wine can become ‘permanently’ resistant to reductive development once a certain amount of Cu fraction 1 has been converted to Cu fraction 3.

6.5.3.3 The variability in Cu fraction 1 and 2 loss during the aging of different wines in ideal conditions.

Previous sections of this report (i.e., 6.5.3.1 and 6.5.3.2) showed that Cu fractions 1 and 2 can protect against reductive aroma development in wine but they decrease during the aging of bottled wine in low oxygen conditions. A combined Cu fraction 1 and 2 concentration of greater than 0.02 mg/L was found in Section 6.3.2 to supress free H_2S accumulation, and concentrations of Cu fraction 1 greater than 0.03 mg/L was found to supress free methanethiol. This link provides confidence for the action of these Cu fractions against reductive aroma development while they remain above the critical concentrations. This section focuses on the rate of change for Cu fractions 1 and 2 across different wines during their aging in low oxygen conditions. If the decay rates for Cu fraction 1 and 2 are similar across all wines, then the duration of protection offered against reductive aroma accumulation may be able to be accurately predicted from the Cu fraction 1 and 2 concentrations at bottling. However, if the decay rates for the Cu fractions are highly variable across different wines, then prediction based upon the average decay rate across different wines would become highly inaccurate. Already, the presence of ascorbic acid in white wine and rosé (Sections 6.4.1 and 6.5.2.1) was observed to accelerate the loss of Cu fractions 1 and 2. This suggested that there was likely to be wine matrix effects that would make the decay rates for Cu fractions 1 and 2 highly variable in wine.

Eight white wines and six red wines (Table 6.12) were aged in low oxygen conditions for a minimum of 12-months. Four of the white wines and two of the red wines were bottled with multiple Cu concentrations.

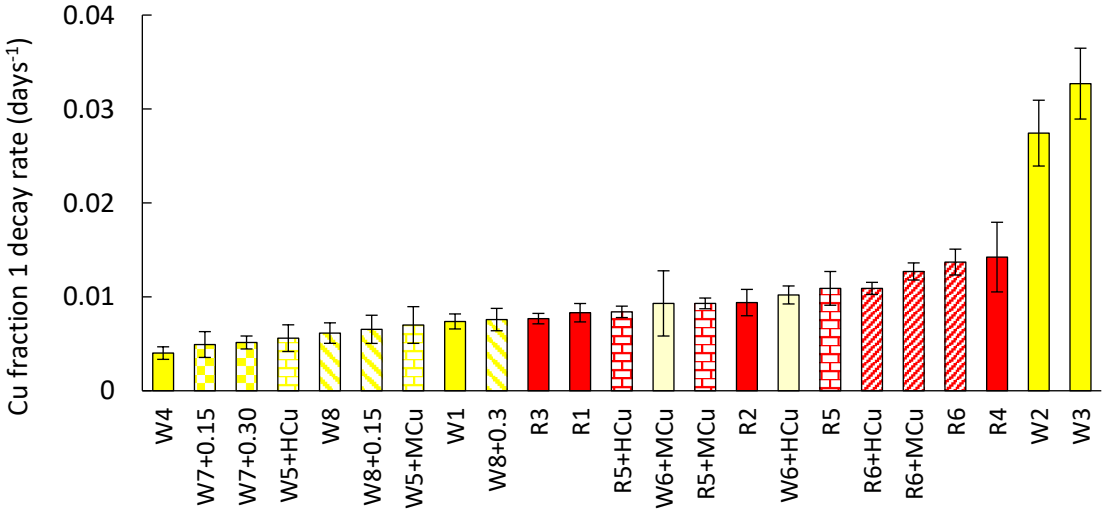


Figure 6.79. The first order decay rates for Cu fraction 1 in 8 white wines and 6 red wines. Four of the eight wines (W5-W8) and two of the red wines (R5-R6) had the rates determined at multiple total Cu concentrations. The error bars represent the 95 % confidence limits and data without the overlap of error bars are deemed significantly different ($p < 0.05$).

Table 6.12 shows the first order Cu fraction 1 decay rates and the associated half-lives for all wines. The half-lives indicate the time required for the Cu fraction 1 to decrease in concentration by a half. In Figure 6.79, the wines are presented in the order of increasing Cu fraction 1 decay rates. The wines with the most contrasting decay rates (i.e., W3 and W4, Figure 6.79) have an 8-fold difference in their corresponding rates, which is also reflected in the half-life values being 21 and 170 days, respectively. Generally, the red wine had a faster rate of Cu fraction 1 loss than white wines, but this was not always

Table 6.12. Data associated with the first-order decay of Cu fractions in white and red wines. The uncertainties quoted are the 95 % confidence limits.

white wine	variety	ascorbic acid at bottling (mg/L)	Cu fraction 1		Combined Cu fraction 1 + 2	
			decay rate ($days^{-1}$)	half-life (days)	decay rate ($days^{-1}$)	half-life (days)
W1	Chardonnay	0	0.0074 ± 0.0008	94 ± 10	0.0044 ± 0.0003	158 ± 12
W2	Chardonnay	0	0.027 ± 0.004	25 ± 3	0.015 ± 0.004	47 ± 12
W3	Sauvignon blanc	20	0.032 ± 0.004	21± 2	0.014 ± 0.003	49 ± 11
W4	Chardonnay	60	0.0040 ± 0.0007	170 ± 30	0.0031 ± 0.0003	224 ± 21
W5+0.3Cu[1]	Chardonnay	0	0.007 ± 0.002	99 ± 30	0.0081 ± 0.0005	86 ± 5
W5+0.6Cu[1]	Chardonnay	0	0.006 ± 0.001	120 ± 30	0.007 ± 0.001	98 ± 15
W6+0.3Cu[1]	Pinot grigio	0	0.009 ± 0.003	75 ± 30	0.007 ± 0.001	96 ± 20
W6+0.6Cu[1]	Pinot grigio	0	0.010 ± 0.001	68 ± 6	0.007 ± 0.002	100 ± 23
W7	Sauvignon blanc	20	not determined (n.d.)	n.d.	0.0075 ± 0.0004	92 ± 4
W7+0.15Cu	Sauvignon blanc	20	0.005 ± 0.001	110 ± 40	0.0051 ± 0.0003	136 ± 7
W7+0.3Cu	Sauvignon blanc	20	0.0051 ± 0.0007	135 ± 18	0.0034 ± 0.0002	202 ± 11
W8	Sauvignon blanc	0	0.006 ± 0.001	110 ± 20	0.0055 ± 0.0009	126 ± 20
W8+0.15Cu	Sauvignon blanc	0	0.007 ± 0.002	106 ± 26	0.0043 ± 0.0004	163 ± 17
W8+0.3Cu	Sauvignon blanc	0	0.008 ± 0.001	91 ± 15	0.0031 ± 0.0003	226 ± 22
average			**0.010 ± 0.009**	**100 ± 40**	**0.007 ± 0.004**	**130 ± 60**

red wine	variety	Cu fraction 1	
		decay rate (days)	half-life (days)
R1	Pinot noir	0.008 ± 0.001	83 ± 10
R2	Shiraz, Cab. Sauv.	0.009 ± 0.001	74 ± 11
R3	Cab. Sauv./Merlot	0.0077 ± 0.0006	90 ± 7
R4	Cab. Sauv./Merlot	0.014 ± 0.004	49 ± 14
R5[2]	Shiraz	0.011 ± 0.002	64 ± 11
R5+0.3Cu[2]	Shiraz	0.0093 ± 0.0006	75 ± 5
R5+0.6Cu[2]	Shiraz	0.0084 ± 0.0006	83 ± 6
R6[2]	Pinot noir	0.014 ± 0.001	51 ± 5
R6+0.3Cu[2]	Pinot noir	0.0127 ± 0.0009	55 ± 4
R6+0.6Cu[2]	Pinot noir	0.0109 ± 0.0006	64 ± 4
average		**0.011 ± 0.02**	**69 ± 15**

[1] From the study of Zhang et al. (2022). [2] From the study of Zhang and Clark (2023).

the case (e.g., W2, W3 and W6). The same wine with different total Cu concentrations at bottling had similar Cu fraction 1 rates despite the variable concentrations at bottling. These results show that the wine matrix has a significant impact on the rate of Cu fraction 1 decay and the variability appears higher for white wines compared to red wines. While the range of half-lives for the white wines varied from 21 to 170 days, the range in red wines was 49-90 days. Ascorbic acid alone could not account for the variability observed for white wines as the wines with the most contrasting Cu fraction 1 decay rates (i.e., W3 and W4) both contained ascorbic acid (Table 6.12).

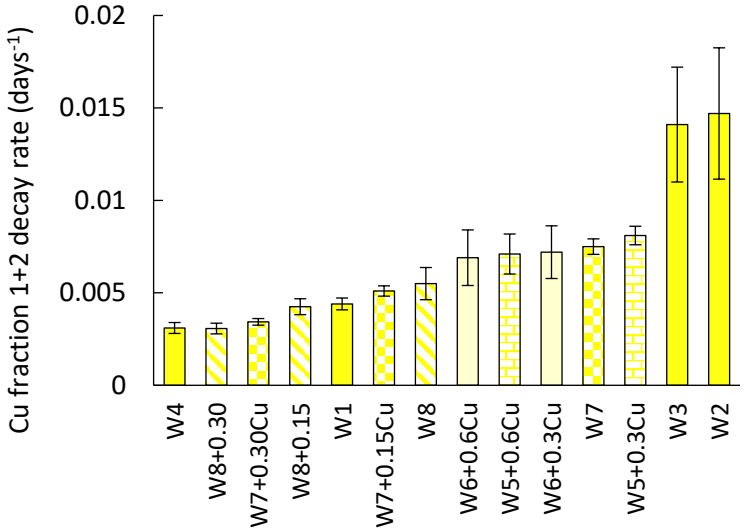


Figure 6.80. The first order decay rates for the combined concentrations of Cu fraction 1 and 2 in 8 white wines. Four of the eight wines (W5-W8) had the rates determined with multiple total Cu concentrations. Note that the y-axis scale is half that of scale in Figure 6.79. The error bars represent the 95 % confidence limits and data without the overlap of error bars are deemed significantly different ($p < 0.05$).

The combined Cu fraction 1 and 2 decay rates are shown in Figure 6.80, and reported in Table 6.12, and were smaller in magnitude than the corresponding Cu fraction 1 rates. As was the case for Cu fraction 1 (Figure 6.79), there was high variability in the decay rates for combined Cu fraction 1 and 2 concentrations across different wines (Figure 6.80). The wines with the most different decay rates for Cu fraction 1 (i.e., W4 versus W2 and W3) also had the largest difference in combined Cu fraction 1 and 2 decay rates (Figure 6.80). For the combined Cu fraction 1 and 2 decay rates, W4 and W2 had a 5-fold rate difference with half-lives of 224 and 47 days, respectively. Unlike the rates for Cu fraction 1 (Figure 6.79), the wines bottled with different Cu concentrations had significant differences in the combined Cu fraction 1 and 2 decay rates. The decay rates for W7, W7+0.15Cu and W7+0.3Cu (Figure 6.80) are good examples of these differences. The fact that the Cu fraction 1 decay rates were similar for W7, regardless of initial Cu concentration, while the combined Cu fraction 1+2 decay rates were different suggested that the Cu fraction 2 concentration was the cause of the variability for the latter decay rates.

These results show that it is not feasible to use the average decay rates across different wines to predict that rate of loss for Cu fractions in a specific wine. Although it may be accurate for some wines, it could be highly inaccurate for others. The variability in precursors for Cu fraction 3 formation in wines would appear to be significantly impacting the rates for Cu fraction 1 and 2 loss. Consequently, providing guidelines for minimum Cu fraction 1 and 2 concentrations at bottling to ensure prevention against reductive aroma accumulation for a certain duration is not currently possible using the determined decay rates. Alternative possibilities for guidelines will be investigated in future sections of this report, including the use of accelerated aging studies, and identifying whether Cu fractions 1 and 2 can offer protection against reductive aroma accumulation even after their depletion (i.e., a legacy effect).

6.5.3.4 Pathways for the change in Cu fractions and accumulation of reductive aroma compounds.

The white and red bottle aging trials provide insight into the pathways allowing reductive aroma compounds to accumulate in wine despite the presence of Cu.

Figure 6.81 shows the proposed pathway for white wines. In low oxygen conditions, Cu fractions 1 and 2 appear to be in equilibrium with each other, and both forms have the potential for conversion to Cu fraction 3. Elevated oxygen concentrations can lead to a decrease in Cu fraction 3 concentration and an increase in Cu fraction 1 (Figure 6.81) (Kontoudakis and Clark, 2020). However, the previous sections of this report showed that wine required saturated oxygen conditions or high oxygen ingress closures to enable significant loss in Cu fraction 3. The presence of some oxygen at bottling (i.e., total packaged oxygen) tended to slow the loss of Cu fractions 1 and 2 in many wines but not lead to extensive increases. The Cu fraction 2 concentrations at bottling appeared to be dependent upon the concentration of thiol compounds in wines capable of complexing Cu(II) and thereby influencing the equilibrium between Cu fractions 1 and 2 (Figure 6.81).

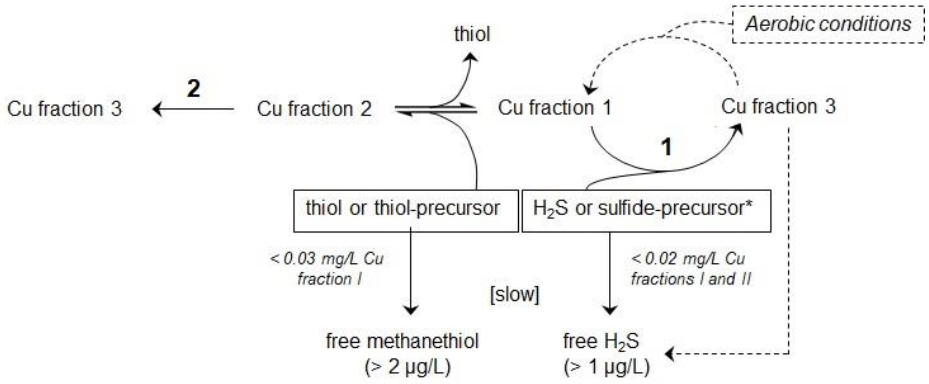


Figure 6.81. The links between Cu fractions and accumulation of free H_2S or free methanethiol during the aging of white wine in low oxygen conditions. Adapted from Zhang et al. (2022).

At low concentrations of Cu fraction 1 (<0.03 mg/L), accumulation of free methanethiol can occur, while at low combined concentrations of Cu fractions 1 and 2 (<0.02 mg/L) accumulation of free H_2S can occur (Figure 6.81). It is proposed that a pool of precursors (Figure 6.81) exist that are responsible for the slow release of the free sulfhydryl compounds when the concentrations of Cu fractions 1 and 2 are low (e.g., < 0.015 or 0.035 mg/L). The identities of these precursors are not certain but could include thiol compounds, disulfides, diorganopolysulfanes and thioacetates (Bekker et al. 2018; Jastrzembski et al. 2017; Kreitman et al. 2019).

In most cases, there was a significant lag period between the time that Cu fractions 1 and/or 2 were below the key indicator concentrations (i.e., 0.015 or 0.035 mg/L) and the time for the free sulfhydryl compounds to accumulate to above odour thresholds. The duration of the lag period is likely to be shortened by lower oxygen concentrations at bottling, the presence of reducing agents (e.g., ascorbic acid) and non-ideal storage conditions. The latter could include elevated temperatures (Franco-Luesma and Ferreira 2016b), light exposure and/or contact with metallic aluminium (i.e., aluminium can containers for wine) that will be investigated further in subsequent sections.

Figure 6.82 shows the proposed pathway for Cu fraction transformations in red wine. As for white wine, sufficiently high oxygen conditions, Cu fraction 3 can be rapidly converted to Cu fraction 1 (Figure 6.82). In low oxygen conditions, Cu fraction 1 present at bottling can readily source sulfide from precursors and undergo first-order decay to form Cu fraction 3 (Figure 6.82). Cu fraction 2, which is common in white

wine but has not been detected in red wine (Clark *et al*. 2020), is not essential for the conversion of Cu fraction 1 to Cu fraction 3. The first-order decay of Cu fraction 1 demonstrated lower stability for precursors in the presence of higher concentrations of Cu fraction 1. Once Cu fraction 1 concentration falls below the critical concentrations of 0.02-0.03 mg/L (Kontoudakis *et al*. 2019), the accumulation of the reductive aroma compounds can occur from a slow degradation of precursors. As for white wine, the lag period between loss of Cu fraction 1 and the accumulation of the reductive aroma compounds requires further investigation.

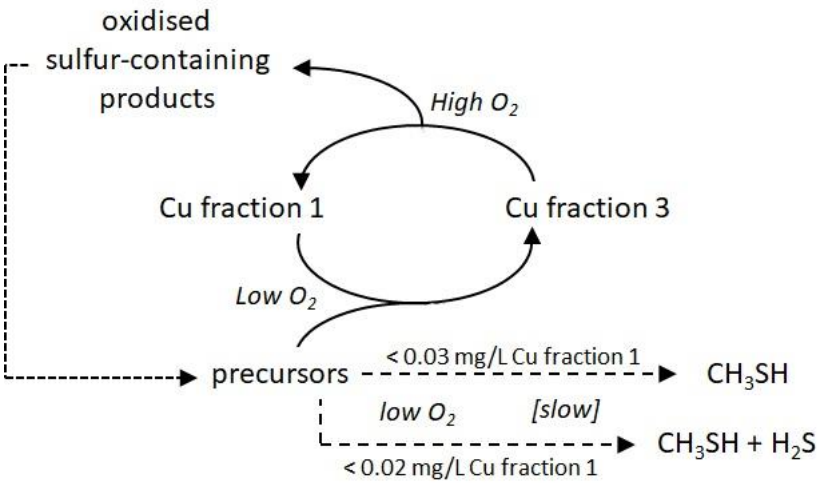


Figure 6.82. The transition between Cu fraction 1 and Cu fraction 3 in red wine, and the potential accumulation of methanethiol (CH_3SH) and hydrogen sulfide (H_2S) under conditions of low Cu fraction 1 concentration.

6.5.4 The ability of polysulfanes to exert an influence on Cu fractions.

Polysulfanes are known precursors for hydrogen sulfide and thiol compounds in wine (Kreitman et al. 2017). They are formed during the oxidation of thiol compounds and hydrogen sulfide in the presence of Cu(II), and have the thiol units at the ends of a sulfur-atom chain (Figure 6.83). Polysulfanes with different sulfur chain lengths can form (Figure 6.84) but they often become less stable with increasing chain length. The thiol compounds found at higher concentrations in wine, such as glutathione, are the most common units attached to the ends of the sulfur atom chain.

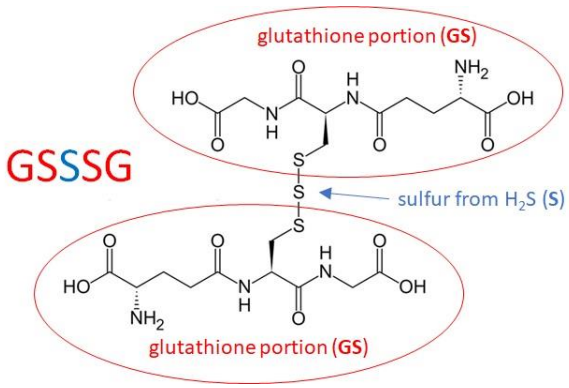


Figure 6.83. An example of a polysulfane. In this case the polysulfane consists of two units stemming from glutathione (GS) capping one sulfur atom stemming from hydrogen sulfide (S) and is represented by 'GSSSG'. Polysulfanes can be formed from any compound containing a thiol group (e.g., cysteine, methanethiol).

An aim of this work was to establish if the glutathione-based polysulfanes (Figure 6.84) or cysteine-based polysulfides could bind Cu(II) sufficiently to prevent its detection by stripping potentiometry. It would also investigate conditions that would induce polysulfane degradation and enable the supply of binding agents to Cu(II). For example, the supply of hydrogen sulfide and certain thiol compounds by polysulfanes could affect a decrease in Cu fraction 1 and an increase in Cu fractions 2 and 3. The interaction between 0.6 mg/L Cu(II) and the polysulfanes were monitored over time (at 25 °C) in anaerobic conditions both with and without 50 mg/L sulfur dioxide present. The experiments were conducted in a model wine system composed of 12 %(v/v) aqueous ethanol and buffered to pH 3.6 with tartaric acid and potassium hydrogen tartrate, and also with 100 mg/L caffeic acid. The polysulfanes were synthesised and purified at Auckland University by Dr Lisa Pilkington from Prof Bruno Fedrizzi's group, and this work was conducted in collaboration with Dr Rebecca Deed. The glutathione-based polysulfane species isolated were those shown in Figure 6.84.

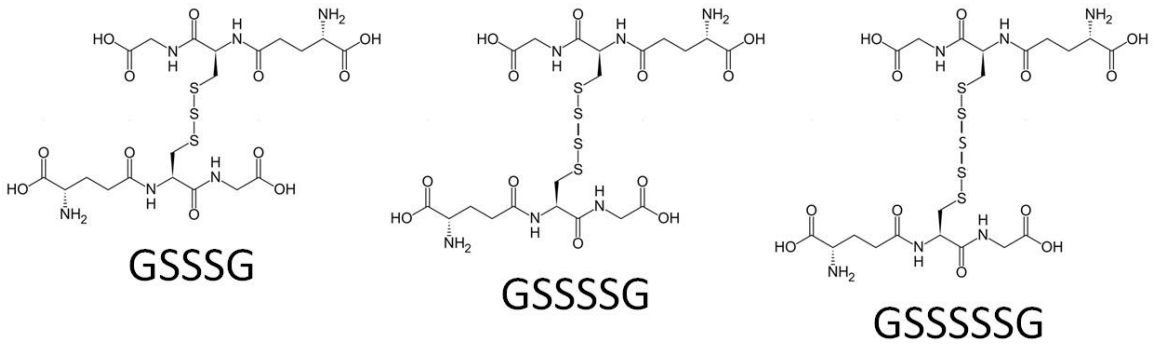


Figure 6.84. The structures of polysulfanes formed from glutathione and hydrogen sulfide with increasing numbers of sulfur atoms between the glutathione units.

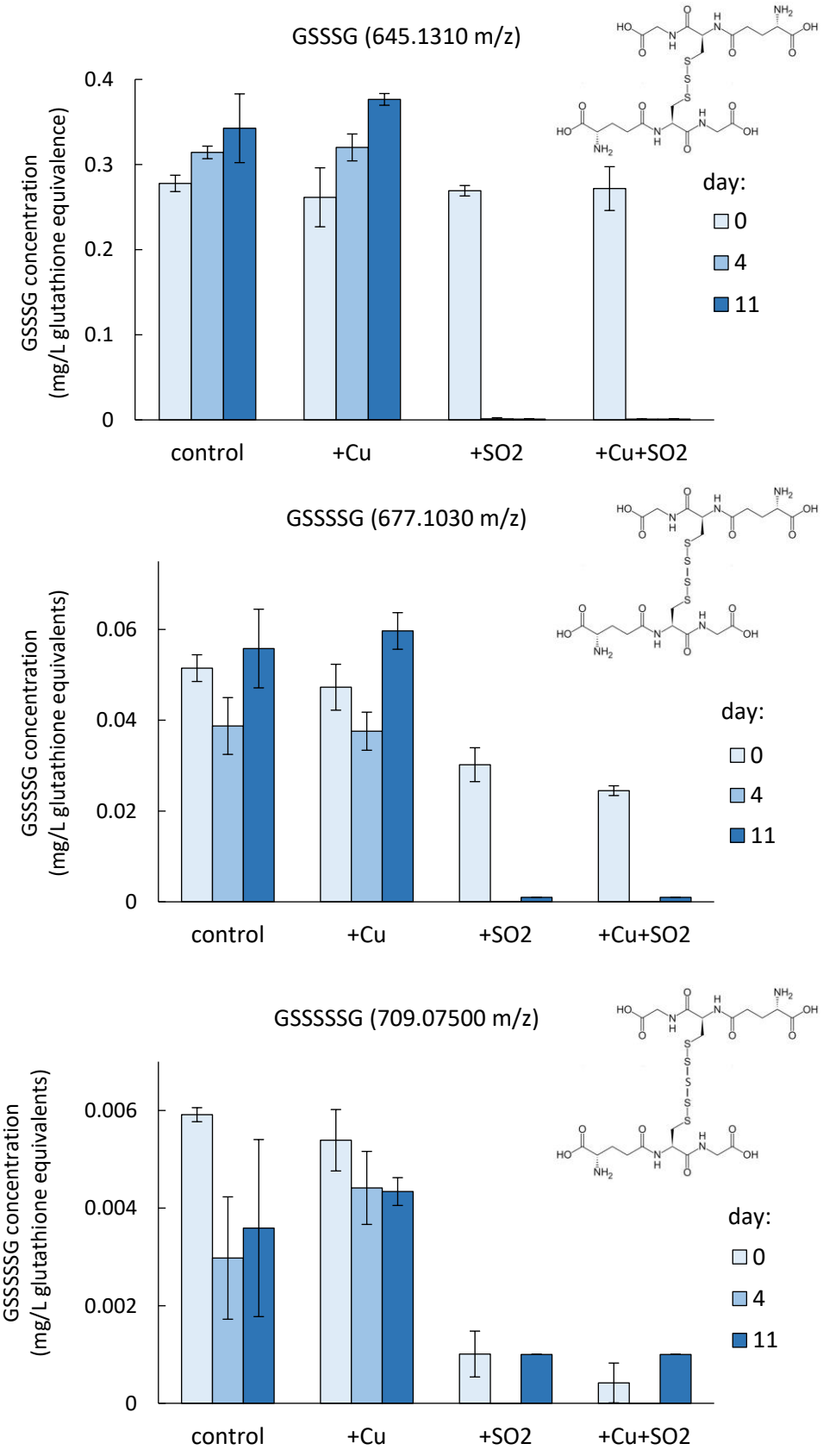


Figure 6.85. The concentrations of the glutathione-based polysulfanes GSSSG (a), GSSSSG (b) and GSSSSSG (c) in model wine samples. +Cu = addition of 0.6 mg/L Cu, +SO2 = addition of 50 mg/L sulfur dioxide.

Samples were analysed by LC-QTOF for polysulfanes, stripping potentiometry for Cu fraction 1 and an automated analyser for sulfur dioxide concentrations. Quantification on the LC-QTOF was conducted by performing an external calibration graph using glutathione and its accurate mass of 308.0910 m/z. The diglutathionylpolysulfanes were quantified based on their accurate mass values, for example, using 645.1310 m/z for GSSSG, 677.1030 m/z for GSSSSG and 709.0750 m/z for GSSSSSG. Confirmation of the identity of the ions was gained from the matching of high-resolution mass fragmentation data to that of Bekker et al. (2018).

Figure 6.85 shows the concentrations for the different chain length glutathione-based polysulfanes in the model wine system either alone (Control) or with 0.6 mg/L Cu(II) (+Cu), 50 mg/L sulfur dioxide (+SO2) or both (+Cu+SO2). The samples were analysed by LC-QTOF on day 0, several hours after their preparation, and then again after 4 and 11 days. The results show that all polysulfanes in the control samples were stable over 11 days, regardless of the polysulfane chain length (Figure 6.84). The same was the case for the polysulfanes in the presence of Cu(II), implying that Cu(II) was not degrading the polysulfanes or binding to them with sufficient strength to impede their detection. In contrast, the samples with sulfur dioxide present ('+SO2' and '+Cu+SO2') had all the polysulfane disappear by day 4. In fact, the larger chain polysulfanes of GSSSSG and GSSSSSG had lower concentrations at day 0 in the sulfur dioxide-containing samples than in the Control, implying that the degradation of the polysulfanes by sulfur dioxide was rapid. It is postulated that polysulfanes may undergo sulfitolysis by sulfur dioxide and yield thiosulfates, whereby the thiol compound from the polysulfane is eliminated with a sulfate unit attached (i.e., GS-SR + HSO_3^- → $GSSO_3^-$ + HSR).

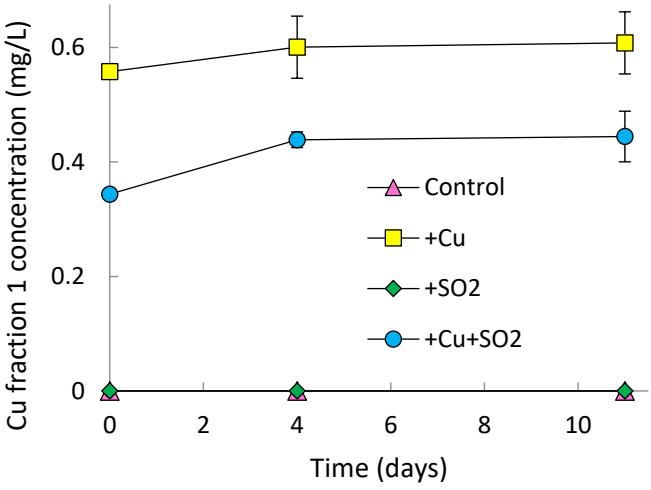


Figure 6.86. The Cu fraction 1 concentrations in the model wine samples. +Cu = addition of 0.6 mg/L Cu, +SO2 = addition of 50 mg/L sulfur dioxide.

Figure 6.86 shows the Cu fraction 1 concentrations in the model wine samples during the 11 days storage period. No Cu is present in the Control and '+SO2' samples as expected, given they had no Cu added to them, while the '+Cu' sample had all the added 0.6 mg/L Cu(II) in the form of Cu fraction 1 throughout the 11-day period. Alternatively, the sample with both 0.6 mg/L Cu and 50 mg/L sulfur dioxide added (i.e., '+Cu+SO2') has a reduced concentration of Cu fraction 1, which is around 0.4 mg/L throughout the storage period. This implies that when the glutathione-based polysulfanes were degraded by sulfur dioxide, some

of the degradation products were able to interact with Cu and transition it from Cu fraction 1. Presumably this was through the release of glutathione to form Cu fraction 2, and/or sulfide to form Cu fraction 3.

A similar experiment was conducted with the cysteine-based polysulfanes (Figure 6.87). In this case, the model wine samples were analysed on day 0 and 4 after the same treatments as for the glutathione-based polysulfanes.

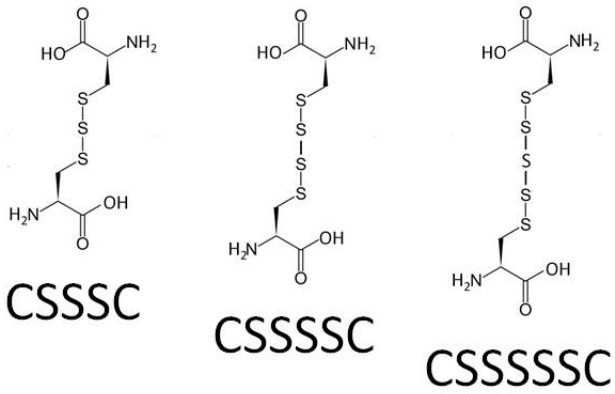


Figure 6.87. The structures of polysulfanes formed from cysteine and hydrogen sulfide with increasing numbers of sulfur atoms between the cysteine units.

Figure 6.88 shows the concentration of the different cysteine-based polysulfanes on day 0 and 4 of the experiment. There was greater uncertainty with the measured concentrations for the cysteine-based polysulfanes (Figure 6.88) compared to the glutathione-based polysulfanes (Figure 6.85) mainly due to the lower concentrations of the former compared to the latter. Similar to the glutathione-based polysulfanes, the cysteine-equivalents were stable in the model wine alone or with Cu(II) but unstable when sulfur dioxide was present (Figure 6.88). When the Cu fraction 1 concentrations were measured on day 4 the '+Cu' sample had all the added 0.6 mg/L Cu(II) present as Cu fraction 1 (i.e., 0.62 ± 0.03 mg/L), while the '+Cu+SO2' sample had a decrease in Cu fraction 1 (i.e., 0.53 ± 0.01 mg/L).

Table 6.13. The concentration of salt-treated hydrogen sulfide in the cysteine-based polysulfanes at day 0 and 4. Where '+Cu' is 0.6 mg/L Cu(II) and '+SO2' is 50 mg/L sulfur dioxide. The LOD for the salt-treated H_2S was 0.3 µg/L.

cysteine-based polysulfane samples	salt-treated H_2S (µg/L)	
	day 0	day 4
control	<LOD	<LOD
+Cu	<LOD	<LOD
+SO2	0.36 ± 0.02	0.34 ± 0.07
+Cu+SO2	0.92 ± 0.06	2.98 ± 0.03

The concentration of salt-treated hydrogen sulfide was also measured in these samples (Table 6.13). The salt-treated hydrogen sulfide is a measure of free hydrogen sulfide as well as a component of sulfide bound to Cu(II). The results showed negligible salt-treated hydrogen sulfide in the control and '+Cu' samples (Table 6.13) implying that the polysulfanes in these samples had not degraded to produce hydrogen sulfide or Cu-bound sulfide.

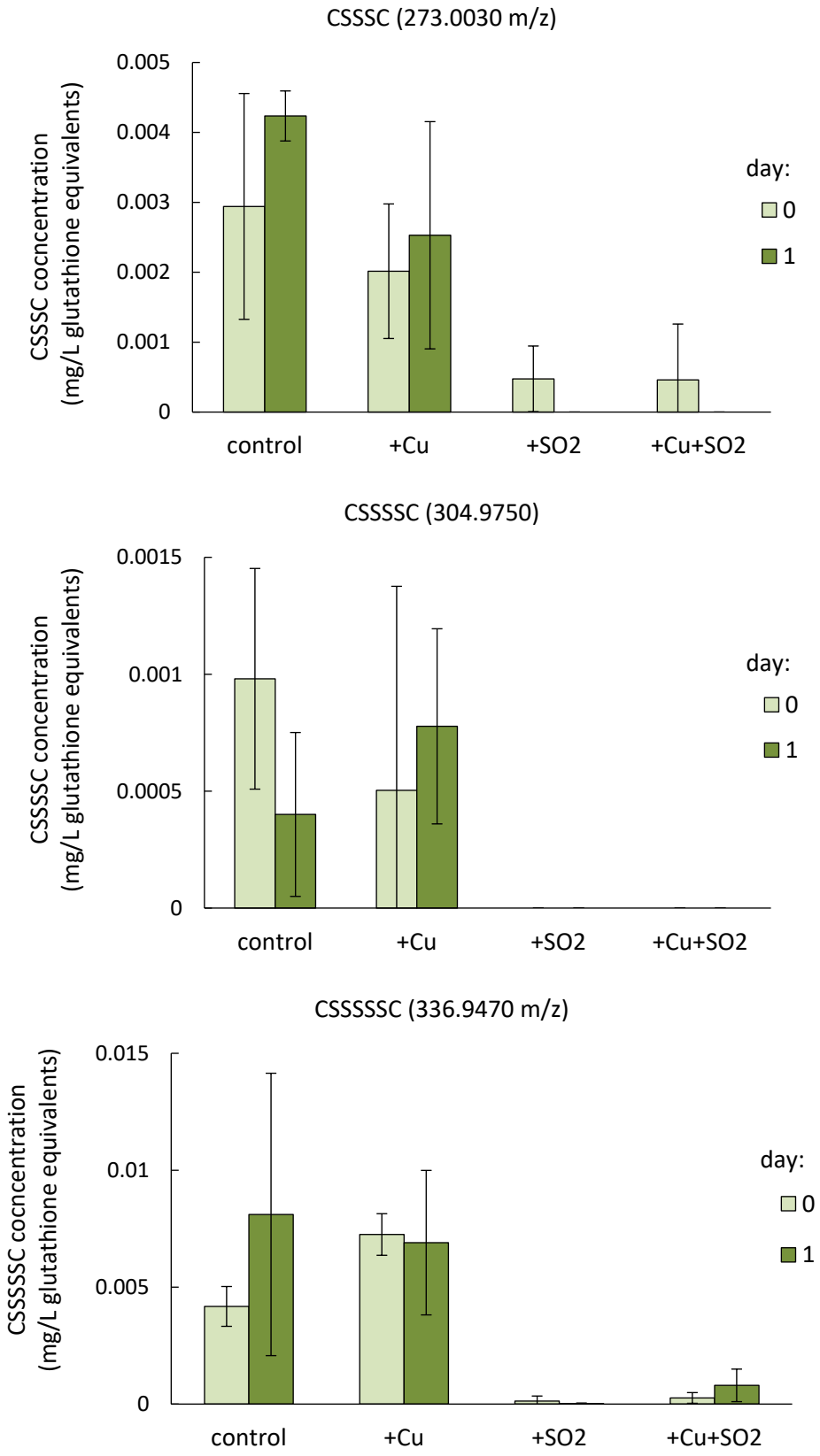
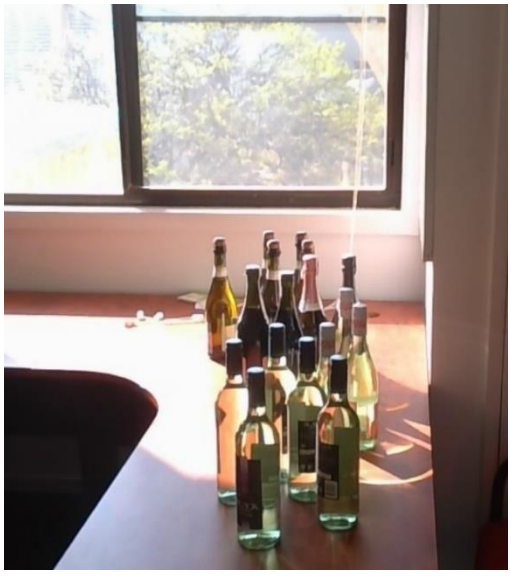


Figure 6.88. The concentrations of the cysteine-based polysulfanes CSSSC (a), CSSSSC (b) and CSSSSSC (c) in model wine samples. '+Cu' = addition of 0.6 mg/L Cu, and '+SO2' = addition of 50 mg/L sulfur dioxide.

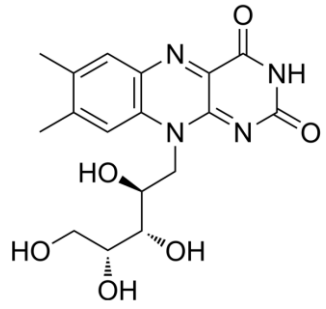
Alternatively, the '+Cu+SO2' sample showed the highest concentration of salt-treated hydrogen sulfide (Table 6.13), which in combination with the decrease in Cu fraction 1, suggests that the treatment of '+Cu+SO2' to the polysulfanes resulted in their degradation and formation of copper sulfides (i.e., Cu fraction 3). The '+SO2' sample had a minor amount of salt-treated hydrogen sulfide but 10-fold lower than observed in the '+Cu' sample. Therefore, Cu(II) can sequester sulfide from polysulfides but only after their degradation by sulfur dioxide. Without Cu(II) present, the yield of hydrogen sulfide from sulfur dioxide-degraded polysulfanes was low. Further work is required to establish if the products formed from the sulfur dioxide-induced degradation of polysulfanes in the absence of Cu(II) may be important sources of slowly released hydrogen sulfide and thiol compounds during the bottle aging of wine.

The glutathione and cysteine-based polysulfanes appeared stable in the model wine system, and also in the presence of Cu(II), during an 4 or 11-day storage period. Sulfur dioxide efficiently degraded the polysulfanes, and when this occurred in the presence of Cu(II), copper sulfides were generated as well as possibly some Cu(I)-thiol complexes.

6.5.5 The impact of light exposure on Cu fractions in white wine.



riboflavin



iron(III) tartrate

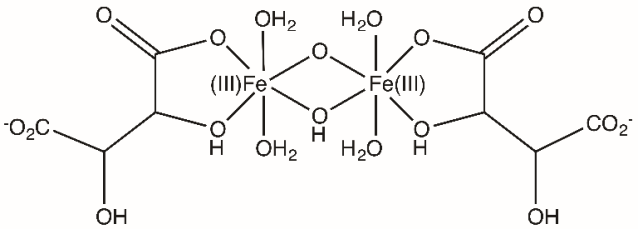


Figure 6.89. The exposure of wine to light during the non-ideal storage of wine and the chemical structures for riboflavin and an iron(III) tartrate complex.

This work was conducted by Charles Sturt University (CSU) PhD student Ms Isara Vongluanngam as part of her PhD project, funded by the Australian Research Council Industrial Training Centre for Innovation Wine Production, and titled 'The impact of light on oxidative and reductive aging of wine'. A section of her PhD overlapped with this current Wine Australia project (CSU 1702-4) on the topic of the changes of Cu fractions in wine with light exposure. The full details of the experiments can be found within Vongluanngam et al. (2024, 2025). Her PhD work was supervised by investigators on this current Wine Australia project (i.e., Dr John Blackman, Prof Andrew Clark, Prof Leigh Schmidtke and Dr Xinyi Zhang all from CSU), as well as by an external supervisor Prof. Kerry Wilkinson (The University of Adelaide).

Light can cause rapid and detrimental compositional changes to wine. Excessive exposure is possible during display of wine bottles in retail outlets or when they are stored in non-ideal conditions by a consumer (Figure 6.89). One outcome is the emergence of a 'light struck' character that can have a range of sensory characters including cooked cabbage, corn nuts, soy/marmite, wet dog and wet wool (Dozon and Nobel 1989).

Riboflavin and iron(III) organic acid complexes are two key photoactive agents in wine that initiate compositional changes in wine (Figure 6.89). Most of the riboflavin present in wine is extracted from yeast during alcoholic fermentation and concentrations greater than 0.05 to 0.08 mg/L can induce light-struck characters (Mattivi et al. 2000). Light within the wavelength range of 300 to 510 nm can excite riboflavin and cause a cascade of reactions leading to the accumulation of spoilage aroma compounds, including the sulfur-containing compounds of methional, methanethiol and dimethyldisulfide.

Alternatively, iron(III) organic acid complexes, such as iron(III) tartrate, have been found to be photoactive at wavelengths below 520 nm. They can produce a range of carbonyl compounds capable of initiating colour and aroma changes to wine.

The impact on light on the Cu fractions in wine is currently unknown. However, given the tendency for thiol compounds to accumulate in wine due to light exposure, it is likely that Cu fractions 1 and 2 concentrations will be impacted. If light exposure significantly accelerated the loss of Cu fractions 1 and 2 then it may have ramifications for the future development of the wine.

This section of work was conducted to address three key questions:

- Does light impact the concentration of the protective Cu fractions in wine (i.e., Cu fractions 1 and 2)?
- Do the Cu fractions in wine offer protection against the light-induced spoilage of wine?
- Does the protection offered by Cu depend on whether the photochemistry is dominated by riboflavin or some other photoactive agent?

The initial set of experiments involved the bottling of a Chardonnay wine in low oxygen conditions with the addition of 0.5 mg/L riboflavin and 0.3 mg/L Cu(II) (as copper(II) sulfate pentahydrate). The final measured concentrations of riboflavin and total Cu in the wine were 0.62 ± 0.03 and 0.54 ± 0.02 mg/L, respectively. The wines were bottled in an anaerobic hood in 750 mL glass Bordeaux-style bottles and sealed with tin liner screw cap closures. Bottle colours used comprised of flint (F), arctic blue (AB), French green (FG), antique green (AG) and amber (A) and their transmission spectra are shown in Figure 6.90. The dissolved oxygen concentrations in the wines after bottling was below 0.3 mg/L.

The irradiation of samples was conducted in a temperature-controlled incubator fitted with internal black-screen coverings to limit light reflection. Wine samples were irradiated with two 18 W florescent lights (Osram Lumilux®) that emitted cool white light (4000 K) with a luminous flux of 1300 lm. Wine samples were placed 13 cm from the light source which gave an average light intensity of approximately 100 µmol/m^2/s, as determined by a LICOR Biosciences LI-185 A photometer. Control samples (i.e., samples stored without light exposure) were covered with aluminium foil before being stored in the same incubator as the light-exposed samples.

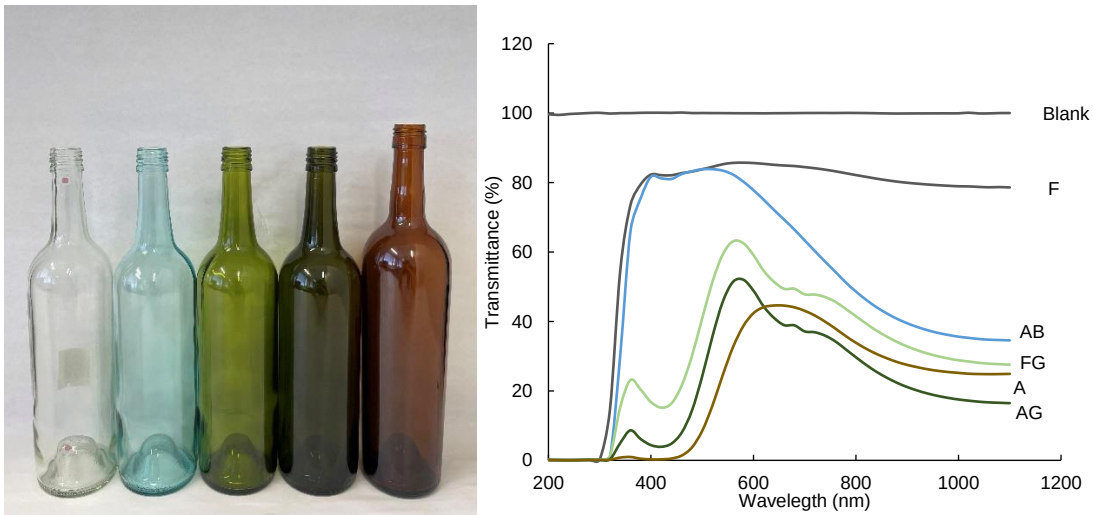


Figure 6.90. Bordeaux-shaped bottles of different colours (flint (F), arctic blue (AB), French green (FG), antique green (AG) and amber (A)) and their corresponding transmission spectra.

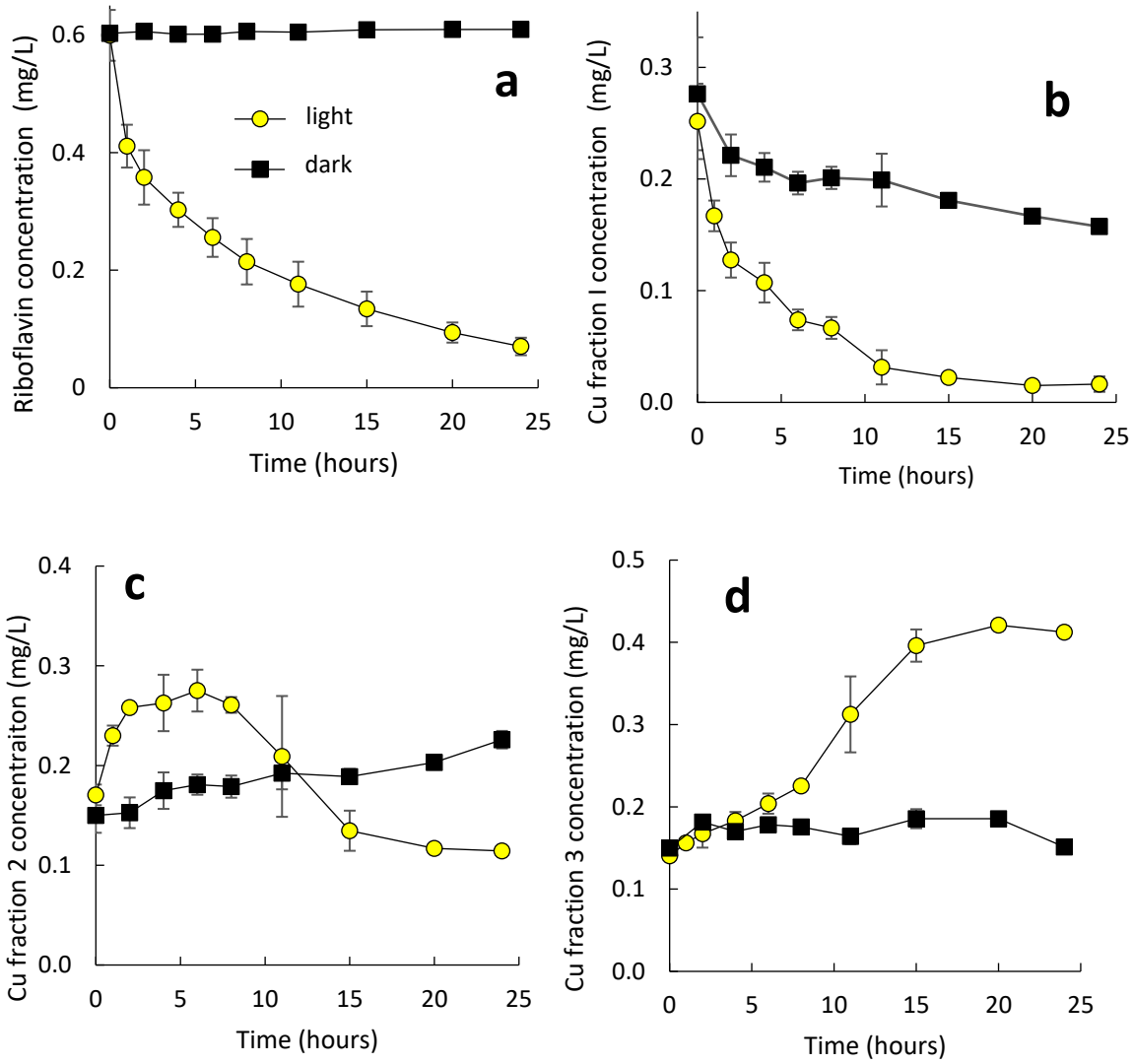


Figure 6.91. The changes in concentration (mg/L) of riboflavin (a), Cu fraction 1 (b), fraction 2 (c) and fraction 3 (d) for Chardonnay wine in flint bottles in low oxygen concentrations. Error bars represent the standard deviation (n=3) for the measurement of triplicate samples within each treatment.

Figure 6.91 shows the concentrations of riboflavin and Cu fractions for the Chardonnay in flint bottles that were either stored in the light or in darkness. As expected, the riboflavin concentration (Figure 6.91a) decreased rapidly, from 0.62 to 0.07 mg/L, during 1 day of light exposure while the sample stored in darkness had a constant concentration over the same time frame.

Like riboflavin, the Cu fraction 1 concentration decreased dramatically with one day of light exposure (i.e., a 97 % loss) (Figure 6.91b). There was also some Cu fraction 1 decrease for the sample stored in darkness but much less than induced by light exposure (Figure 6.91b). Cu fraction 2 concentration initially increased but then decreased to 60 % of its initial value with light exposure (Figure 6.91c), while the sample stored in darkness had a gradual increase in Cu fraction 2. Cu fraction 3 concentration increased significantly with light exposure (Figure 6.91d) but remained relatively stable for the sample stored in darkness.

Table 6.14. Decay rates and half-lives for the combined Cu fraction 1 and 2 concentrations in Chardonnay stored in low oxygen concentrations and different coloured bottles.

Treatment	Combined Cu fraction 1 and 2		
	1^{st} order rate[2] ($days^{-1}$)	R^2	Half-life (days)
Flint	1.49 ± 0.07^{a}	0.933	0.47 ± 0.02^{f}
Arctic blue	1.13 ± 0.02^{b}	0.980	0.62 ± 0.01^{e}
French green	0.26 ± 0.02^{c}	0.967	2.7 ± 0.3^{d}
Antique green	0.101 ± 0.005^{d}	0.925	6.9 ± 0.3^{c}
Amber	0.031 ± 0.005^{e}	0.796	23 ± 3^{b}
Dark	0.012 ± 0.001^{f}	0.866	63 ± 8^{a}

Results are expressed as mean ± standard deviation and different letters in columns mean significant difference ($p < 0.05$) using Dunnett's T3 test for post hoc multiple comparison. Although first-order rates are shown for all samples, the samples with slowest decay (i.e., 'Amber' and 'Dark' treatments) had better fitting of data with second-order kinetics rather than first order. The second order rates and half-lives can be viewed in Vongluanngam et al. (2024).

The first order decay rate for the combined Cu fraction 1 and 2 concentrations was 120 times higher in the wine stored in light compared to the sample stored in darkness (see Flint versus Dark in Table 6.14). These rates meant that the combined concentrations of Cu fraction 1 and 2 would halve every 11.5 hours when this wine was exposed to light and every 63 days when in darkness.

Table 6.14 also shows the comparative decay rates and half-lives for the wine when stored in different coloured bottles and exposed to the same light as the flint bottle. The rates decrease in the order of bottle colour intensity of the bottles, which corresponded to glass that had the lowest transmission of light for wavelengths below 500 nm: flint > artic blue > French green > antique green > amber. Therefore, the darker coloured glass bottles could protect the light-induced loss of Cu fractions 1 and 2. However, even the darkest coloured glass (i.e., amber) still had a decay rate for Cu fractions 1 and 2 that was more than twice the rate observed for the sample stored in darkness (Table 6.14). Therefore, darker glass offered some protection against light but not complete protection.

A second set of experiments were conducted to assess whether light with very low riboflavin concentrations (i.e., << 0.05 mg/L) would also impact the Cu fractions in white wine. The light exposure experiments were conducted over a 6.5-month period, with light exposure for 12 hours per day, and included free hydrogen sulfide and free methanethiol measurements.

For this set of experiments, a Chardonnay wine was used with a concentration of 0.013 ± 0.006 mg/L riboflavin and 0.020 ± 0.006 mg/L total Cu. The experimental design consisted of eight treatments including the addition of 0.3 mg/L of Cu(II) and/or 0.5 mg/L of riboflavin (RF) or no additions, either exposed to light or stored in darkness (as the control).

Experimental factors were coded as:

- **light** - for wine exposed to light without additions,
- **light+riboflavin** - for wine exposed to light with riboflavin addition,
- **light+Cu** - for wine exposed to light with Cu(II) addition,
- **light+Cu+riboflavin** - for wine exposed to light with riboflavin and Cu(II) addition,
- **dark** - for wine stored in darkness without additions,
- **dark+riboflavin** - for wine stored in darkness with RF addition,
- **dark+Cu** - for wine stored in darkness with Cu(II) addition, and
- **dark+Cu+riboflavin** - for wine stored in darkness with RF and Cu(II) addition.

After bottling, wine samples were stored vertically on shelves inside a temperature-controlled room 20 ± 0.2°C (Figure 6.92). Each sample was exposed to four 36W fluorescent light tubes (Osram Lumilux) that emitted daylight (6500 K) with a luminous flux of 3250 and had an emission spectrum as shown in Figure 6.92. During the storage experiment, samples were placed 20 cm from the light source giving the average incident light intensity of 100 µmol/m^2/s. The positions of samples were randomised every second day to ensure that all samples received a uniform light exposure. Wines were irradiated for 12 hours per day (i.e., 9 am to 9 pm) for 190 days, with the timing of exposure automated with a digital timer. The 'dark' samples were covered completely with aluminum foil before being placed inside the same temperature-controlled room as the light-exposed samples. During the storage period, triplicate samples for each treatment underwent chemical analysis at 6 time-points (i.e., 0, 2, 7, 30, 60, 190 days).

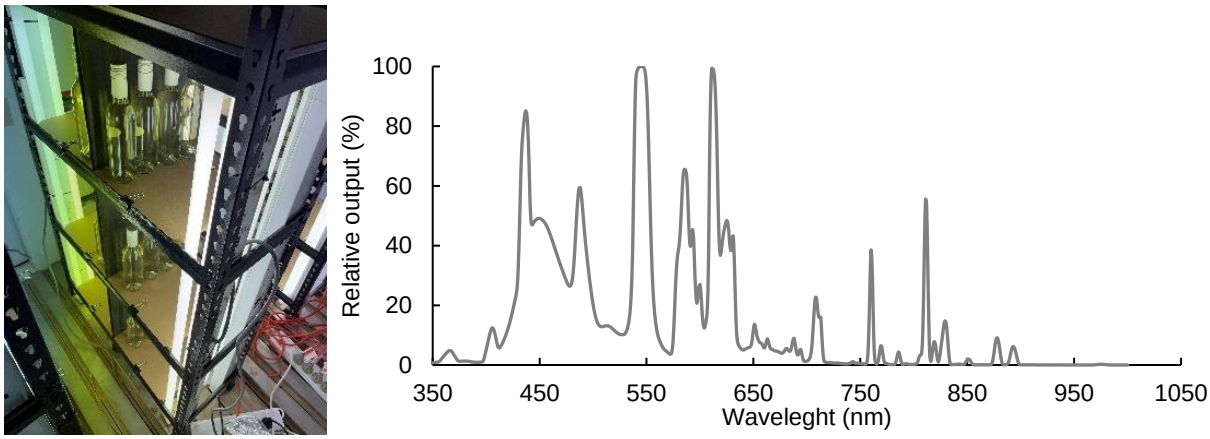


Figure 6.92. The set up for the irradiation of wine samples with fluorescent light and the emission spectra for the fluorescent tubes used in the experiment.

For all samples containing added riboflavin (0.5 mg/L), light exposure caused its concentration to drop below 0.05 mg/L by the first measurement timepoint (i.e., day 2), while darkness provided negligible loss (data not shown).

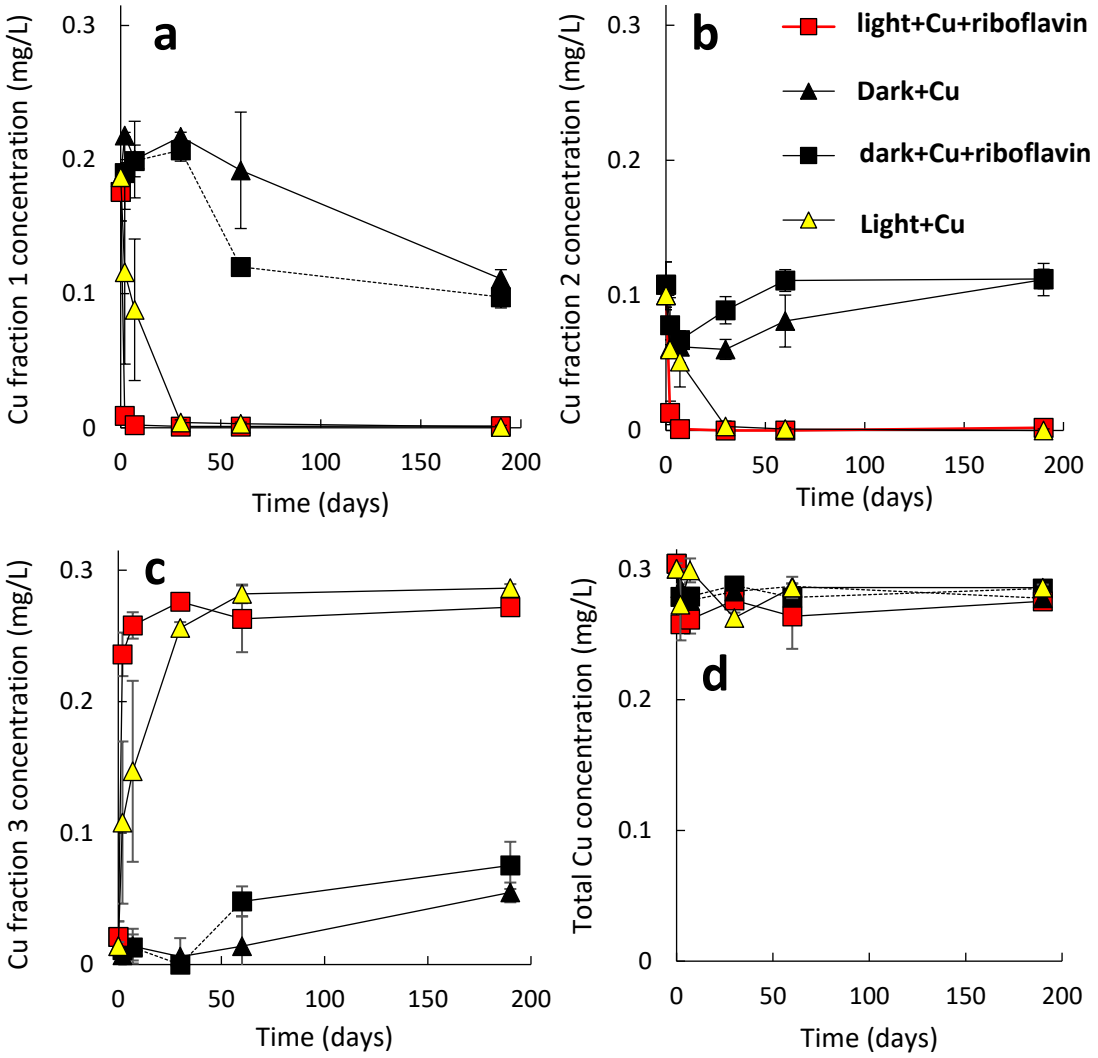


Figure 6.93. The concentrations (mg/L) of Cu fraction 1 (a), fraction 2 (b), fraction 3 (c), and total Cu (d) in the white wines with and without added riboflavin. Error bars represent the standard deviation (n = 3) for the measurement of triplicate samples. 'riboflavin' = addition of 0.5 mg/L at bottling.

For the samples with high initial riboflavin concentrations (i.e., 0.5 mg/L) the results were like those of the previous experiment (Figure 6.91), whereby light exposure caused most of Cu fractions 1 and 2 (**light+riboflavin** in Figure 6.93a,b) to be lost by day 2 along with a concomitant increase in Cu fraction 3 (Figure 6.93c). For the high riboflavin samples stored in darkness (**dark+riboflavin** in Figure 6.93a-d) the changes in Cu fractions were much slower with around 50 % of Cu fraction 1 remaining after 190 days.

For the samples without riboflavin added at bottling, light exposure caused similar changes in Cu fractions 1 and 2 as for the high riboflavin samples but the changes occurred at a slower rate (**light** versus **light+riboflavin** in Figure 6.93). That is, there were still appreciable levels of Cu fractions 1 and 2 present at the 7-day timepoint for the **light** samples, but there was none in the **light+riboflavin** samples. Similarly, Cu fraction 3 increased slower in the lower riboflavin sample exposed to light. These results confirmed light-induced conversion between Cu fractions still occurred even with low initial concentrations of riboflavin (~0.01 mg/L) and continued when the riboflavin concentration was gone.

Immediately after bottling (day 0 in Figure 6.94), the free H_2S concentration was negligible (< 1 µg/L) in all treatments, while the free MeSH concentration was higher in the samples with lower Cu concentrations (i.e., **dark** versus **dark+Cu** at day 0 in Figure 6.94b). The samples stored in darkness had no increase in H_2S and MeSH throughout the 190-day period.

The light-exposed samples with high riboflavin concentrations showed a rapid increase in MeSH concentration within the first seven days regardless of the addition of Cu at bottling (**light+riboflavin** and **light+Cu+riboflavin** in Figure 6.94a). This period coincided with the accelerated loss of Cu fractions 1 and 2 (Figure 6.93a,b) and riboflavin in the same samples. By day 30, there was around 30 µg/L of free MeSH in the **light+riboflavin** and **light+Cu+riboflavin** samples which was around 6-fold higher than the aroma threshold for methanethiol. After this time the free MeSH continued to gradually increase in the **light+riboflavin** sample but remained relatively constant in the **light+Cu+riboflavin** sample. Therefore, Cu addition at bottling could offer little protection from light-induced free MeSH production in samples with high riboflavin, even with a short period of exposure (i.e., 2 days), and this was due in part to the rapid loss of Cu fraction 1 under such conditions (Figure 6.93a).

For samples with low riboflavin concentrations, light exposure caused a more gradual increase in free MeSH concentrations than for the high riboflavin samples (**light** versus **light+Cu** in Figure 6.94b), reaching only 8 µg/L after 190 days, regardless of Cu concentration. The samples with a higher Cu concentration (**light+Cu** versus **light**) had significantly lower MeSH concentrations at 2 and 7 days, and a trend to lower concentrations at day 30, but the same concentration at day 90 and 190 as the lower Cu sample (Figure 6.94b). This was consistent with the samples with higher total Cu concentrations having sufficient Cu fraction 1 present to inhibit the accumulation of MeSH at day 2 and 7. However, by day 30, the Cu fraction 1 was depleted and therefore by the day 90 and 190 time-points there was no significant effect of Cu on the free MeSH accumulation. These results suggest a protective effect of Cu fraction 1 against MeSH accumulation during shorter-term light exposure (i.e., 12 h exposure per day for 0 - 30 days) only if the riboflavin concentration was low (i.e., ≤ 0.01 mg/L).

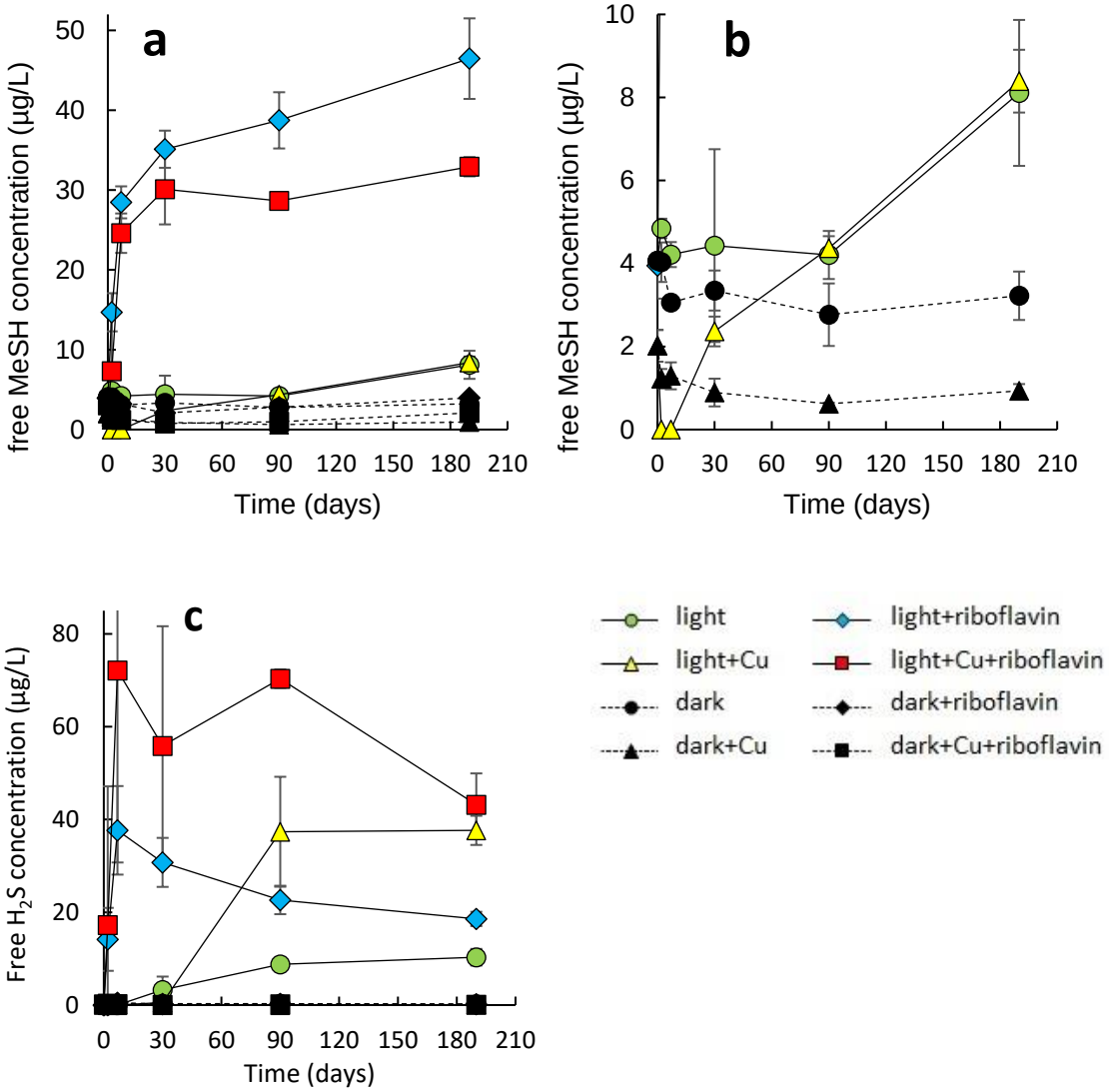


Figure 6.94. The concentrations (µg/L) of free MeSH (a, b) and free H_2S (c) during the storage experiment. Panel (b) shows a zoomed in version of free MeSH without the high riboflavin samples. The coloured symbols represent samples exposed to light while the black symbols represent samples stored in darkness. Error bars represent the standard deviation (n = 3) for the measurement of triplicate samples. 'riboflavin' = addition of 0.5 mg/L at bottling; 'Cu' = addition of 0.3 mg/L at bottling.

The light-exposed samples that contained a high riboflavin concentration had rapid accumulation of free H_2S, reaching maximum concentrations by the 7-day time-point (**light+riboflavin** and **light+Cu+riboflavin** in Figure 6.94c). Cu(II) did not inhibit this H_2S production, and in fact, higher concentrations of H_2S were observed with increased Cu in the light-exposed high riboflavin samples (i.e., **light+Cu+riboflavin**). The samples with low riboflavin generated considerably less H_2S than the corresponding samples with high riboflavin. Cu combined with the low riboflavin concentration proved some protection against free H_2S accumulation to day 30 (Figure 6.94c), but then enhanced its production at day 90 and 190. Overall, this outcome demonstrates that riboflavin photoreactions can significantly accelerate free H_2S formation in wine.

Figure 6.95 summarises the observed inter-relationships between Cu fractions, riboflavin and sulfhydryl compounds during the exposure of white wine to light. Initially, light exposure accelerated the acquisition of sulfide from sulfur-containing precursors by Cu fractions 1 and 2 to form Cu fraction 3 (pathway A, Figure 6.95). This pathway occurred irrespective of the presence of riboflavin, but the rate of conversion was faster with higher riboflavin concentrations. Once Cu fractions 1 and 2 were depleted, pathway B could occur, leading to accumulation of free MeSH and free H_2S. This pathway was also accelerated in the presence of riboflavin, but it could still eventuate in the absence of riboflavin. Cu fraction 3 (copper sulfide) was found to enhance the accumulation of H_2S in light-exposed wine but only after the depletion of Cu fractions 1 and 2. To date, this is the first report of the Cu-induced production of free H_2S during the exposure of wine to light. Given that light rapidly removes the Cu fractions associated with reductive aroma inhibition in wine, it is possible that the exposure of bottled wine to light may have a legacy effect whereby it becomes more prone to reductive spoilage even if it is aged under ideal conditions.

To answer the questions posed at the start of this Section:

- Does light impact the concentration of the protective Cu fractions in wine (i.e., Cu fractions 1 and 2)?
  ANSWER: Yes. Light significantly accelerates the Cu fractions 1 and 2 concentration decreases in wine.

- Do the Cu fractions in wine offer protection against the light-induced spoilage of wine?
  ANSWER: Yes. Cu fractions 1 and 2 can inhibit free H_2S and free MeSH accumulation induced by light but they can only offer protection while they remain in the wine (i.e., transient protection).

- Does the protection offered by Cu depend on whether the photochemistry is dominated by riboflavin or some other photoactive agent?
  ANSWER: Yes. In wines of low riboflavin content (i.e., ~0.01 mg/L), Cu fraction 1 and 2 can offer protection against free H_2S and free MeSH during short-term light exposure. In wines of high riboflavin concentration, Cu fraction 1 and 2 disappear so rapidly that they cannot offer protection against light exposure and the generated Cu fraction 3 readily promotes free H_2S accumulation.

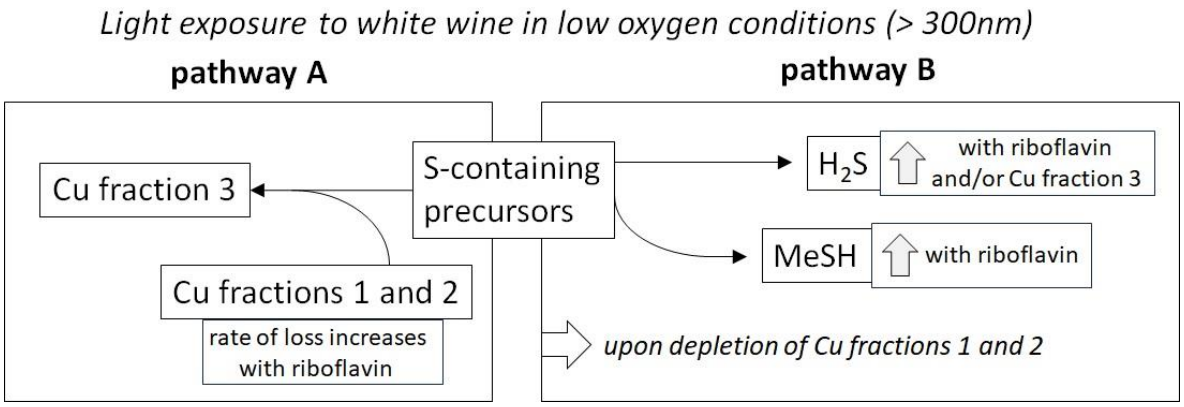


Figure 6.95. The inter-relationships between Cu fractions, riboflavin and the accumulation of volatile sulfur compounds in white wine exposed to light.

6.5.6 The impact of wine storage temperature on Cu fractions.

This section investigates the impact of wine storage temperature on the concentrations of Cu fractions. A previous study had shown that storage of wine at elevated temperatures could accelerate the accumulation of H_2S and MeSH in wine (Franco-Luesma and Ferreira, 2016b), hence, it is likely that it would impact the concentrations of all Cu fractions in wine.

Four red wines and four white wines (Table 6.15) were either aged in darkness at 14 °C for one year or at 40 °C for twelve days. The wines were obtained directly from the bottling line of two commercial wineries and were all sealed under screw cap. The wines were either placed in a cellar for aging at 14 °C, or the bottles were placed in a 40 °C incubator. Three bottles of each wine were analysed at each time-point and Cu fractions were determined by BCA colorimetric analysis for white wines and stripping potentiometry for red wines.

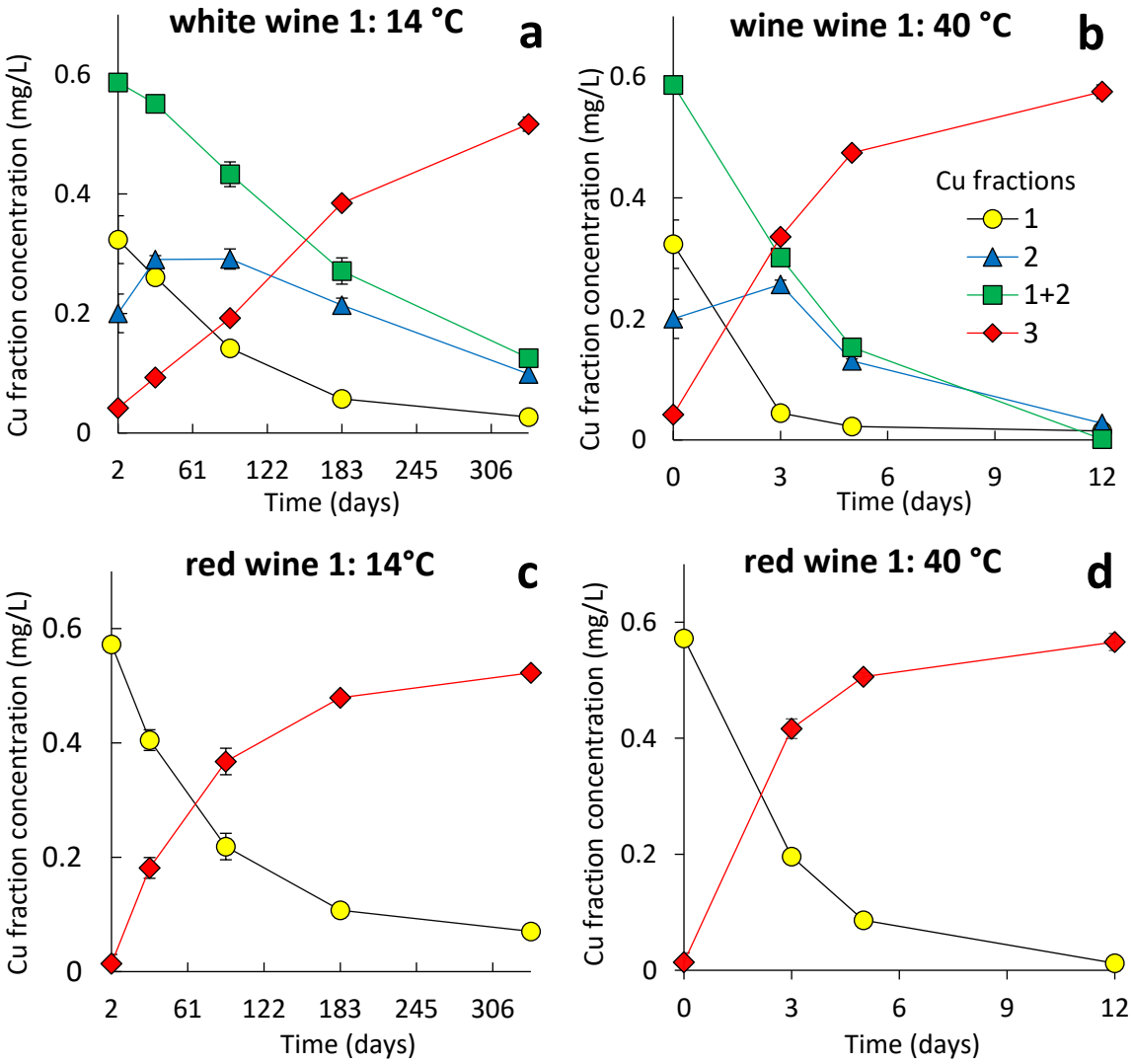


Figure 6.95. The concentrations of Cu fractions in a white wine at 14 °C (a) and 40 °C (b) and a red wine at 14 °C (c) and 40 °C (d). The error bars indicate the standard deviation (n=3) and where not visible are smaller than the symbol size. White wine 1 was a Chardonnay and red wine 1 was a Pinot noir.

At 14 °C the Cu fractions changed as previously described (Section 6.5.3), that is, overall decreases for Cu fractions 1 and 2 in white wine (Figure 6.95a), and decrease for Cu fraction 1 for red wine (Figure 6.95c), with a concomitant increase in Cu fraction 3 in both wines. The same decay profile occurred when the wines were stored at 40 °C but within a much shorter time frame (Figure 6.95b,d). The changes observed in one year at 14 °C occurred in less than two weeks at 40 °C.

The accelerated change in Cu fractions with elevated temperature was observed for all four red and white wines albeit with variation in the rates of change. The first order decay rates for Cu fraction 1 and/or 2 are shown in Figure 6.96. The first order decay rates for Cu fraction 1 (Figure 6.96a) were at least 20-fold lower for wine storage at 14 °C versus 40 °C. Similarly, the decay rate for the Cu fraction 1 and 2 concentrations (Figure 6.96b) were 10-fold lower for wine storage at 14 °C versus 40 °C. For the four red wines, the decay rates for Cu fraction 1 at 40 °C had good agreement with each other (i.e., 1.86 ± 0.03 day^{-1}), but this was not the case for rates at 14 °C (Figure 6.96, Table 6.15). This suggested the matrix effects of red wine had less impact on the decay of Cu fraction 1 at higher temperatures. The white wine had high variability in the rates determined at both storage temperatures.

Table 6.15. Data for the decay of Cu fractions in white and red wines at 14 and 40 °C. The uncertainty quoted is the standard deviation.

		Cu fraction 1			combined Cu fraction 1 + 2		
wine	variety	half-life at 14 °C ($days^{-1}$)	half-life at 40 °C ($days^{-1}$)	activation energy (E_a) (kJ/mol)	half-life at 14 °C ($days^{-1}$)	half-life at 40 °C ($days^{-1}$)	activation energy (E_a) (kJ/mol)
white							
W1	Chardonnay	94 ± 5 b	1.22 ± 0.09 b	125	158 ± 5 b	3.2 ± 0.1 c	112
W2	Chardonnay	25 ± 1 c	1.30 ± 0.07 b	85	47 ± 5 c	5.1 ± 0.4 a,b	85
W3	Sauv. blanc	21± 1 c	1.14 ± 0.07 b	84	49 ± 4 c	3.9 ± 0.3 b,c	89
W4	Sauv. blanc	173 ± 13 a	3.2 ± 0.2 a	131	224 ± 10 a	6.1 ± 0.2 a	118
average		*78 ± 72*	*2 ± 1*	*100 ± 25*	*120 ± 87*	*5 ± 1*	*100 ± 15*
red							
R1	Pinot noir	83 ± 4 a	1.84 ± 0.05 a	110			
R2	Shiraz, CabS	74 ± 5 a	1.84 ± 0.04 a	106			
R3	CabS, Merlot	90 ± 3 a	1.85 ± 0.04 a	112			
R4	CabS, Merlot	49 ± 5 b	1.9 ± 0.2 a	93			
average		*74 ± 18*	*1.86 ± 0.03*	*105 ± 9*			

Cu fraction 1 half-lives with different letters are significantly different (P = 0.05), and Cu fraction 1 and 2 half-lives with different letters are significantly different (0.05). [1] This average value excludes the data from R4.

The decay rates in Figure 6.95 allowed determination of the activation energy associated with the loss of the different Cu fractions. The activation energy value allows the prediction of decay rates at a given temperature (i.e., in this case between 14 to 40 °C) and its magnitude can also provide some mechanistic insights. Based on the data within Figure 6.95, the activation energies could only be estimated as accurate determination requires collection of data from at least three different temperatures rather than two (i.e., 14 and 40 °C). Three temperatures of storage would have enabled the linearity between measured decay rates and the storage temperature (i.e., inverse temperature in units of kelvin) to be confirmed rather than assumed. Table 6.15 shows the estimated activation energies for Cu fraction 1 decay in all wines, and for the combined Cu fraction 1 and 2 decay in the white wines. It is evident that the activation energies

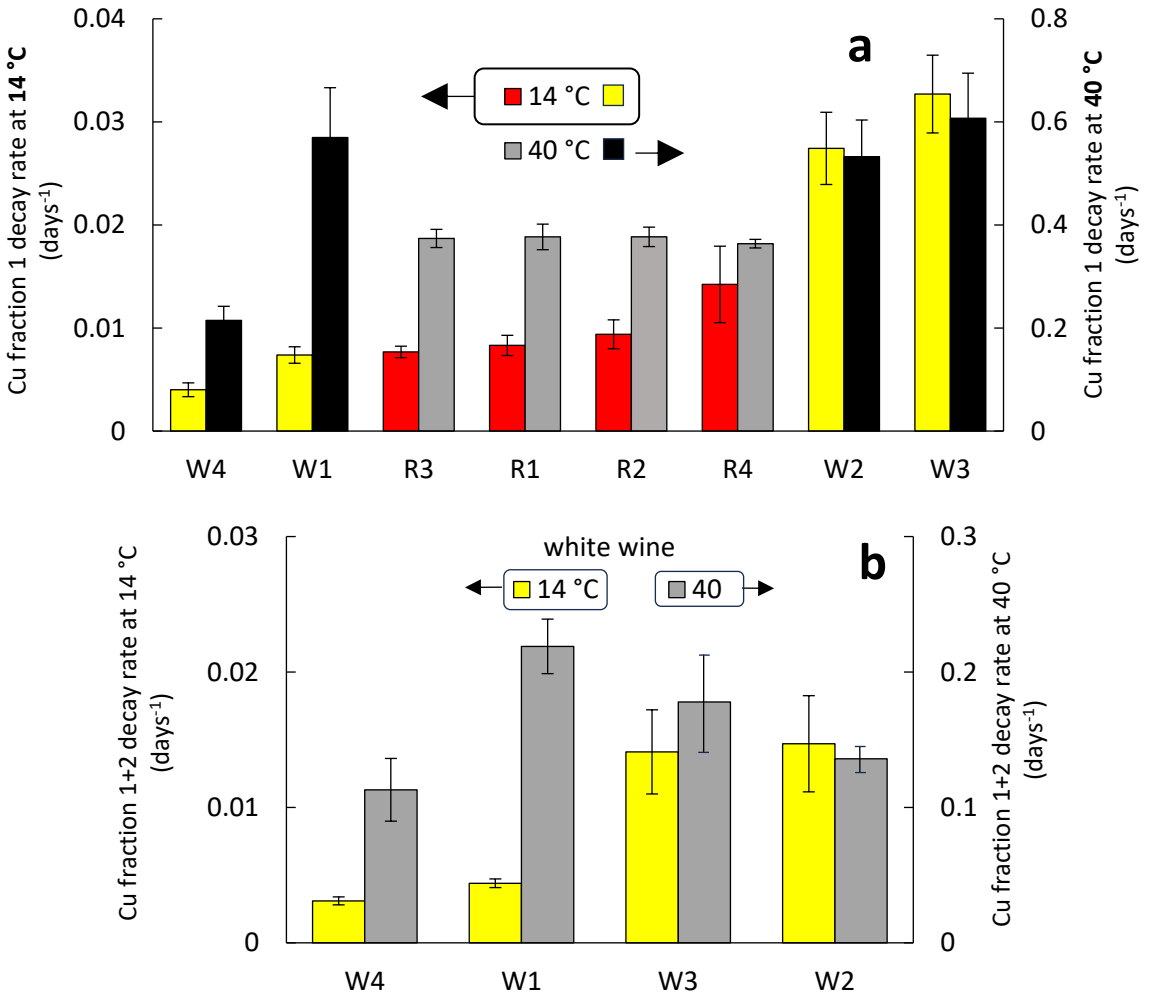


Figure 6.96. The first order decay rates for Cu fraction 1 concentration (a) and combined Cu fraction 1 and 2 concentrations (b) at 14 °C (coloured bars and left y-axis) and 40 °C (grey and black bars and right y-axis). Error bars are the 95 % confidence limits (n=3) and significant difference is taken as the non-overlap of error bars for rates determined at a given temperature.

vary considerably between the different wines, with the standard deviation being 15 – 25 % of the average values in white wine and 9 % in red wine. As found for the decay rates at 14 °C in Section 6.5.3, the activation energies seem to be influenced considerably by the wine matrix. This means that using the overall average activation energy to predict a decay rate in a wine would not be accurate.

The results of this study show that wine storage temperature has a profound impact on the changes in Cu fractions during wine aging. The protective Cu fractions 1 and 2 decrease in concentrations at rates that are more than 10 to 20-fold faster at 40 °C compared to 14 °C. The magnitude of change in the decay rates of Cu fractions 1 and/or with temperature are wine dependent, as reflected by estimated activation energy values, and cannot be accurately predicted based on an overall average activation energy value.

6.5.7 The use of accelerated aging and Cu fraction measurements to predict the onset of reductive wine development.

Earlier sections of this report have shown that while the Cu fraction 1 concentration is above 0.03 mg/L there can be reasonable confidence that free methanethiol will not accumulate to detrimental levels in wine. Similarly, when the combined Cu fraction 1 and 2 concentrations are above 0.02 mg/L then free hydrogen sulfide will not accumulate. However, during bottle aging these protective Cu fractions decrease in concentrations and their rate of decrease is impacted by the wine matrix and wine storage temperature. Attempts to use decay rates averaged across different wines to predict the time at which the Cu fractions would fall below the critical concentrations proved unfeasible. There was too much variation in the decay rates across different wines.

In any case, most wines showed a considerable lag period between the depletion of the Cu fractions and the emergence of free hydrogen sulfide and free MeSH. If this lag period was sufficiently large enough, then the time at which the protective fractions fell below the critical concentrations would be less important than the time of eventual accumulation of free hydrogen sulfide and free methanethiol. Even low concentrations of Cu fractions 1 and 2 at bottling may be sufficient to prevent free hydrogen sulfide and methanethiol accumulation for the intended shelf-life of many wine (e.g., 1 to 2 years) regardless of their depletion soon after bottling.

This section of work was conducted to assess if Cu fraction measurements in combination with the accelerated aging of wine could identify the wines at risk of reductive development. Also, it was intended to provide insight into the length of time between Cu fraction 1 and 2 depletion and free H_2S and methanethiol accumulation.

Ten white wines were aged in darkness at 14 °C for 18 months in a cellar and at 40 °C for one month in an incubator. The wines were obtained directly from the bottling line of three commercial wineries and were all sealed under screw cap. Three bottles of each wine were analysed at each time-point and Cu fractions were determined by BCA colorimetric analysis for white wines and stripping potentiometry for red wines. Oxygen and sulfur dioxide measurements showed that oxygen ingress through the closures during the storage period was negligible (data not shown).

The purpose of the accelerated aging protocol was to allow identification of problematic white wines that were likely to exceed a critical threshold of free H_2S or free MeSH during bottle aging for 12 to 18 months in ideal storage conditions. The identification of such wines prior to bottling may enable interventions in the winery to improve the bottle aging outcome for the wine. The test was not intended to predict that absolute amount of free H_2S or free MeSH that would accumulate in a wine but rather whether the wine would be likely to exceed threshold concentrations. For the data presented, the following colour coding is used:

- **Red data (true positive)**: the 40 °C storage of wines correctly predicts that free H_2S or methanethiol will accumulate in wines aged in ideal conditions (i.e., 12 – 18 months at 17 °C in darkness).
- **Yellow data (false negative)**: the 40 °C storage of wines incorrectly predicts that free H_2S or methanethiol will not accumulate in wines aged in ideal conditions.
- **Black data (false positive)**: the 40 °C storage of wines incorrectly predicts that free H_2S or free methanethiol will accumulate in wines aged in ideal conditions.
- **Green data (true negative)**: the 40 °C storage of wines correctly predicts that free H_2S or free methanethiol will not accumulate in wines aged in ideal conditions.

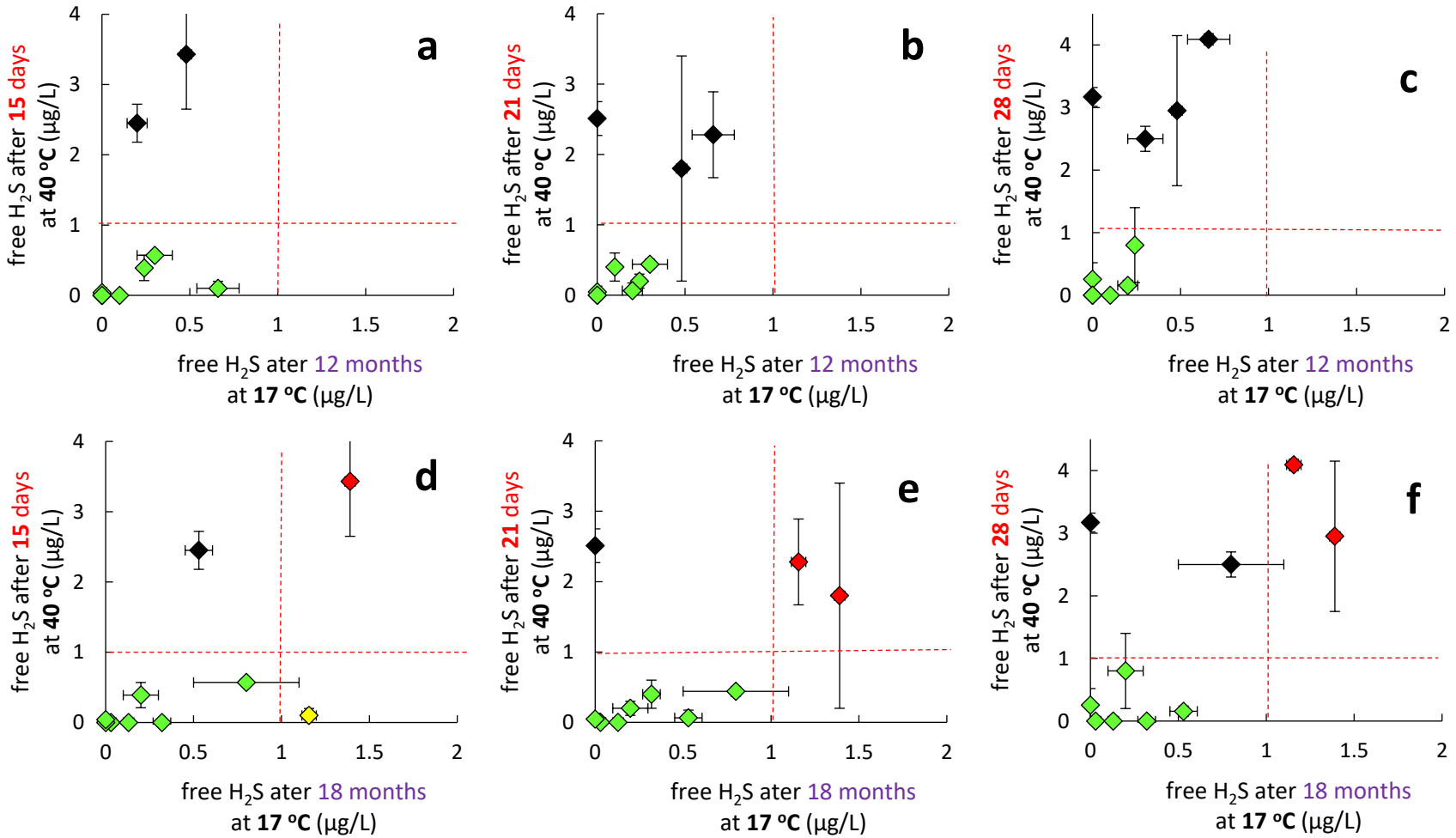


Figure 6.97. The free H_2S concentrations in white wine samples incubated at 40 °C for 15 (a,d), 21 (b,e) and 28 (c,f) days compared to the free H_2S concentration in samples aged at 17 °C for 12 (a-c) and 18 (d-f) months. The dashed red line indicates the aroma threshold for H_2S. The red data points are true positives, the green data points are true negatives, the yellow data points are false negatives and the black data points are false positives. Error bars indicate the standard deviation (n = 3).

Figure 6.97 shows comparisons of free H_2S concentrations in the wines when stored at 40 °C for 15, 21 or 28 days against the wines stored at 17 °C for 12 or 18 months. The red dashed lines show the aroma threshold for H_2S (1 µg/L) and these were used as the critical threshold concentrations. No wines exceeded 1 µg/L free H_2S after 12 months aging at 17 °C (Figure 6.97a-c), but when stored at 40 °C for 15-28 days, there were 2-4 wines that exceeded this threshold. These wines were deemed false positives by exceeding 1 µg/L free H_2S when stored at 40 °C but not at 17 °C (black data points in Figure 6.97a-c). This showed that storing wines at 40 °C tended to overestimate the free H_2S that would form in these wines after 12 months at 17 °C (Figure 6.97 a-c).

After 18-months storage at 17 °C, two wines exceeded 1 µg/L free H_2S (red data points, Figure 6.97d-f). These same two wines accumulated free H_2S above 1 µg/L when stored at 40 °C for 21 or 28 days but not when stored for 15 days. However, storage for 21 days provided one false positive and 40 °C storage for 28 days provided two false positives (black data points in Figure 6.97e,f). No other threshold level at 40 °C could separate the false positives (black data points) from the true positives (red data points) in Figure 6.97. Therefore, based on this data set, the use of a 40 °C accelerated aging test proved to be of limited value to predict free H_2S accumulation in white wine.

The methional concentration accumulated to above the published aroma threshold (> 3 µg/L) in most wines after aging for 12 or 18 months at 17 °C (Figure 6.98). Consequently, it was considered apt to use a threshold concentration for methanethiol higher than the aroma threshold. A concentration of 5 µg/L was used given that this was around the 4.8 µg/L methanethiol concentration that were assigned by the sensory panel as being wines with most reductive character in the bottle aging trial from Section 6.3.1.3. In comparing the data from the wines stored at 40 °C compared to those aged in bottle at 17 °C for 12-months (Figure 6.98a-c), there appeared to be reasonable agreement in the wines exceeding 5 µg/L. Heating the wines for 40 °C for 28 days provided the highest number of true positive assignments (red data points in Figure 6.98a), with no false negative or false positive assignments (yellow or black data points). A similar outcome was evident in the relationship between the wines stored at 40 °C and those aged for 18 months at 17 °C (Figure 6.98d-f).

These results show the potential feasibility in using an accelerated aging test to predict free MeSH accumulation in bottle aged wine stored under ideal conditions. However, the results also show that the same test is not likely to be successful for the prediction of free H_2S accumulation. From Section 6.3.1.3, of the 9 wines deemed as having the highest reductive character from a survey of 50 white wines, all but two had elevated concentrations of both free H_2S (i.e., > 1.8 µg/L) and free MeSH (i.e., > 4.8 µg/L). Consequently, the identification of wines that accumulate free MeSH may also highlight most wines that will also tend to accumulate free H_2S. A larger scale study would be required to confirm the value of this accelerated aging methodology for the prediction of reductive tendencies of wine.

Based on the relationship described in Figure 6.98, it was decided to assess any commonalities in the Cu fraction 1 concentrations for the wines that exhibited > 5 µg/L methanethiol accumulation. Figure 6.99 shows that only the wines with less than 0.015 mg/L Cu fraction 1 concentration at bottling accumulated methanethiol above 5 µg/L. Although, this relationship is only shown for the set of wines in Figure 6.98, it suggested a potential link between Cu fraction concentration at bottling and the potential for free MeSH to accumulate during bottle aging.

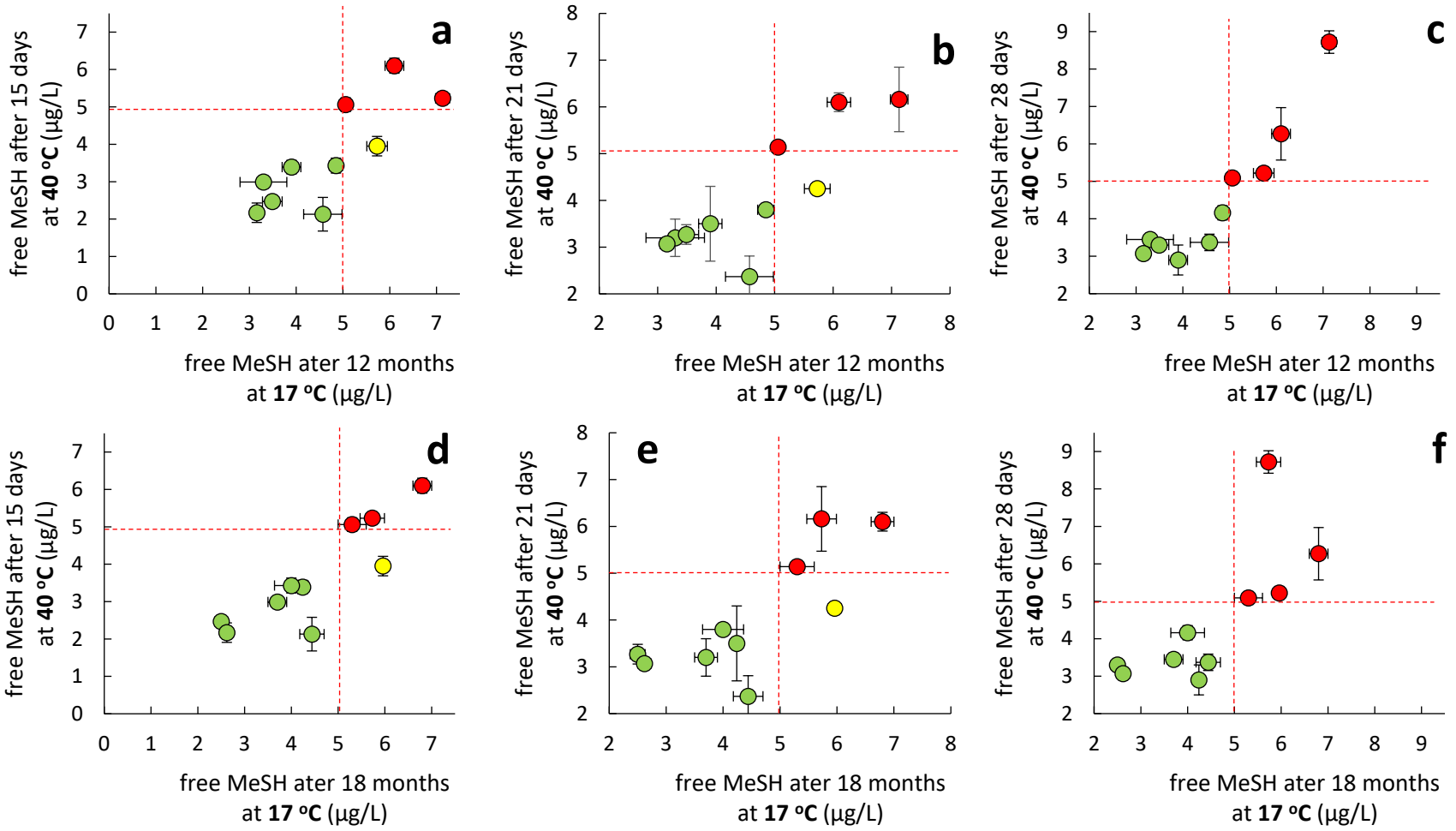


Figure 6.98. The free methanethiol concentrations in white wine samples incubated at 40 °C for 15 (a,d), 21 (b,e) and 28 (c,f) days compared to the free methanethiol concentration in samples aged at 17 °C for 12 (a-c) and 18 (d-f) months. The red dashed line is the assigned allowable threshold level for free methanethiol. The red data points are true positives, the green data points are true negatives and the yellow data points are false negatives. Error bars indicate the standard deviation (n = 3).

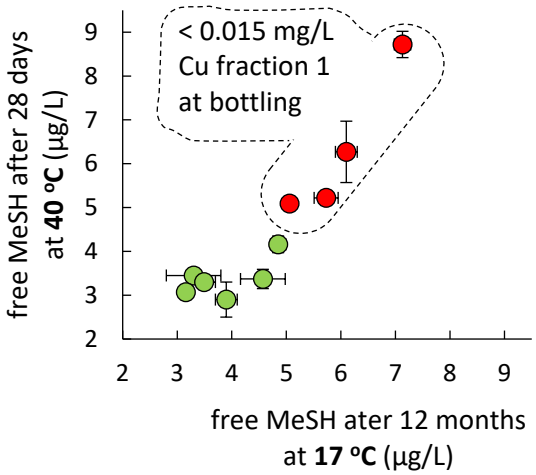


Figure 6.99. The concentration of free methanethiol in wines after storage at 40 °C for 28 days and 17 °C for 12 months. The wines with less than 0.015 mg/L Cu fraction 1 at bottling are circled.

To investigate this further, the data was plotted to show the Cu fraction 1 concentration at bottling versus the free methanethiol concentration after 12 to 15 months. A total of 43 wines were used in the data set including 14 red, 26 white and 3 rosé wines. Figure 6.100 shows that the wines that accumulated more than 5 µg/L methanethiol after 12 to 15 months of bottle aging had less than 0.04 mg/L of Cu fraction 1 at bottling. This value was only marginally above the 0.03 mg/L Cu fraction 1 threshold that could ensure that methanethiol would not also be present above the aroma threshold. The result suggests that a Cu fraction 1 concentration at bottling slightly above the critical level of 0.03 mg/L to ensure inhibition of methanethiol can provide good protection against free methanethiol accumulation for 1 year of bottle storage in ideal conditions. Given Cu fraction 1 is known to decrease during wine aging, it suggests a significant lag period occurs between Cu fraction 1 loss and the accumulation of free methanethiol occurs.

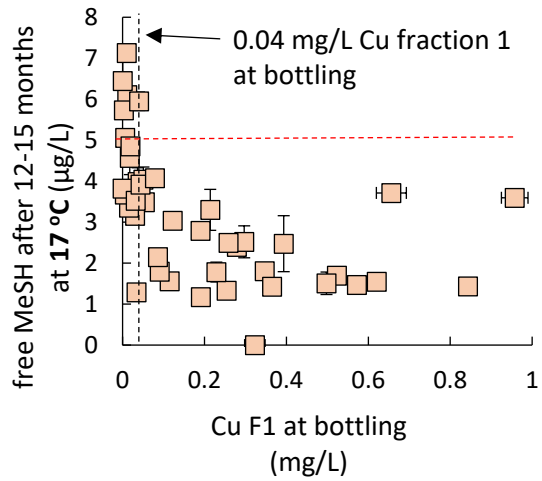


Figure 6.100. The concentration of free methanethiol after 12 to 15 months bottle aging versus the concentration of Cu fraction 1 at bottling. The red dashed line represents the designated threshold for methanethiol concentration, and the black line indicates the Cu fraction 1 concentration at bottling that did not allow free methanethiol accumulation in these wines above the designated threshold. Included in the data set are 26 white wines, 3 rose wines and 14 red wines.

Of the wines that accumulated more than 5 µg/L methanethiol in Figure 6.100, the lag period between Cu fraction 1 loss and free methanethiol accumulation above 5 µg/L was determined. Figure 6.101 shows that the average time between Cu fraction 1 loss (i.e., <0.03 mg/L) and free methanethiol accumulation (> 5 µg/L) was 370 ± 70 days. Consequently, given the large lag time, it follows that a Cu fraction 1 concentration marginally above the critical 0.03 mg/L concentration required for methanethiol inhibition is sufficient for longer term protection of the wine (i.e., ~ 1 year).

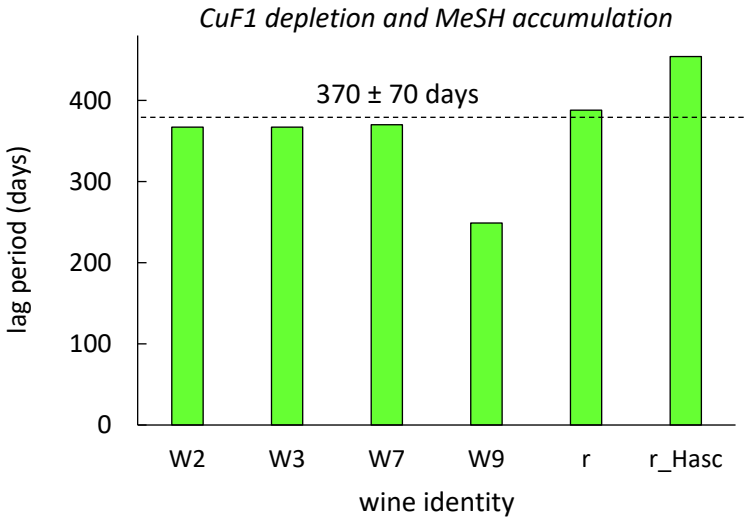


Figure 6.101. The lag period between Cu fraction 1 concentration reaching less than 0.03 mg/L and the concentration of methanethiol exceeding 5 µg/L. Wine identity key: 'w' indicates white wine, 'r' indicates rosé and 'Hasc' indicates the addition of ascorbic acid at bottling.

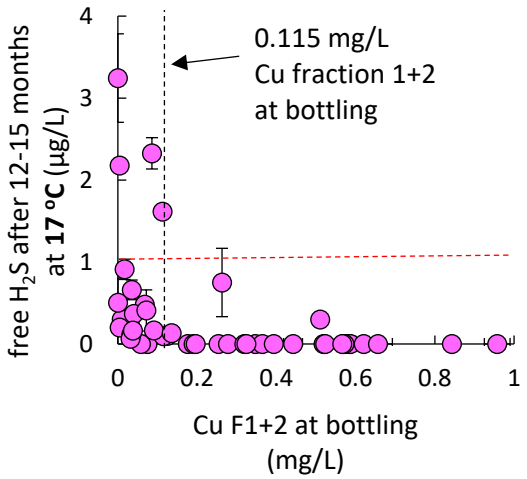


Figure 6.102. The concentration of free H_2S after 12 to 15 months bottle aging versus the combined concentrations of Cu fraction 1+2 at bottling. The red dashed line represents the designated threshold for H_2S concentration, and the black line indicates the Cu fraction 1+2 concentration at bottling that did not allow free H_2S accumulation in these wines above the designated threshold. Included in the data set are 26 white wines, 3 rose wines and 14 red wines.

A similar approach can be taken for free H_2S, whereby the concentration in wines after 12-15 months bottle aging in ideal storage conditions can be plotted against the combined Cu fraction 1 and 2 concentration at bottling. In this case, no wines that had a combined Cu fraction 1+2 concentration at bottling of 0.115 mg/L, or above, accumulated hydrogen sulfide above the aroma threshold (1 µg/L). However, given that Section 6.3.1.3 showed that wines were deemed reductive with concentrations around 2 µg/L, then a slightly lower Cu fraction 1+2 concentration of 0.09 mg/L could be used with this less conservative threshold. The 0.115 mg/L or 0.09 mg/L concentration of Cu fraction 1+2 required at bottling for long term inhibition of free H_2S is much higher than the 0.04 mg/L of Cu fraction 1 required for long term methanethiol inhibition. In terms of the time between the combined Cu fraction 1+2 concentration falling below 0.02 mg/L and the free hydrogen sulfide concentration exceeding 1 µg/L, Figure 6.103 shows that the average lag time was 380 ± 100 days.

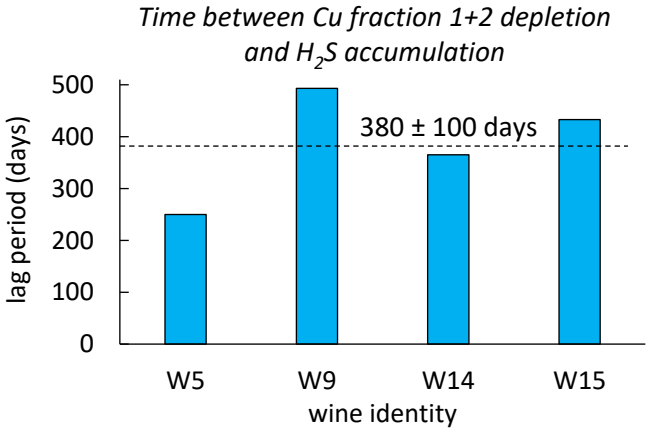


Figure 6.103. The lag period between the combined Cu fraction 1+2 concentration reaching less than 0.02 mg/L and the concentration of H_2S exceeding 1 µg/L. Wine identity key: 'w' indicates white wine.

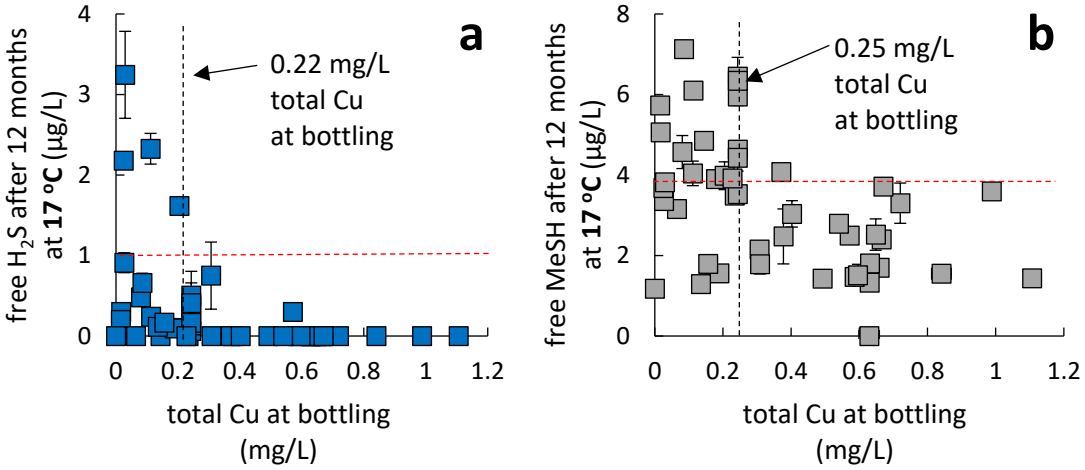


Figure 6.104. The concentration of free H_2S (a) and free methanethiol (b) after 12 to 15 months bottle aging versus the total Cu concentration at bottling. The red dashed line represents the designated threshold for H_2S or methanethiol concentration, and the black line indicates the total Cu concentration at bottling that did not allow sulfhydryl accumulation in these wines above the designated threshold. Included in the data set are 26 white wines, 3 rose wines and 14 red wines.

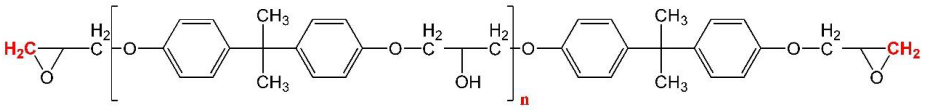
Similar plots could also be devised for total Cu at bottling, although the chemical link between total Cu and free H_2S and free MeSH is less direct than for the Cu fractions. In this case, a concentration of 0.22 mg/L total Cu at bottling inhibited free H_2S accumulation and 0.25 mg/L total Cu at bottling inhibited free methanethiol accumulation. However, given the lack of direct chemical link between total Cu and these free sulfhydryl compounds, there are likely to be many exceptions to the use of threshold levels of total

Cu for free sulfhydryl inhibition. Given that total Cu does not tend to change much in wines bottled and stored in ideal conditions, the total Cu concentration measured at bottling will be the same as the total Cu in the wine throughout aging. Consequentially, the plots in Figures 6.40c, 6.41c, 6.42c and 6.43 show the relationship of total Cu and free sulfhydryl compounds for 413 to 893 bottles of wine. These figures show no free H_2S or free methanethiol accumulation in wines with total Cu concentrations higher than 0.27 to 0.28 mg/L. The exception is those wines that have excessive light exposure or contact metallic aluminium.

The results of this section show that there is some feasibility in the use of an accelerated aging test to indicate the likelihood of free methanethiol accumulation during the bottling aging of wine for 12-months. Although it would be useful to investigate a larger bank of wine samples to strengthen this link. This test may also be of use for indicating free H_2S accumulation given that often both H_2S and methanethiol are present at elevated concentrations in reductively aged wine. Given the large lag period between the depletion of protective Cu fractions and the appearance of free H_2S or free methanethiol, the Cu fraction 1 and 2 concentrations at bottling may provide some assistance to inhibit the incidence of reductive development of wine. Such guidelines would be best used in combination with a test that would identify the propensity for a wine to undergo reductive development (i.e., accelerated aging). The results on the 43 wines assayed showed that Cu fraction 1 concentrations above 0.04 mg/L at bottling tended to provide longer term (i.e. 12 months) inhibition of free methanethiol, and concentrations of Cu fractions 1 and 2 above 0.115 mg/L provided long term inhibition of free hydrogen sulfide. This would only be the case for wines aged in ideal conditions , including low temperature (i.e., 17 °C) and in darkness.

6.6 The impact of Al and other metal ions on Cu fractions in wine

Aluminium cans are a relatively recent packaging innovation for the wine industry. They are composed of an aluminium alloy, containing mainly metallic aluminium in conjunction with minor amounts (e.g., 1 %) of manganese and magnesium. A major challenge for the use of aluminium cans for wine has been the rapid emergence of reductive characters in some wines upon storage that is bought about through the contact of wine with metallic aluminium. Although aluminium cans contain an internal, 1-10 µm thick, lacquer coating, to separate the wine from the metallic aluminium and its oxide layer, often slight imperfections or pitting over time allow contact between the wine and the aluminium alloy. A common lacquer used for wine cans is the bisphenol A-diglycidyl ether epoxy resin:



The application of the liner is best described by Coetzee (2001): 'After the application of the polymer coating, cans are exposed to elevated temperatures which enable proper curing. During this process, any solvents in the liner evaporate and chemical cross-linking of the coating is established providing the optimum barrier properties. If the coating is under cured, it will have weak physical and chemical properties, while over curing will result in a burnt and brittle lining.'.

If contact occurs between metallic aluminium and wine, hydrogen sulfide can be produced. A key mechanism involved is the reaction between molecular SO_2 with metallic aluminium (Coetzee 2001):

$$2Al\ (s) + SO_2\ (aq) + 6H^+\ (aq) \longrightarrow 2Al^{3+}\ (aq) + H_2S\ (g) + 2H_2O \qquad \text{equation 1}$$

To date research has shown that high chloride and/or high free SO_2 (especially when combined with low pH) can promote the reductive development of wine either through the deterioration of the can liner or directly accelerating the reaction shown in equation 1. Due to the gradual degradation of the liner, and other factors, the maximum shelf-life of canned wine is generally considered to be 12 months.

Hydrogen sulfide production will have ramifications for the stability of Cu fractions in canned wine, especially the enhanced conversion of fractions 1 and 2 to fraction 3 (i.e., copper sulfides). This section of the report investigates the impact of metallic aluminium on the fractions of Cu in model wines and real white wines. In addition, the impact of other metal ions, such as Al(III), Zn(II) and Fe(III), on Cu fractions will be investigated when they are alone or in combination with metallic aluminium. This work was completed as part of a collaboration with Dr Eric Wilkes (AWRI).

6.6.1 The impact of Al foil and/or Al^{3+} and Zn^{2+} on Cu fractions in model wines.

This set of experiments conducted in a model white wine system aimed to increase understanding of compositional factors that influence H_2S production from sulfur dioxide, and also the resulting impacts on Cu fractions. Commercial food-grade aluminium foil was utilised as a source of metallic aluminium, which is typically composed of 98.5 % aluminium and small amounts of silicon, iron and other trace metals. The compositional parameters to be assessed in experiments included the influence of the following in the model wine system: amount of Al foil, pH, Al^{3+}, Zn^{2+} and alternative sulfur-sources for H_2S production (i.e., besides SO_2).

The base model wine system consisted of 12 % (v/v) aqueous ethanol buffered to pH 3.2 (with tartaric acid and potassium hydrogen tartrate). The model wine also contained 50 mg/L of total sulfur dioxide and 200 mg/L caffeic acid, with the latter to mimic a typical phenolic compound concentration in a white wine. The Al foil was used in the form of 0.84 ± 0.03 mg discs cut with a hole punch (Figure 6.105).

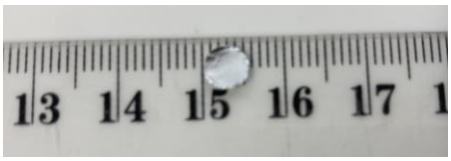


Figure 6.105. The size of aluminium disc cut from aluminium foil that was used in model wine experiments.

Samples were prepared in an anaerobic hood in 40 mL vials and were sealed without any ullage. Once prepared, the samples were stored in anaerobic jars that could be frequently sparged with nitrogen to ensure low oxygen concentrations. Oxygen sensors on the anaerobic jars allowed measurement of any accumulating oxygen and it was removed via sparging with nitrogen if it exceeded 1 % air saturation. The storage conditions were darkness and 25 ± 1 °C. Samples underwent periodic analysis of free and total SO_2 (automated analyser), Cu fraction 1 (BCA-colorimetry), Al^{3+} and total Cu (ICP-OES). The free H_2S concentration was measured by both gas detection tubes and GC-SCD but only data from the latter will be shown in this report.

The first set of experiments investigated the influence of the number of aluminium discs placed within the 40 mL model wine samples, which were varied from none to five. All samples contained 0.6 mg/L Cu(II) (added as copper(II) sulfate pentahydrate) apart from one additional sample that contained 3 Al discs and 0 mg/L total Cu. Figures 6.106a,b show that the aluminium discs led to a decrease in both free and total sulfur dioxide with most loss occuring within the first 5-days of storage. Cu did not appear to have an influence on the amount of sulfur dioxide loss based on the similar losses in the 3-disc Al samples with 0 or 0.6 mg/L Cu(II).

Free H_2S was detected in all the samples containing aluminium discs confirming the ability of sulfur dioxide to be converted to free H_2S in this model wine system (Figure 6.106c). The free H_2S increased with the number of alumnium discs and the presence of Cu in the 3-disc Al sample had no signficant impact (p < 0.05) on the free H_2S concentration apart from inducing a lower concentration of free H_2S at day 9. The magnitude of free H_2S was large ranging from 13.5 to 270 µg/L in the Al containing samples. The Cu fraction 1 concentration also decreased rapidly in all samples containing Al which is consistent with the production of hydrogen sulfide and the conversion of Cu organic acids (Cu fraction 1) to copper sulfides (Cu fraction 3). Finally, Figure 6.106e showed that the concentration of Al^{3+} in the model wine system increased over the storage period in samples containing Al discs, with higher concentrations with more discs present.

These results are consistent with equation 1, whereby sulfur dioxide can react with metallic aluminium in the acidic environment of wine and produce Al^{3+} and hydrogen sulfide. The hydrogen sulfide binds with Cu(II) present in the model wine as Cu(II) tartrates (Cu fraction 1) and forms copper sulfides (Cu fraction 3). These results show that if wine comes into contact with metallic aluminium it will cause the rapid loss of Cu fraction 1. A second experiment with sample pH variation (ranging from 3.2 to 4.0) and the presence of 5 aluminium discs showed no signficant impact (p < 0.05) of pH on concentrations of free and total SO_2, free H_2S, Cu fraction 1 or Al^{3+} (data not shown).

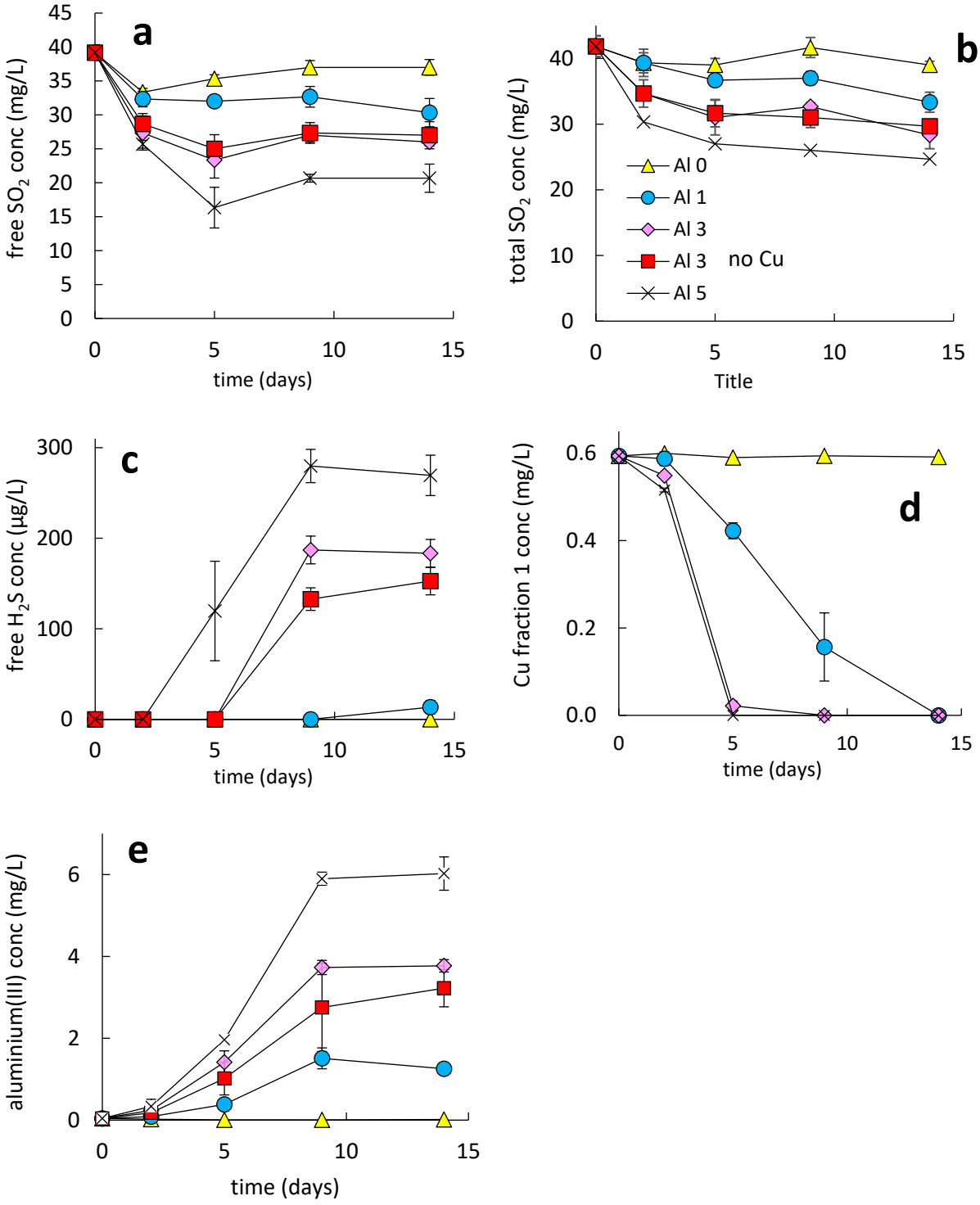


Figure 6.106. The concentration of free SO_2 (a), total SO_2 (b), free H_2S (c), Cu fraction 1 (d) and Al^{3+} (e) in the model wine samples. Error bars indicate the standard deviation of triplicate samples.

The next set of experiments investigated the impact of Zn^{2+} and Al^{3+} both alone and in combination with five aluminium discs on the Cu fractions and free H_2S in the model wine system. This was conducted to assess whether Zn^{2+} could bind H_2S and thereby potentially slow the loss of Cu fraction 1 through competitive binding. Given the inclusion of Al^{3+} in equation 1, it was of interest to determine whether wine Al^{3+} concentration could influence this reaction for free H_2S production. In this experiment the concentrations of Cu^{2+}, Zn^{2+} and Al^{3+} used were 0.6, 3 and 2 mg/L as these were within the concentration ranges commonly found for the metal ions in commerical wines.

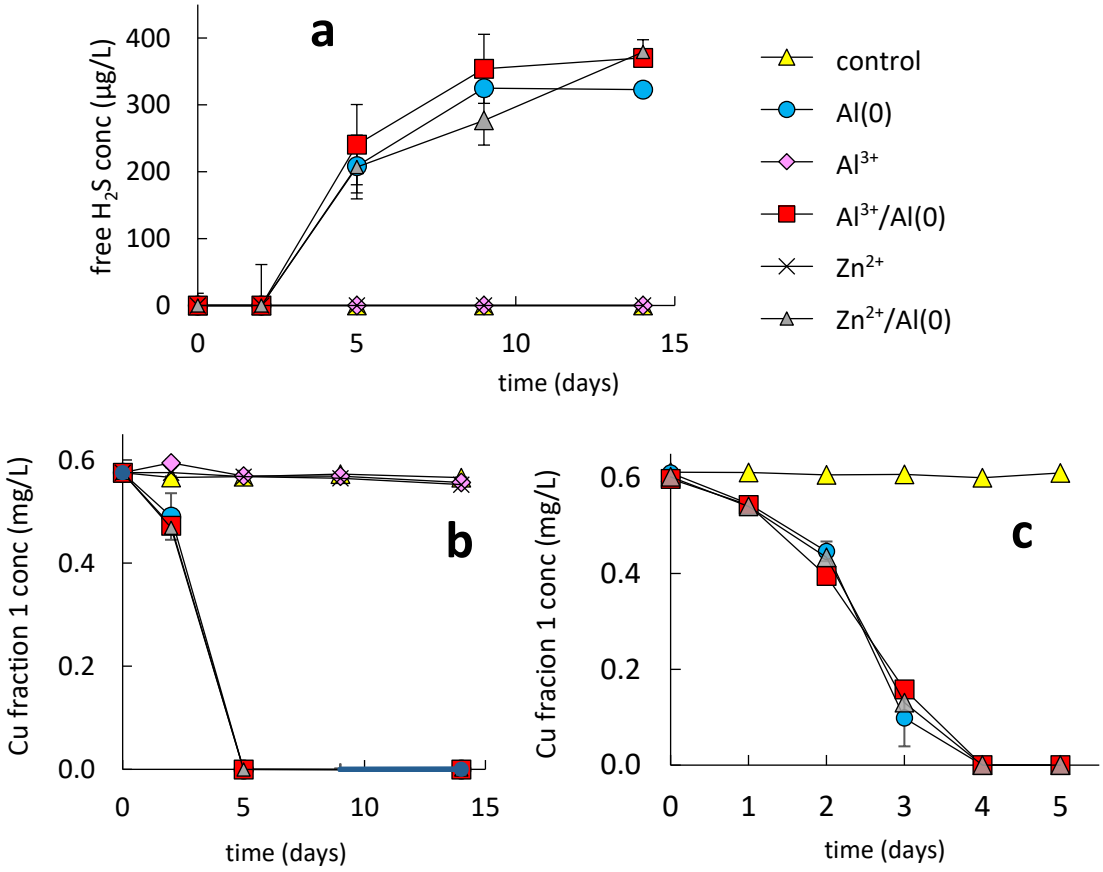


Figure 6.107. The concentration of free H_2S (a), Cu fraction 1 (b,c) in the model wine system. Panel c is the repeated experiment with more time points between days 2 and 5.

Figure 6.107a shows that free H_2S was generated in samples containing the Al discs but none was formed in the samples containing Al^{3+} or Zn^{2+} alone (i.e., without Al discs). Also, there was no impact by Al^{3+} or Zn^{2+} on the amount of free H_2S in the samples containing Al discs. The same was the case for the rate of Cu fraction 1 loss (Figure 6.107b). However, due to the rapid decrease in Cu fraction 1 concentration the experiment was repeat with more Cu fraction 1 measurements performed between days 2 and 5 of the experiment. From Figure 6.107c it is evident that neither Zn^{2+} or Al^{3+} impacted the loss of Cu fraction 1 during the reaction of SO_2 with Al to produce hydrogen sulfide.

The final experiment using the model wine system assessed the potential for Cu fraction 1 to decrease when sulfate (SO_4^{2-}) was the only sulfur-source for H_2S in the model wine system. In this case, a set of samples utilised either 50 mg/L SO_2, which equated to 0.78 mM, or 0.78 mM sodium sulfate. Figure 6.108 shows the free H_2S and Cu fractions in the samples. The SO_2 treatment showed the typical production of

free H_2S and consequent decrease in Cu fraction 1. The samples with SO_4^{2-} did not show free H_2S but a significant decrease in the concentration of Cu fraction 1 was measured. The lack of free H_2S in the SO_4^{2-} samples is consistent with the significant amount (> 0.1 mg/L) of Cu fraction 1 still present by day 14. Free H_2S can only accumulate once the concentration of Cu fraction 1 falls to much lower concentrations (typically below 0.02 mg/L). This result shows that the Cu fraction 1 concentration can still be lowered by metallic Al even in the absence of SO_2, albeit at a slower rate. This is either SO_4^{2-} in the presence of metallic aluminium generating a strong binding compound, such as hydrogen sulfide, for Cu fraction 1, or reactions are occurring which precipitate Cu onto the metallic aluminium.

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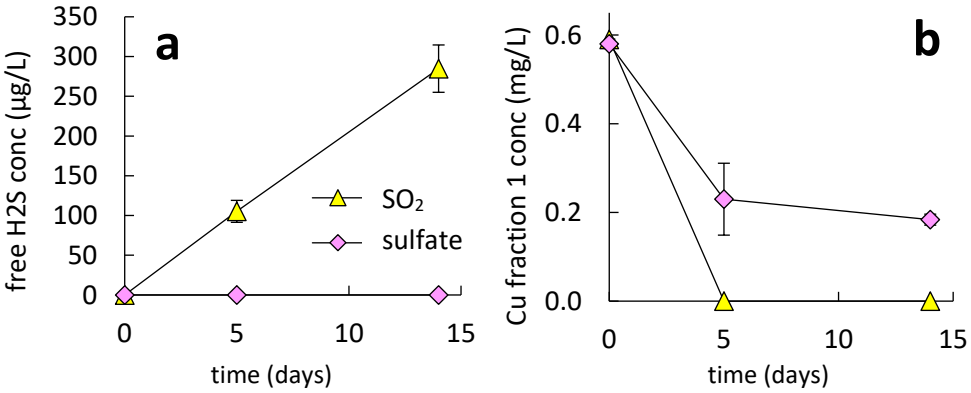


Figure 6.108. The concentration of free H_2S (a) and Cu fraction 1 (b) in the model wines system with either sulfur dioxide or sulfate as a substrate for reduction by metallic Al.

The results of the model wines studies show that SO_2 can be reduced by metallic aluminium and result in the formation of hydrogen sulfide that can bind Cu fraction 1 and accelerate its loss. Increasing the amount of aluminium led to an increased loss of Cu fraction 1 while neither wine pH, within the range of 3.2 to 4.0, or the presence of other metal ions, such as Zn^{2+} or Al^{3+}, had any impact. The loss of Cu fraction 1 also occurred when sulfur dioxide was substituted with SO_4^{2-}, implying that the presence of SO_2 is not critical for Cu fraction 1 loss by metallic aluminium in wine. However, Cu fraction 1 loss was much faster in the presence of SO_2 compared to SO_4^{2-}.

6.6.2 The impact of Al foil with Al(III), Fe(II) or Zn(II) on Cu fractions in white wine.

The study on the impact of metallic Al in combination with 2 mg/L Al^{3+} or 3 mg/L Zn^{2+}, that was performed in the model wine system (Section 6.6.1), was repeated in a Chardonnay wine. In addition, the influence of 1 mg/L Fe^{2+} was also assessed. The experimental conditions are identical to those used in the model wine system, including the use of 5 metallic aluminium discs for all samples.

Figure 6.109 shows the concentrations of sulfur dioxide (a,b), free hydrogen sulfide (c), Cu fraction 1 (d), Cu fraction 2 (e) and Cu fraction 3 (f) in the Chardonnay during the two-week storage period. In this case, there was no significant impact of metallic Al concentration on the free or total sulfur dioxide concentrations (Figure 6.109a,b). There was only free H_2S production in the wines containing metallic aluminium and no impact of Al^{3+}, Fe^{2+} or Zn^{2+} on the amount of free H_2S generated (Figure 6.109c). The concentration of free H_2S generated in the white wine (Figure 6.109c) was around half the concentration observed for the model wine system (Figure 6.106b). The samples with metallic aluminium all had a faster decrease in Cu fraction 1 concentration (Figure 6.109d) and an earlier depletion of Cu fraction 2 (Figure

6.109e) than the control sample without metallic aluminium. Cu fraction 3 was dominant in all the wines with metallic Al by day 5 and was significantly higher than in the control wine in most samples by day 2 (Figure 6.109f). There were no significant impacts ($p < 0.05$) of Al^{3+}, Fe^{2+} or Zn^{2+} on the influence of metallic Al on Cu fractions although in the Al^{3+} samples there was a trend on day 2 to a slower transition from Cu fractions 1 and 2 to Cu fraction 3 (Figure 6.109d-f).

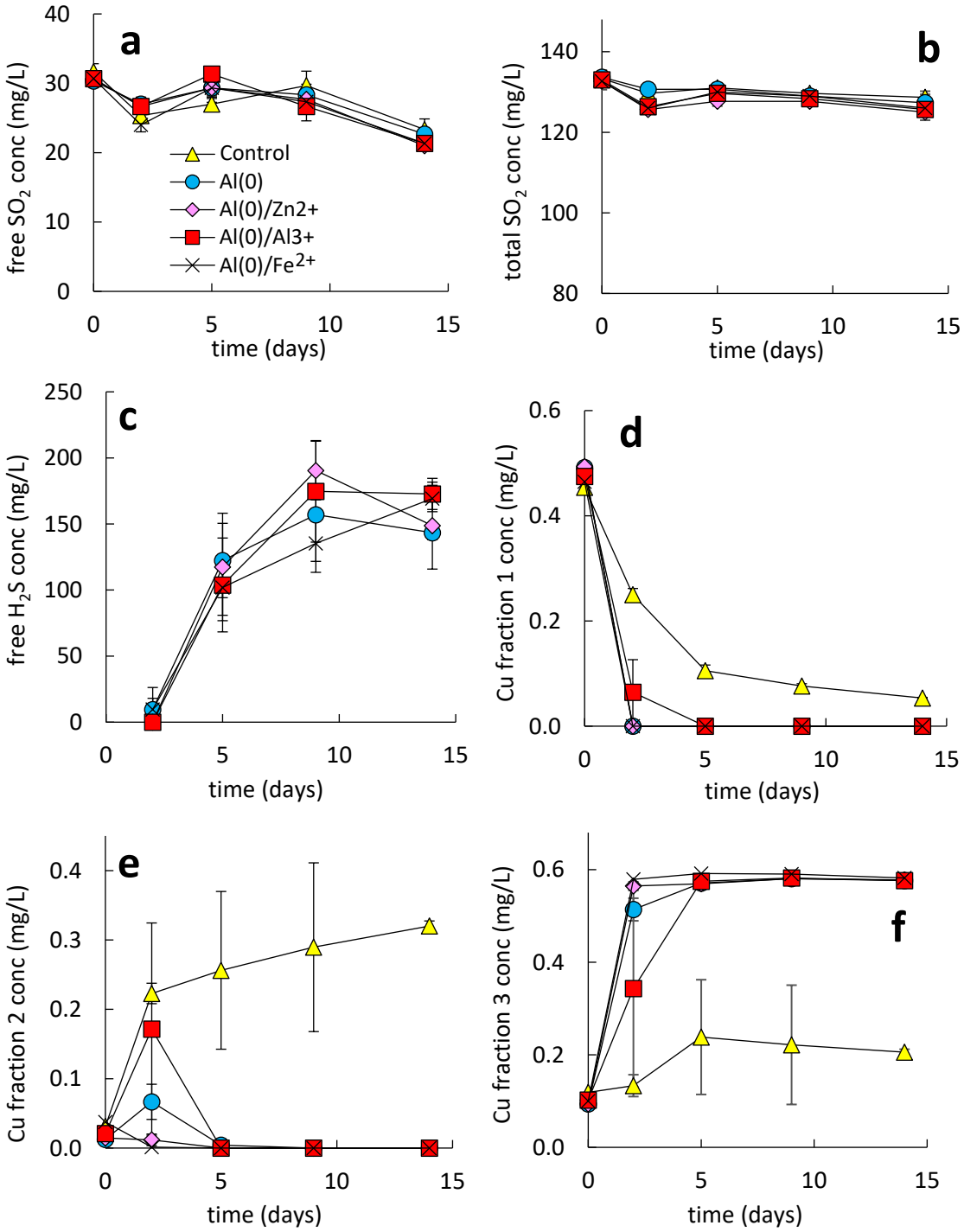


Figure 6.109. The concentration of free SO_2 (a), total SO_2 (b), free H_2S (c), Cu fraction 1 (d), Cu fraction 2 (e) and Cu fraction 3 (f) in the Chardonnay during storage alone (control) or in combination with Al(0), or Al(0) and Zn^{2+}, or Al(0) and Al^{3+}, or Al(0) and Fe^{2+}.

The results demonstrated that metallic Al has less impact on SO_2 concentrations in a white wine compared to the model wine system. However, significant concentrations of free H_2S were still formed in the white wine by metallic Al. This implied that the metallic aluminium could source sulfur from other sources besides SO_2 in real wine samples. The metallic aluminium accelerated the loss of Cu fractions 1 and 2 in white wine along with the accelerated production of Cu fraction 3, consistent with the formation of copper sulfides. Other metal ions such as Al^{3+}, Fe^{2+} and Zn^{2+} had negligible impact on the concentrations of Cu fractions or free H_2S.

6.6.3 A Pinot Grigio can aging trial with variable Cu and/or damaged liners.

An aging trial was conducted using Pinot Grigio in 250 mL aluminium cans to assess the impact on Cu fractions. The treatments were: i) different Cu(II) concentrations at the time of filling the cans with wine, and ii) a level of damage to the internal liner of the can. The aims were to follow the loss of Cu fractions 1 and 2 during aging in the can, as well as establishing the impact of liner damage on the decrease of Cu fractions 1 and 2 and the production of any free H_2S.

The cans used were those recommended for wine packaging and had bisphenol A-diglycidyl ether epoxy liners. To a portion of the cans, a pin was used to inflict three single point indentations down the length of the can on the internal surface and for each can this was performed four times with the indentations made equidistant from each other (Figure 6.110). This treatment was termed *damaged*. The other treatment was addition of 0.3 mg/L Cu(II) (as copper(II) sulfate pentahydrate) to the wine as it was being filled into the cans (more detail below).

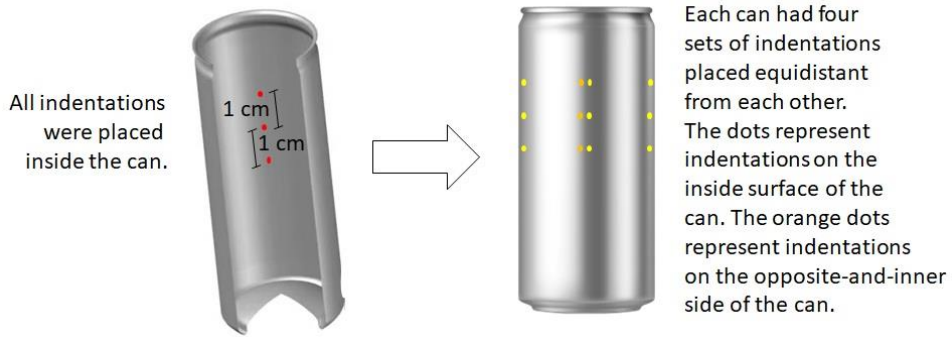


Figure 6.110. A schematic of the indentations used for the *damage* treatment of the cans.

Figure 6.111 shows the cans and the canning process. During the automated filling process, the cans were sparged with carbon dioxide, filled with wine, dosed with liquid nitrogen and then capped (Figure 6.111). For the cans with Cu(II) addition, 0.15 mL of 500 mg/L Cu(II) was added to the 250 mL Pinot Grigio in the can between the liquid nitrogen dosing step and before capping, which afforded a Cu(II) addition of 0.3 mg/L. Just prior to canning, the ascorbic acid concentration was 58 ± 1 mg/L, the pH was 3.10 ± 0.05, and the free and total SO_2 concentrations were 31 ± 2 mg/L and 73 ± 1 mg/L, respectively. The base Pinot Grigio wine was measured with ICP-OES and had a total Cu concentration of 0.403 ± 0.002 mg/L, and the wine with the addition of 0.3 mg/L had a measured concentration of 0.680 ± 0.008 mg/L (standard deviation, n=3). The concentration of other metals in the wine were 0.81 ± 0.01 mg/L for Al^{3+}, 78 ± 1 mg/L



Figure 6.111. The automated can filling equipment. The complete filler unit (upper left), the stock of incoming empty cans (upper right), cans entering the filling unit where they are sparged with CO_2, filled with wine, dosed with liquid nitrogen and then capped (lower right), the cans exiting the filler after capping (lower left).

for Mg^{2+}, 1.69 ± 0.03 mg/L for Mn^{2+}, 0.58 ± 0.01 mg/L for Zn^{2+} and 0.39 ± 0.01 mg/L for Fe^{2+}. Once canning was completed the wines were stored at 25 ± 1 °C.

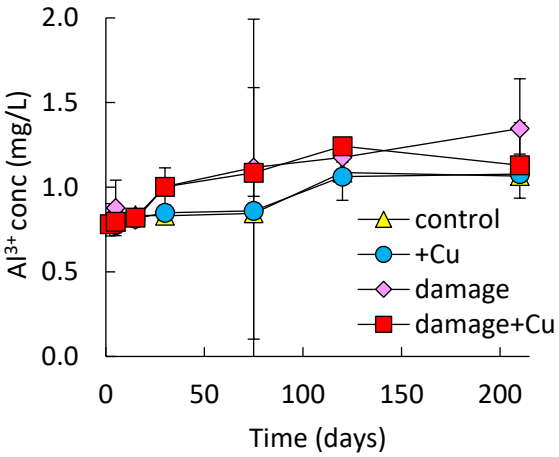


Figure 6.112. The concentration of Al^{3+} in the canned Pinot Grigio over 4-months of storage at 25 °C.

The Al^{3+} concentration in the samples was used as a measure of the effectiveness of the *damaged* treatment (i.e., the multiple indentations of the inner wall of the cans). It was expected the *damaged* cans would allow more contact between wine and metallic Al in the can wall, and thereby enhance extracton of Al^{3+} into the wine. Figure 6.112 shows that this was generally the case with trends to higher concentatrations of Al^{3+} for wines in the damaged cans from the day 30 timepoint. However, the uncertainty in the measured Al^{3+} concentrations were large (see the error bars in Figure 6.112) implying that the extraction of Al^{3+} into the wine from cans was highly variable. A contributor to this variability may have been differences in the depth of the indentations on the cans. Conversely, at the day 75 and day 120 time points the samples with the highest uncertainty in measured Al^{3+} were the samples in undamaged cans (i.e., '+Cu' and 'control' samples, respectively).

The SO_2 and ascorbic acid concentrations had large decreases in all samples during the first two weeks of storage (Figure 6.113), which suggested elevated total packaged oxygen concentrations in the sealed cans. This would be expected to delay the appearance of free hydrogen sulfide compared to a wine of lower total packaged oxygen and also potentially slow the transition of Cu fractions 1 and 2 to Cu fraction 3.

Free hydrogen sulfide was not detected in any samples until 120 days of storage (Figure 6.114). At the subsequent analysis time point (210 days), all samples contained free hydrogen sulfide and the concentrations of 3 to 25 µg/L were relatively high compared to the published aroma threshold of hydrogen sulfide (i.e., 1 µg/L). The highest concentration by day 210 was still 5 times lower than observed for the white wine with the Al foil discs (Figure 6.109c). By day 210, there was a trend to higher concentrations of free hydrogen sulfide in the control wine compared to the other treatments (Figure 6.113), but due to a large uncertainty in the measured values the difference was not statistically significant ($p < 0.05$). Such large uncertainty in the free hydrogen sulfide generated in cans was consistent with the large uncertainty observed for the Al^{3+} concentrations in the wines. Other studies have also shown large uncertainity in free hydrogen sulfide measured in cans which was attributed to random imperfections of liners within the cans.

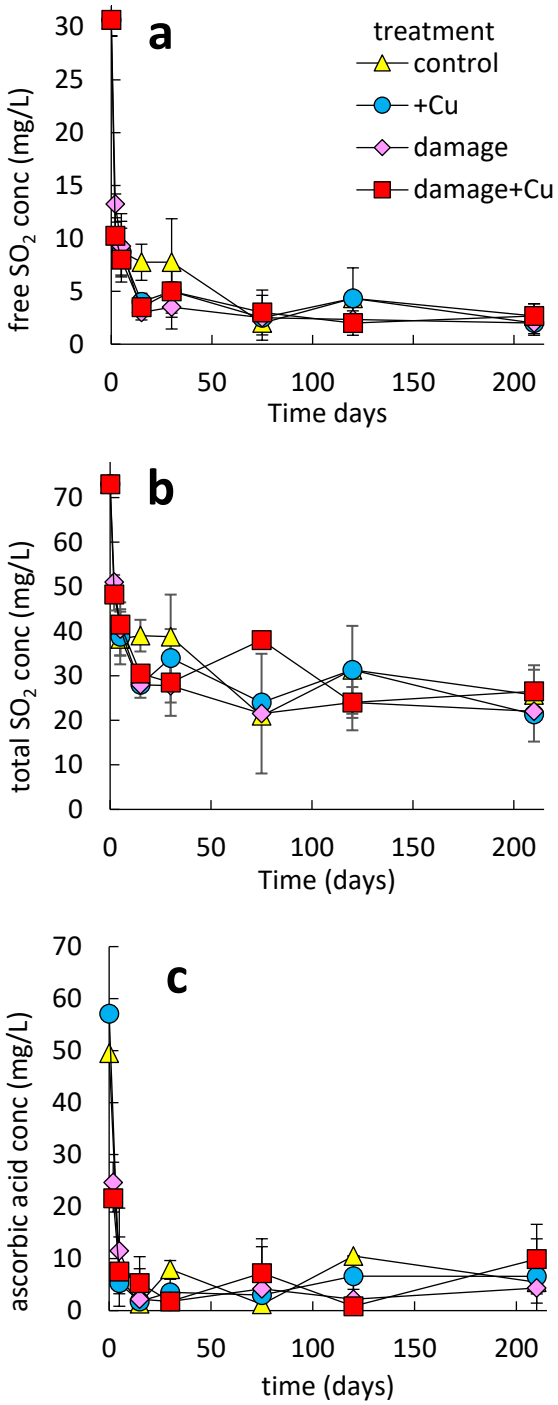


Figure 6.113. The concentrations of free SO_2 (a), total SO_2 (b), and ascorbic acid (c) in the canned Pinot Grigio during aging. The error bars are the standard deviation (n=3).

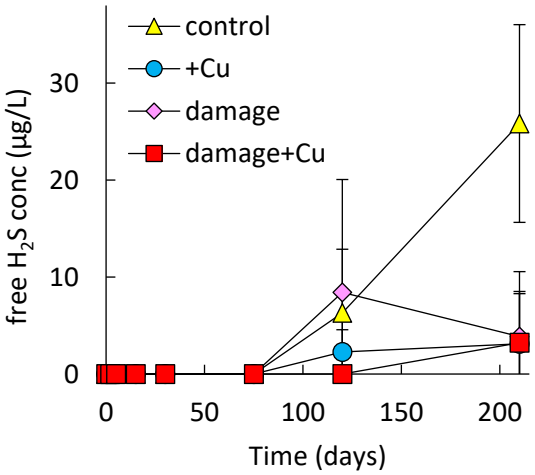


Figure 6.114. The concentration of free hydrogen sulfide in the Pinot Grigio during 210 days of storage at 25 °C. The error bars indicate the standard deviation (n=3).

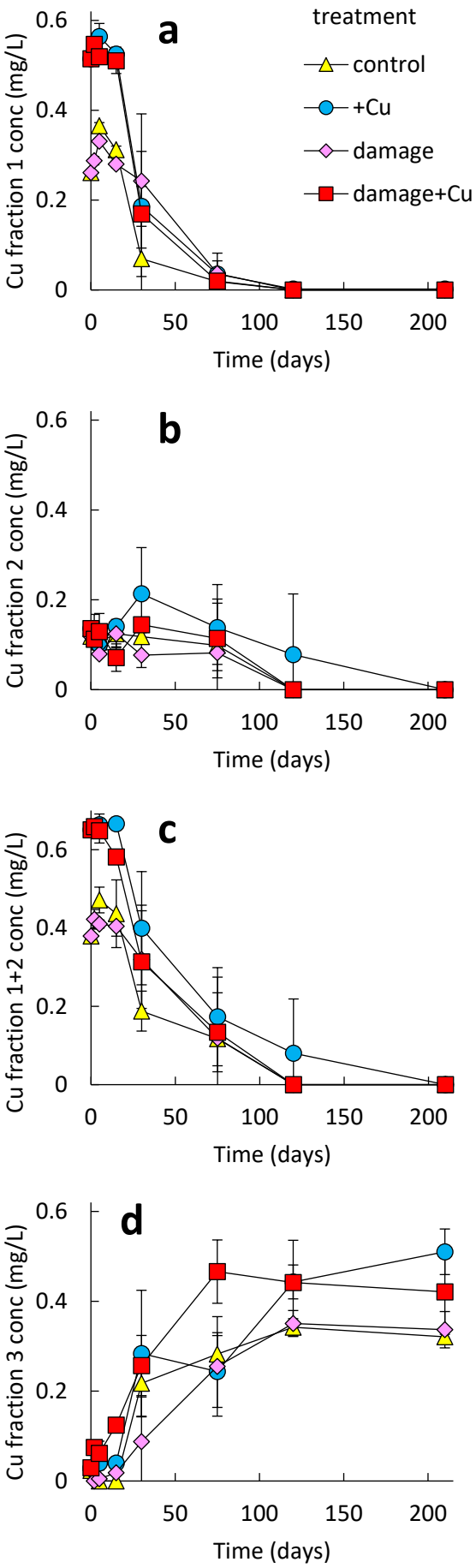


Figure 6.115. The concentrations of Cu fraction 1 (a), Cu fraction 2 (b), combined Cu fractions 1+2 (c), and Cu fraction 3 (d) in the canned Pinot Grigio during aging.

The Cu fraction 1 and 2 concentrations decreased during the aging of the wine in can (Figure 6.115a-c). As expected, the wines with 0.3 mg/L Cu added during the canning process contained higher Cu fraction 1 and 2 concentrations in the early stages of the storage experiment but by 75 days the Cu fraction 1 and 2 concentrations were similar across all samples. The Cu fraction 1 and 2 first order decay rates were also similar across all samples, regardless of treatments, and was 0.020 ± 0.02 $days^{-1}$ giving a half-life of 34 days. This rate of decay was much faster than for white wines aged in ideal conditions (i.e., Table 6.15, shortest half-life 47 days) but not as fast as white wines aged at 40 °C (Table 6.15, average half-life 5 ± 1 days). There were only minor differences caused by the *damage* treatment of the cans on Cu fraction concentrations but none were signficant ($p < 0.05$).

The results of this study show that Cu fractions 1 and 2 are lost more rapidly in wine stored in cans than wine stored in bottle in ideal conditions. The increased concentration of Cu at the filling of wines in cans did not cause any significant increase in free H_2S and there were trends to less free H_2S. The damaged cans did lead to some increased extraction of Al^{3+} but the inherent variability in Al^{3+} extraction among both damaged and undamaged cans meant that the difference was not significant. The damaged cans did not lead to any significant differences in the change of Cu fractions or accumulation of free H_2S.

6.5 'How to' guide for winemakers

The 'How to' guide was successfully completed. See the attached files: '*HowToCuGuide_2025.pdf*', and '*BCA_Cu_calculator.xlsx*'.

6.6 Teaching materials

1. The book chapters listed below draw upon research generated in this project and both were utilised in the Charles Sturt University subjects WSC115 Wine Science 1 and WSC317 Wine Science 2. These subjects are in the Bachelor of Viticulture, Bachelor of Wine Business and/or Bachelor of Wine Science courses.

- Clark, A.C. and Bekker, M. (2021) Ch. 9. Metal ions and complexes. *In* Theory and concepts of chemical, physical and sensory analyses and test of grapes and wine. Editors: Iland, P., Bruer, D., Bruer, N., Ford, C. and Wilkes, E. (Publisher: Patrick Iland Promotions, Adelaide). 1st Edition. pp. 99-103.
- Clark, A.C. and Bekker, M. (2021) Ch. 10. Gases. *In* Theory and concepts of chemical, physical and sensory analyses and test of grapes and wine. Editors: Iland, P., Bruer, D., Bruer, N., Ford, C. and Wilkes, E. (Publisher: Patrick Iland Promotions, Adelaide). 1st Edition. pp. 104-119.

2. In addition, the following recorded presentation and power-point slides are utilised within WSC319 Wine Chemistry:
Clark. A.C. (2024) Managing copper in the lead up to bottling: Cu fractions and other things you should know. *ASVO Seminar: Achieving clarity and getting wine into bottle.* (online webinar) 20 June.

3. The 'How-to-guide' (Section 6.5) and BCA_Cu_calculator (Appendix 6) have also been integrated into the subject materials for WSC319 Wine Chemistry (within the Cu fining component and the laboratory manual).

4. The attached PowerPoint slides (see '*Cu_WSC319_WineChem.pptx*') are utilised in the WSC319 Wine Chemistry subject.

7 OUTCOMES AND CONCLUSIONS

The objectives of this project were to:

1. Determine the influence of metal speciation and wine composition on the amount of sulfur dioxide consumed per mg/L oxygen in red and white wine.
2. Assess reversibility of key Cu speciation forms and their activity on mechanisms directly relevant to the development of red and white wines.
3. Establish the influence of ascorbic acid on the stability and activity of copper(I) sulfide.
4. Determine the impact of metal speciation and metal concentration ratios on mechanisms that contribute to colour and flavour development in wine.
5. Establish a link between metal speciation and steps in the wine production process that allow efficient removal of metals from wine.
6. Trial several large-scale applications of the most viable novel winery operations identified in small scale wine production.
7. Communicate the novel wine production techniques to winemakers, including the preparation of a 'How to' guide.
8. Prepare teaching notes for use in oenology subjects at Charles Sturt University and The University of Adelaide.

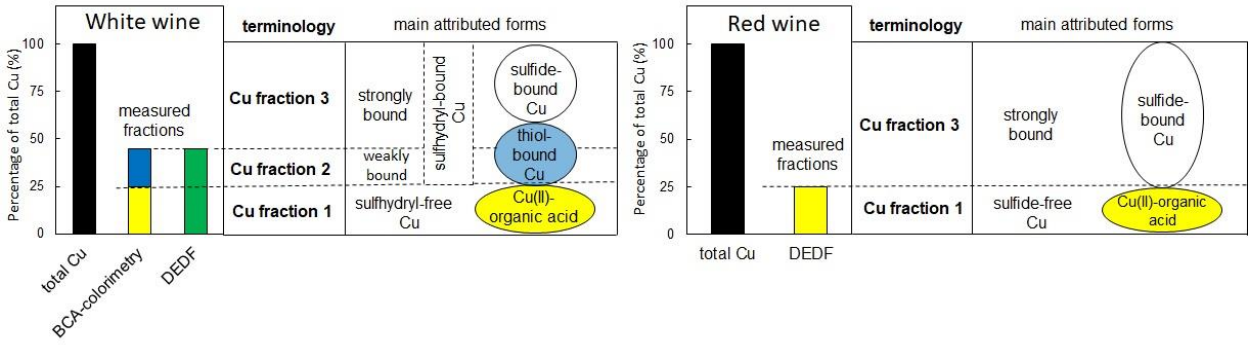
The specified outputs and activities of the project are addressed in the various sections of this report as indicated in mega-Table 4.1.

7.1 Measurement of total Cu and its fractions in wine

New methods were created to measure total Cu and its fractions in wine. This included the use of BCA-colorimetry for total Cu measurement in red wine utilising equipment commonly found in wineries. The accuracy of total Cu results on an automated analyser, using a commercially available Cu analysis kit, was improved with the inclusion of Ag(I) in the analysis methodology.

BCA-colorimetry and diatomaceous earth depth filtration (DEDF) were successfully applied to the determination of Cu fractions in wine. Three fractions of Cu (Figure 7.1) were measurable using equipment commonly found in wineries. Preliminary work was performed to enable automated analysers to quantify Cu fractions 1 and 2 in white wine. The results show good outcomes and with futher work the automated measure of Cu fractions should be readily achievable.

Figure 7.1. Different Cu fractions developed in this project.



7.2 The removal of Cu and its fractions from wine

Procedures for the removal of Cu from wine were investigated on both small and large wine volumes (Table 7.1). For the procedures that lowered total Cu concentration, the fraction of Cu generally removed was Cu fraction 3 (i.e., copper sulfides and aggregated Cu species), which is the least beneficial Cu fraction in wine. PVI/PVP was the exception, allowing efficient removal of all Cu fractions from white wine. The diatomaceous earth depth filtration and PVI/PVP methods investigated were the most efficient for the removal of Cu fraction 3 from wine in both small and large volumes. However, in terms of cost and integration into current wine production practises, bentonite has some clear advantages over PVI/PVP and diatomaceous earth depth filtration for Cu removal from white wine.

Table 7.1. Procedures assayed for Cu removal from wine. Symbols representing the percentage of total Cu removed by the different procedures:
✗ < 10 %, ✓ 10-40 %, ✓✓ 40-70 %, ✓✓✓ >70 %, ✓✓ > 40 % removal of total Cu based on past studies,[a] variable: loss, gain or no change of total Cu. Grey shading highlights the procedures not performed as part of this project.

procedure	pore size (µm)	treatment of small volume (laboratory scale)		treatment of large volume (winery scale)		main Cu fraction removed (1, 2 or 3)
		white	*red*	*white*	*red*	
membrane filtration						
cellulose	0.2-0.45	✗	✓	✗	✗	3
nylon	0.2-0.45	✓✓	✓✓	✗	✗	3
PES	0.2-0.45	✓✓✓	✓	✗	✗	3
PVDF	0.2-0.45	✗	✓	✗	✗	3
PTFE	0.2-0.45	✗	✓	✗	✗	3
depth filtration						
cellulose (CDF)	2-3	✓	✓			3
	1-2	✓	✓			3
	0.1-0.3	✓	✓	✓		3
diatomaceous earth (DEDF)	2-3	✓	✓			3
	1-2	✓✓	✓✓			3
	0.1-0.3	✓✓✓	✓✓✓	✓✓✓	✓✓✓[b]	3
bentonite						
calcium bentonite	n/a	✓✓		✓✓[a]		3
PVI/PVP						
PVI/PVP+ silicon dioxide	n/a	✓✓✓	✓✓✓			white: all
PVI/PVP+ chitosan	n/a	✓✓✓	✓✓✓	✓✓	✓✓	red: 3
crossflow filtration						
crossflow alone	0.2			variable	variable	3
with lenticular+membrane	0.2-0.45			variable	✓	3

cellulose, regenerated cellulose; PES, polyethersulfone; PTFE, polytetrafluroethylene; PVDF, polyvinylidene fluoride
[a] Rousseva et al. (2016) and Catarino et al. (2006).
[b] Based on medium scale experiments extrapolated to large scale results.

7.3 The impact of Cu species on wine aroma

This project was the first to assess the analysis methodologies best suited for measuring the key contributors to reductive aroma characters in wine. This study utilised data from sensory analysis in combination with quantitative data from free and salt-treated GC-SCD protocols for H_2S and methanethiol, and from the free H_2S gas detection tubes analysis. The GC-SCD and GDT measure of free H_2S concentration and the GC-SCD measure of free methanethiol and salt-treated methanethiol related best to reductive characters (i.e., sewage, rotten egg etc.) in wine. The salt-treated measure of H_2S did not relate to the reductive sensory characters in wine.

Cu fractions 1 and 2 were found to inhibit free H_2S and free methanethiol accumulation and therefore supressed the emergence of reductive aromas. This link was confirmed in the survey of over 800 bottles of white wine using the BCA colorimetric technique. A concentration of 0.02 mg/L for the combined concentrations of Cu fractions 1 and 2 was able to inhibit free H_2S to concentrations below its aroma threshold, while a concentration of 0.03 mg/L for Cu fraction 1 could inhibit methanethiol to concentrations below its aroma threshold. If H_2S was produced in the presence of Cu fractions 1 and 2 then a key mechanism to prevent its accumulation was the formation of copper sulfides (i.e., Cu fraction 3). Alternatively, if methanethiol was produced in the presence of sufficient Cu fraction 1 then oxidation of methanethiol to a disulfide was the more prevalent mechanism than complex formation. In red wines, Cu fraction 1 concentrations above 0.03 mg/L appeared to inhibit both free H_2S and free methanethiol inhibition. Although Cu fractions linked more directly to inhibition of the reductive aroma compounds than total Cu, it was observed that wines that developed reductive aroma characters generally had lower total Cu concentrations (i.e., < 0.12-0.25 mg/L).

Given the impact of Cu fractions 1 and 2 on sulfhydryl compounds (i.e., H_2S and methanethiol), their impact on the varietal thiol aroma compounds common to Sauvignon blanc were investigated. Cu(II) addition at bottling reduced the concentrations of 3-sulfhydrylhexanol (3SH), 3-sulfhydrylhexyl acetate (3SHA) and 4-methyl-sulfanylpentan-2-one (4MSP) compounds in two Sauvignon blanc wines. These compounds are generally associated with desirable aromas such as passionfruit, boxwood and grapefruit zest. The impact of Cu was immediate and in the case of 3SH remained evident throughout 18-months of aging. For 3SHA and 4MSP there was a diminishing influence of Cu(II) on their concentrations during aging, which mirrored the decreasing concentration of Cu fraction 1 during the bottle aging of the wine. Interestingly, Cu(II) addition did not always lead to wines being less associated with the aroma descriptors associated with the varietal thiol compounds. In fact, in some cases, Cu(II) addition led to wines being perceived to have more *tropical* and *fruit* character than control wines, possibly due to the ability of Cu(II) to remove potential masking compounds, such as free H_2S and methanethiol.

7.4 The impact of Cu speciation on the amount of sulfur dioxide consumed by oxygen

Cu fractions 1 and 2 accelerated the rate of loss of oxygen, free sulfur dioxide, total sulfur dioxide and ascorbic acid in white wines containing ascorbic acid. However, the distribution of Cu fractions did not impact any of these parameters in white wine without ascorbic acid or red wines. Ascorbic acid appeared to stabilise Cu fractions against the rapid transition to Cu fraction 1 and 2 induced by oxygen, and thereby allow the Cu fraction distributions at bottling to dictate oxygen

reaction rates in white wine. In all wine types, the distribution of Cu between fractions 1, 2 and 3 did not impact the amount of sulfur dioxide lost for a given amount of oxygen consumed by a wine. Instead, ascorbic acid led to a lower amount of total sulfur dioxide lost for a given amount of oxygen consumed by a white wine. The following summarise the different amounts of total sulfur dioxide lost for a given amount of oxygen consumed by the wine investigated (where the quoted uncertainty is the standard deviation (n=3)):

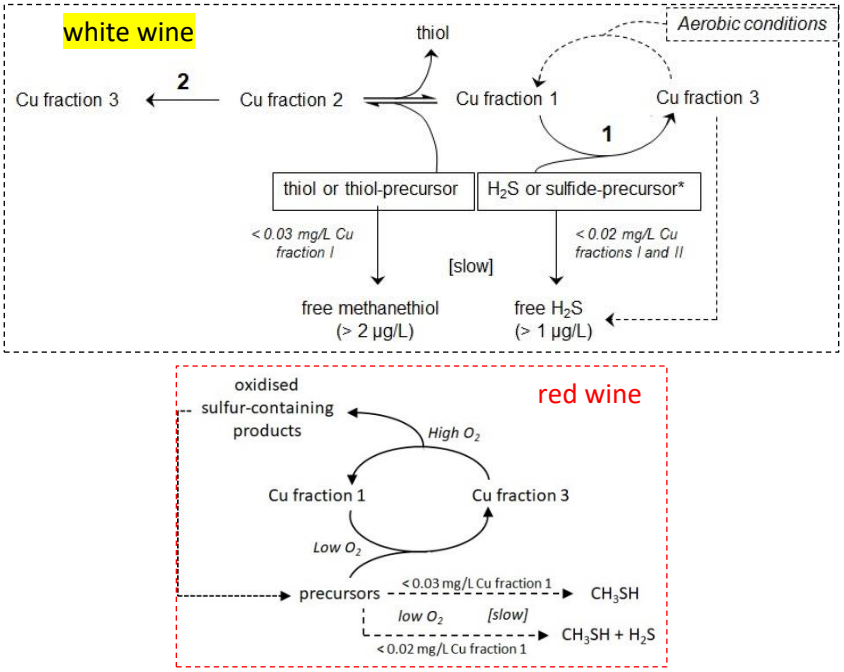
white wine = 3.6 ± 0.6 mg/L total sulfur dioxide for each 1 mg/L oxygen
white wine + ascorbic acid = 2.0 ± 0.2 mg/L total sulfur dioxide for each 1 mg/L oxygen
red wine = 2.8 ± 0.4 mg/L total sulfur dioxide for each 1 mg/L oxygen

7.5 The transition between Cu fractions in wine

After the addition of Cu(II) to wine, its distribution among the different fractions depended on whether the wine had reductive characters (i.e., a reactive addition) or had no reductive characters (i.e., a preventative addition). For preventative additions, the Cu fraction 1 and 2 forms are favoured after the addition, while for reactive additions, a significant amount of Cu fraction 3 is formed.

Figure 7.2 summarises the transition between Cu fractions in white wine (upper) and red wine (lower) during wine aging. With exposure to high oxygen conditions, Cu fraction 3 readily transitions to Cu fraction 1 and/or 2, while in low oxygen conditions Cu fractions 1 and 2 transition to Cu fraction 3. Upon depletion of Cu fraction 1 and 2 to less than 0.02 and 0.03 mg/L, respectively, free H_2S and free methanethiol can accumulate if the wine has a propensity for this to occur.

Figure 7.2 Proposed mechanisms for Cu fraction transitions in white wine (upper) and red wine (lower) stored in ideal conditions (darkness, 14-17 °C). CH_3SH: methanethiol.



Studies in model wines confirmed that glutathione-based and cysteine-based polysulfanes could act as precursors for sulfide and thiol compounds able to convert Cu fraction 1 to Cu fraction 2 or

3. In the model wine system, the presence of sulfur dioxide rapidly degraded polysulfanes and in the presence of Cu fraction 1 allowed the production of copper sulfides (Cu fraction 3). The yield of salt-treated H_2S from the cysteine-based polysulfanes was much higher in the presence of both Cu fraction 1 and SO_2 combined rather with SO_2 or Cu fraction 1 alone.

There were four conditions that were found to accelerate the loss of the protective fractions of Cu (i.e., 1 and 2) and increase Cu fraction 3:

i) ascorbic acid in white wine and rosé –

Ascorbic acid accelerated the loss of Cu fractions 1 and 2 in high or low oxygen conditions but did not appear to accelerate free H_2S or free methanethiol accumulation.

ii) light exposure to white wine –

Light exposure rapidly decreased Cu fraction 1 and 2 concentrations especially at higher riboflavin concentrations (Figure 7.3). Once Cu fraction 1 and 2 decreased below 0.02-0.03 mg/L, free H_2S and free methanethiol rapidly accumulated with continued light exposure. Cu fraction 3 (i.e., copper sulfides) appeared to enhance free H_2S production in wines exposed to light after Cu fraction 1 and 2 were depleted (i.e., < 0.02-0.03 mg/L). Darker bottles slowed the Cu fractions 1 and 2 loss induced by light. Cu fraction 1 and 2 offered transient protection from short-term light exposure in wines of low riboflavin concentration, but gave virtually no protection in wines which had high riboflavin concentration.

iii) elevated storage temperature for red and white wines –

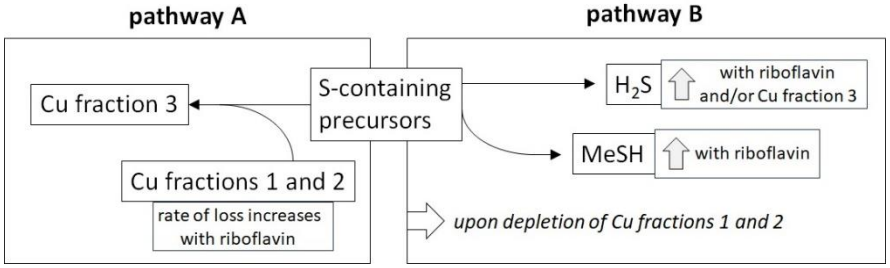
Cu fraction 1 and 2 concentration decreased at rates that were 10 to 20-fold faster when wine was stored at 40 °C compared to 14 °C.

iv) the contact of white wine with aluminium.

Cu fraction 1 and 2 loss and Cu fraction 3 (i.e., copper sulfide) production was accelerated as H_2S was generated from SO_2, and other precursors, by metallic aluminium in wine. Increased Cu(II) added at the canning of Pinot Grigio cans did not lead increase the amount of free hydrogen sulfide generated after 7-months of aging.

The decay rates for the protective Cu fractions 1 and 2 were highly variable in red and white wines despite storage in ideal conditions (14-17 °C). This meant that using the concentrations of Cu fraction 1 and 2 at bottling to predict the time it would take to fall below the critical concentrations (0.02-0.03 mg/L) was not possible based on the average decay rates of Cu fraction 1 and 2 across different wines. Alternatively, 28-day accelerated aging tests showed some potential in identifying those wines that would accumulate free methanethiol above 5 µg/L after being stored for 12-18 months in ideal conditions. The same test was not successful in highlighting wines capable of accumulating free hydrogen sulfide above 2 µg/L, but it was noted that for the wines analysed to date, the vast majority accumulating free methanethiol also accumulated free hydrogen sulfide.

Figure 7.3 The Cu fraction transitions in white wine of low O_2 concentration exposed to light (> 300 nm).



A relatively large lag period was evident between Cu fraction 1 and 2 depletion and the accumulation of free H_2S and free methanethiol for wines stored in ideal conditions. This meant that guidelines using concentrations of Cu fraction 1 and 2 marginally above their critical inhibition concentrations at bottling may be sufficient for longer term protection against reductive development of wine stored in ideal conditions. Such guidelines would be best used for wines highlighted by the accelerated aging test as being likely to accumulate free methanethiol. The results from 43 red, white and rosé wines stored in ideal conditions showed that Cu fraction 1 concentrations above 0.04 mg/L at bottling tended to provide long term (i.e., 12-month) inhibition of free methanethiol, and Cu fraction 1 and 2 above 0.115 mg/L provided long term inhibition for free hydrogen sulfide.

7.6 The impact of Al and other metal ions on Cu fractions in model wines and white wine

The outcomes for this work have already been summarised in the preceding section (Section 7.5). Metal ions such as Al^{3+}, Fe^{2+} and Zn^{2+} had no impact alone, or in combination with metallic aluminium, on Cu fractions or the production of free methanethiol.

7.7 'How to' guide for winemakers

This guide summarises the novel measurement techniques for total Cu and Cu fractions in wine, along with the guidelines for Cu fraction 3 removal and recommended Cu fraction 1 and 2 concentrations at bottling to limit the incidence of reductive wine development. Attached to the guide is an excel calculator to convert measured absorbances to Cu concentrations.

7.8 Teaching materials

Various aspects of this project have been incorporated into subjects within the CSU courses of Viticulture, Wine Science and Wine Business.

8 RECOMMENDATIONS

The following are the recommendations from this project:

Broad Industry Practises

- Given the influence of Cu on wine development, it would be worthwhile for wineries to have a greater understanding of the importance for total Cu measurement in wine. Total Cu concentrations can be routinely measured in red and white wines utilising a bench-top spectrophotometer and the BCA colorimetric methodology. The only requirement of the spectrophotometer is its ability to hold a glass 40 mm cuvette.
- Prior to making preventative Cu additions to wine, it is recommended to measure the concentrations of Cu fraction 1 and 2 already present. Cu fractions (i.e., 1, 2 and 3) in white wines can be routinely measured in white wines using a bench-top spectrophotometer and the BCA colorimetric methodology. The analysis can be performed quantitatively, with calibration during each analysis, or semi-quantitatively and more rapidly if calibration is extrapolated from typical past calibration responses. Alternatively, Cu fractions can be measured in red and white wines using diatomaceous earth depth filtration to adsorb Cu fraction 3 and allow detection of Cu fractions 1 and 2 combined in the filtrate. Detection of Cu fractions 1 and 2 can be performed with routine measures of Cu (e.g., BCA colorimetry, ICP-OES, FAAS).
- If total Cu concentrations are high in a wine, and the Cu is predominantly in the form of Cu fraction 3, then the recommended approaches for the removal of the Cu from large volumes of wine are: i) depth filtration using cellulose sheets incorporated with diatomaceous earth, ii) PVI/PVP or iii) bentonite (in the case of white wine). If the Cu is in the form of Cu fraction 1 and 2 (i.e., after a mistaken addition of too much Cu(II)) then PVI/PVP is likely to have more success than the other two methods.
- Free GC-SCD measures of H_2S and methanethiol or free gas detection tube measures of free H_2S should be used for quantifying the aroma compounds directly responsible for reductive aroma characters in wine. The salt-treated GC-SCD measures are not an accurate measure for the contribution of H_2S to reductive aroma characters.
- Preventative Cu(II) additions to wines with desired varietal thiol aromas (i.e., Sauvignon blanc) should be carefully considered with efforts to balance risks against excessive H_2S and methanethiol accumulation against losses of beneficial aromas. Cu addition at bottling leads to a decrease in the concentration of varietal thiol aroma compounds but if masking agents, such as free H_2S and methanethiol are removed, it can still lead to wines being perceived to have more *tropical* and *fruit* characters.
- If the loss of SO_2 is to be minimised after white wine or rosé bottling, due to its reaction with total packaged oxygen, then ascorbic acid can assist in this role. The amount of total SO_2 lost for a given amount of oxygen consumed can be nearly halved if ascorbic acid is present.

- To maximise the concentrations of Cu fraction 1 and 2 in a wine during bottle aging, then Cu(II) additions to wine should be made close to the bottling of the wine and in the absence of any reductive characters.
- Wine producers and retailers should be more cognisant that the exposure of wine to light and storage at elevated temperatures will accelerate the loss of Cu fraction 1 and 2 and hasten the appearance of reductive aromas.
- It is recommended that Cu fraction 1 and 2 be used as a measure of non-ideal storage conditions for a wine. That is, winery hold-back-samples stored in ideal conditions (i.e., darkness and 14-17 °C) and compared to commercial off-the-shelf samples for Cu fraction profiles. The extent of conversion of Cu fractions 1 and 2 to Cu fraction 3 relative to the winery retained sample will determine the extent of gross light exposure and elevated temperature that the commercial off-the-shelf sample has encountered.
- Winemakers should already be aware of the benefits in storing wine in darker bottles to protect them from the effects of light. However, it is recommended that particular care be taken for wines that have elevated riboflavin concentrations and/or Cu fraction 3 as both can promote free H_2S and free methanethiol formation with light exposure.
- Wine makers should be aware that free H_2S will not accumulate in wine unless the combined Cu fraction 1 and 2 concentration is below 0.02 mg/L, and free methanethiol will not accumulate unless the Cu fraction 1 concentration is below 0.03 mg/L. This does not mean that wines with Cu fraction concentrations below these levels will have problems with free H_2S or free methanethiol accumulation. Instead it means that wines with a propensity for free H_2S or free methanethiol to be produced can only accumulate these compounds once the Cu factions drop below these specific concentrations.
- Based on the bottle aging studies of 43 red, white and rosé wines, the current recommendations for Cu fraction 1 and 2 levels at bottling to reduce the likelihood of reductive aroma accumulation for wine stored in ideal conditions are:
    - > 0.04 mg/L Cu fraction 1 to prevent free methanethiol
    - > 0.115 mg/L for the combined concentrations of Cu fraction 1 and 2 to prevent free H_2S
- A more efficient test needs to be designed to highlight those wines with a high probability of accumulating free H_2S and free methanethiol than currently available. At the moment, based on the study of 10 white wines, an accelerated aging study was found to have success in flagging the white wines most at risk from developing elevated methanethiol concentrations (> 5 µg/L) after 12-18 months of bottle aging in ideal conditions. An important aspect of this test was aging them at 40 °C for 21-28 days in a screw cap sealed wine bottle with a similar total packaged oxygen as would be present in the finished wine. Although the same test was not successful in highlighting wines that would accumulate free H_2S, it was evident that in most wines that accumulated free methanethiol in ideal storage conditions, they also accumulated free H_2S. This test would require the measurement of free or salt-treated methanethiol concentration by GC-SCD, and therefore would most likely require commercial testing (e.g., AWRI Commercial Services).

Future research

- An assessment is required for commercially available Cu kits used by automated analysers for their accuracy in determining total Cu in red and white wines. For example, a past Cu kit (no longer commercially available) showed more accurate results for total Cu in white wine with the inclusion of Ag(I) whilst another Cu kit overestimated total Cu if Ag(I) was included in the methodology. Research assessing the accuracy of several commercially available kits would be worthwhile and allow wineries greater confidence in using such kits for routine wine analysis.
- Validating a methodology for measuring Cu fractions on an automated analyser in both red and white wine is critical. The adoption of Cu fraction measurement by larger wineries, and commercial wine analysis providers, would be much greater if their measurement can be automated. Preliminary results show a high probability of successful validation of the measure if a future investigation was carried out.
- More detailed studies are required on the impact of cross flow filtration, and its use in series with other filtration techniques, to better understand the variability for its effect on total Cu and Cu fractions in wine.
- Studies would be worthwhile to confirm or disprove a causative link between Cu fraction 1 and the Cu-induced decrease in concentration of varietal thiol aroma compounds.
- When SO_2 is added to a solution of polysulfanes, the presence of Cu fraction 1 results in a higher yield of hydrogen sulfide emanating from the polysulfane (i.e., in the form of copper sulfides). More research is required to establish if the products formed from polysulfanes and SO_2, in the absence of Cu fraction 1, can act as quantitatively important precursors for reductive aroma compounds during the bottle aging of wine.
- A more practical test is necessary to identify those wines at risk from undergoing reductive development during bottle aging. This would be more readily adopted by wineries than a 21-28-day heating test at 40 °C. Combining the addition of reducing agents (as has already been investigated by other researchers) with heating, and/or light exposure, may be a worthwhile approach. This research will require a long-term project enabling 12-18 months for bottle storage trials to validate short-term testing approaches.

9 Appendix 1: Communications

Publications in international peer review journals:

1. Clark, A.C. and Kontoudakis, N. (2018) Increasing the efficiency and accuracy of labile Cu measurement in wine with screen-printed electrodes. *Chemosensors* **6**, 35.
2. Kontoudakis, N., Schmidtke, L.M., Bekker, M.Z., Smith, M., Smith, P.A., Scollary, G.R., Wilkes, E.N. and Clark, A.C. (2019) Analytical strategies for the measurement of different forms of Cu and Fe in wine: Comparison between approaches in relation to wine composition. *Food Chemistry* **274**, 89-99.
3. Zhang, X., Kontoudakis, N., Blackman, J.W and Clark, A.C. (2019) Copper(II) and sulfur dioxide in Chardonnay juice and Shiraz must: Impact on volatile aroma compounds and Cu forms in wine. *Beverages* **5**, 70.
4. Clark, A.C., Zhang, Z. and Kontoudakis, N. (2020) Simplified measures of copper fractions in wine: colorimetric and filtration-based approaches. *The Australian Journal of Grape and Wine Research* **26**, 399-409.
5. Kontoudakis, N. and Clark, A.C. (2020) Sulfide-binding to Cu(II) in wine: Impact on oxygen consumption rates. *Food Chemistry* **316**, 126352.
6. Kontoudakis, N., Smith, M., Smith, P.A., Wilkes, E.N. and Clark A.C. (2020) The colorimetric determination of copper in wine: total copper. *The Australian Journal of Grape and Wine Research* **26**, 121-129.
7. Zhang, X., Kontoudakis, N., Wilkes, E., Scrimgeour, N., Hirlam, K. and Clark, A.C. (2021) The removal of Cu from wine by copolymer PVI/PVP: Impact on Cu fractions and binding agent. *Food Chemistry*, **357**, 129764.
8. Zhang, X., Blackman, J.W., Prenzler, P.D. and Clark, A.C. (2022) Suppression of reductive characters in white wine by Cu fractions: Efficiency and duration of protection during bottle aging. *Food Chemistry* **393**, 133305.
9. Zhang, X., Bowyer, P., Scollary, G.R., Clark, A.C. and Kontoudakis, N. (2022) Sulfide-bound copper removal from red and white wine using membrane and depth filters: Impacts of oxygen, H2S-to-Cu ratios, diatomaceous earth and wine volume. *Food Chemistry* **377**, 131758.
10. Zhang, X., Blackman, J.W. and Clark, A.C. (2023) Ascorbic acid addition to rosé: Impact on oxidative and reductive development of bottled wine.
11. Zhang, X. and Clark, A.C. (2023) Cu fractions in Shiraz and Pinot noir wines during bottle aging: rates of changes and capacity for conversion. *OenoOne* **57**, https://doi.org/10.20870/oeno-one.2023.57.4.7425
12. Vongluanngam, I., Zhang, X., Blackman, J.W., Schmidtke, L.M., Wilkinson, K.L. and Clark, A.C. (2024) Impact of light on protective fractions of Cu in white wine: Influence of oxygen and bottle colour. *Food Chemistry* **452**, 139504.
13. Zhang, X., Bekker, M.Z., Kulcsar, A.C., Espinase Nandorfy, D. and Clark, A. C. (2024) Comparison of techniques for the quantitation of reductive aroma compounds in white wine: Links to sensory analysis and Cu fractions. *Journal of Agricultural and Food Chemistry* **72**, 11050-11061.
14. Vongluanngam, I., Blackman, J.W., Zhang, X., Schmidtke, L.M., Wilkinson, K.L. and Clark, A.C. (2025) The impact of Cu fractions on the light induced spoilage aromas of Chardonnay wine at variable riboflavin concentration. *Journal of Agricultural and Food Chemisry* (**accepted**) In press.
15. Zhang, X., Langford, K.L., Vongluanngam, I., Kariyawasam, T. and Clark, A.C. (2025) The suppression of free H2S and methanethiol by Cu fractions in wine: Evaluation across diverse wine-types and storage conditions. *Food Chemistry* (In progress).

Book Chapters:

1. Clark, A.C. and Bekker, M. (2021) Ch. 9. Metal ions and complexes. *In* Theory and concepts of chemical, physical and sensory analyses and test of grapes and wine. Editors: Iland, P., Bruer, D., Bruer, N., Ford, C. and Wilkes, E. (Publisher: Patrick Iland Promotions, Adelaide). 1st Edition. pp. 99-103.

2. Clark, A.C. and Bekker, M. (2021) Ch. 10. Gases. *In* Theory and concepts of chemical, physical and sensory analyses and test of grapes and wine. Editors: Iland, P., Bruer, D., Bruer, N., Ford, C. and Wilkes, E. (Publisher: Patrick Iland Promotions, Adelaide). 1st Edition. pp. 104-119.

Publications in journals dedicated to the wine industry:

1. Zhang, X., Blackman, J.W., Clark, A.C. and Scollary, G.R. (2022) The duration of protection offered by Cu against reductive character development during bottle aging of white wine. *Wine and Viticulture Journal* **37**, 28-31.

2. Zhang, X., Kontoudakis, N., Clark, A.C., Bowyer, P. & Scollary, G.R. (2022) Sulfide-bound copper removal from red and white wine by depth filtration. *Wine and Viticulture Journal* **37**, 32-34.

3. Clark, A.C., Kontoudakis, N., Zhang, X. and Scollary, G.R. (2021) An efficient method for the rapid assessment of free copper in white wine. *Wine and Viticulture Journal* **36**, 34-38.

4. Zhang, X. Kontoudakis, N., Scollary, G.R. and Clark, A.C. (2021) Simplified method for the rapid quantification of copper fractions in wine. *Vine and Wine Technical Reviews.* https://doi.org/10.20870/IVES-TR.2021.4698

5. Clark, A.C. and Kontoudakis, N. (2019) The colorimetric measurement of free and bound Cu in white wine. *In:* Conference proceedings: 17th Australian Wine Technical Conference (Adelaide, Australia). Editors: Beames, K., Robinson, E., Dry, P. and Johnson, D. (Publisher: AWITC Inc.) pp. 100-103.

6. Danenberg, E. and Clark, A.C. (2019) A new, easier methodology for assessing copper in white wine. *The Australian and New Zealand Grapegrower and Winemaker magazine*. November 2019.

Oral communications at conferences:

1. Zhang, X., Bekker, M.Z., Kulcsar, A.C., Espinase Nandorfy, D. and Clark, A.C. (2024) Reductive aroma compounds in white wine: Comparison of measurement techniques and their relation to sensory analysis and copper fractions. *13th In Vino Analytica Scientia* (UC Davis, USA), 9-12 July.

2. Vongluanngam, I., Zhang, X., Blackman, J., Schmidtke, L., Wilkinson, K.L. and Clark, A.C. (2024) The influence of copper fractions of light induced spoilage of white wines. *13th In Vino Analytica Scientia* (UC Davis, USA), 9-12 July.

3. Vongluanngam, I., Zhang, X., Blackman, J., Schmidtke, L., Wilkinson, K.L. and Clark, A.C. (2023) Changes in Cu fractions and riboflavin in white wines during short-term light exposure: Impacts of oxygen and bottle colour. *ŒNO Macrowine.* (Bordeaux, France) 10-13 July.

4. Zhang, X., Langford, K. and Clark, A.C. (2022) The inhibition of hydrogen sulfide and methanethiol accumulation in wine by Cu(II): The influence of temperature on the duration of protection. 12th *In Vino Analytica Scientia* (Neustadt an der Weinstrasse, Germany), 3-7 July.

5. Zhang, X., Kontoudakis, N., Blackman, J. and Clark, A.C. (2021) Inhibition of reductive characters in wine by Cu-organic acids: Predicting the duration of protection. *Macrowine 2021* (Verona, Italy), 23-30 June.

6. Zhang, X., Kontoudakis, N., Suklje, K., Antalick, G., Blackman, J.W. and Clark, A.C. (2019) Changes in red wine composition during bottle aging: Impacts of viticultural conditions and oxygen availability. ŒNO *In Vino Analytica Scientia* (Bordeaux, France) 25-28 June.

7. Kontoudakis, N., Hirlam, K., Smith, M., Smith, P., Scrimgeour, N., Bowyer, P., Wilkes, E. and Clark, A.C. (2019) Simplifying the measurement of different forms of Cu in wines and strategies for efficient removal. ŒNO *In Vino Analytica Scientia* (Bordeaux, France) 25-28 June.

Oral communications for the industry:

1. Clark. A.C. (2024) Managing copper in the lead up to bottling: Cu fractions and other things you should know. *ASVO Seminar: Achieving clarity and getting wine into bottle.* (online webinar) 20 June.

2. Zhang, X., Blackman, J.W. and Clark, A.C. (2022) Reductive development of white wine during bottle ageing: The time-line of protection offered by specific Cu fractions. *Fresh Science A, 18th Australian Wine Industry Technical Conference. (*Adelaide, Australia) 26-29 June.

3. Clark, A.C. and Zhang, X. (2022) Cu fractions in wine: Changes after bottling and implications for wine. *Workshop 17, The 18th Australian Wine Industry Technical Conference*. (Adelaide, Australia) 26-29 June.

4. Zhang, X. and Clark, A.C. (2022) The removal of specific fractions of Cu from wine. *Workshop 17, The 18th Australian Wine Industry Technical Conference*. (Adelaide, Australia) 26-29 June.

5. Clark, A.C. and Zhang, X. (2021) Measurement and behaviour of Cu in wine. *Enoforum USA.* (online forum, USA) 4-5 May.

6. Zhang, X., Kontoudakis, N., Scollary, G.R. and Clark, A.C. (2021) The impacts of free and bound forms of copper in wine. *Interwinery Analysis Group Annual Technical Seminar 2021.* (Adelaide, Australia) 18 June.

7. Kontoudakis, N., Scollary, G.R., Clark, A.C., Smith, M., Smith, P.A., Wilkes, E.N. (2018) A simple colorimetric method to measure copper in white wines. *CRUSH – the grape and wine science symposium.* (Adelaide, Australia) 25-26 September.

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1. Zhang, X., Blackman, J.W., Prenzler, P.D. and Clark, A.C. (2022) Reductive development of white wine during bottle ageing: Time-line of protection from specific Cu fractions. *18th Australian Wine Industry Technical Conference* (Adealide, Australia) 26-29 June.

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10. Appendix 2: Intellectual property

All IP resulting from this project has been, or will be published, and is in the public domain.

11. Appendix 3: References

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12. Appendix 4: Staff

In 2022, the National Wine and Grape Industry Centre (NWGIC) became part of The Gulbali Institute at Charles Sturt University (CSU).

Post-doctorate research fellows (principal investigators 1.0 FTE):

Dr Nikolaos Kontoudakis (NWGIC/CSU)	- 2018 to 2019.
Dr Xinyi Zhang (The Gulbali Institute/NWGIC/CSU)	- 2019 to 2023.
Dr Thiloka Kariyawasam (The Gulbali Institute/CSU)	- 2023-2024.

In kind staff (NWGIC/The Gulbali Institute, CSU)

Dr John Blackman	- 2018 to 2024.
Prof Andrew Clark	- 2018 to 2024.
Prof. Leigh Schmidtke	- 2018 to 2024.
Dr Xinyi Zhang	- 2023 to 2024.

13. Appendix 5: Collaborators

See the acknowledgements on page 10 (Section 2.1).

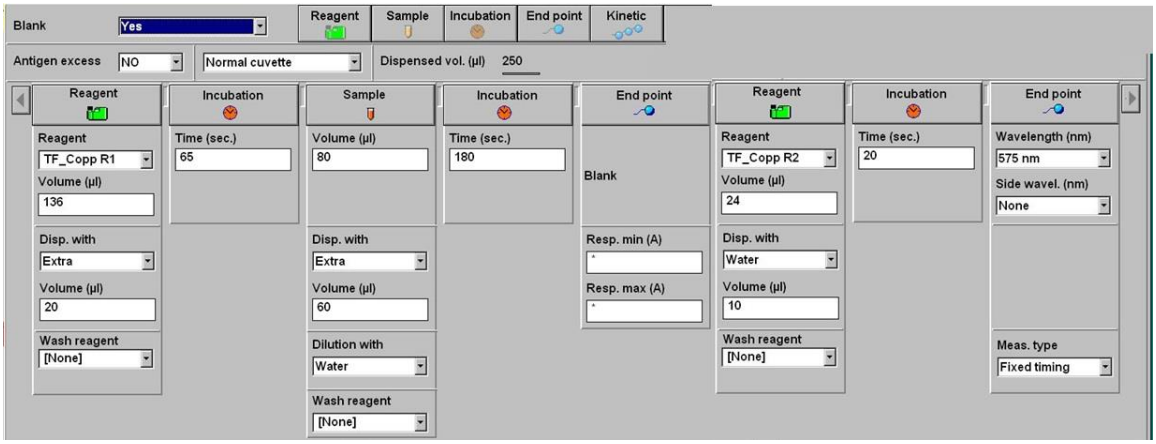
14. Appendix 6: Calculation tool for the determination of total Cu and Cu fractions by the BCA colorimetric analysis.

See the attachment file: '*BCA_Cu_calculator.xls*'.

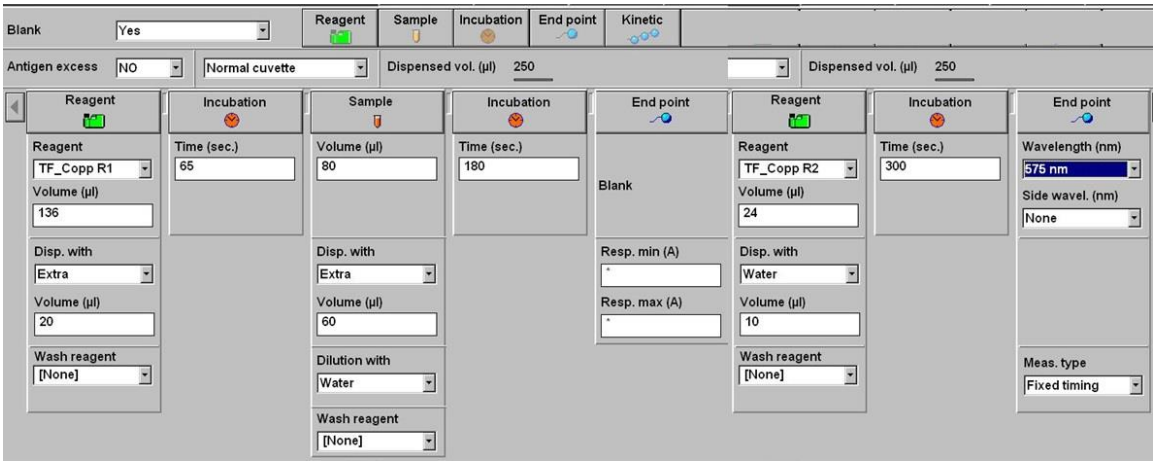
14 Appendix 7: Test flows for the determination of Cu fraction 1 and Cu fractions 1+2 on the automated analyser.

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A.

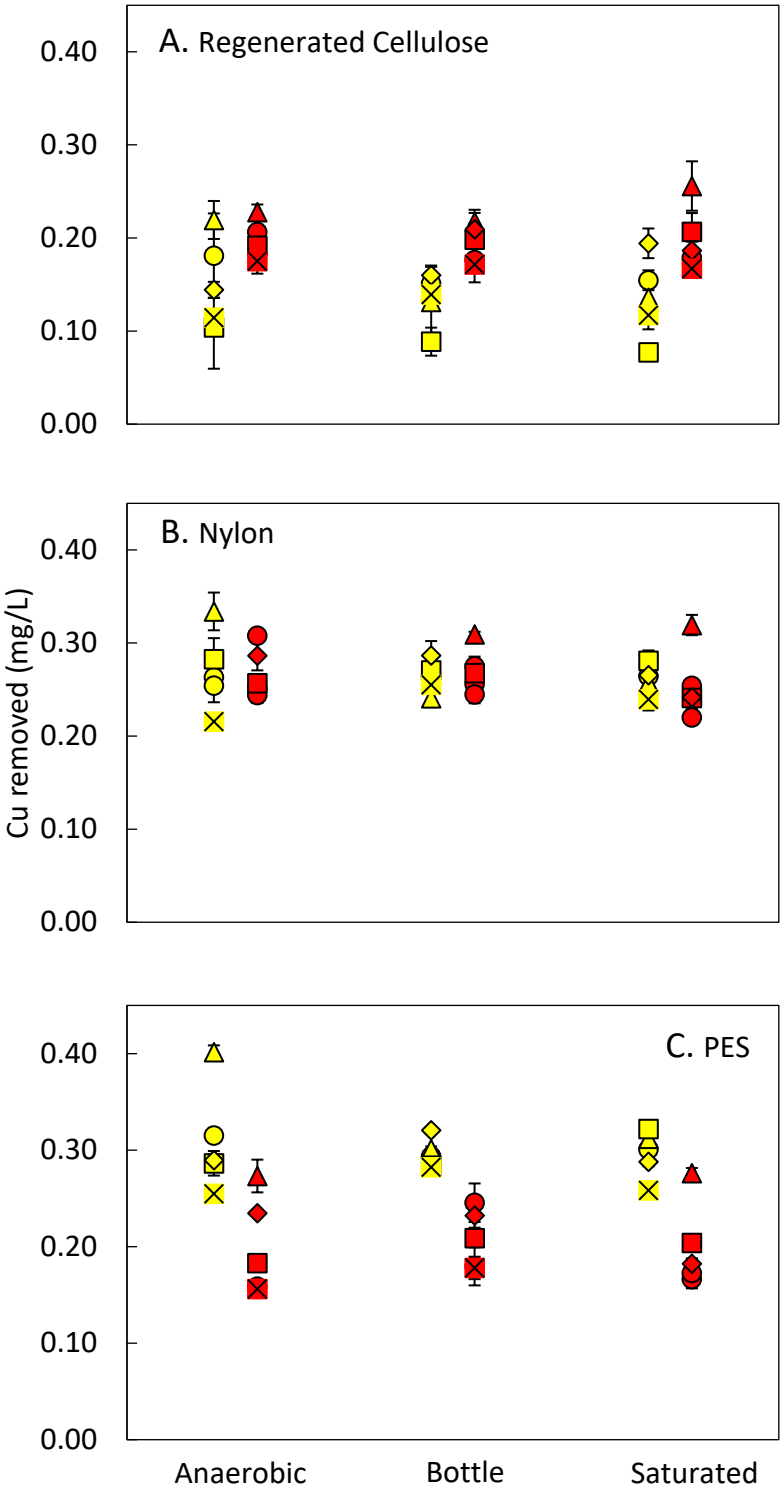


B.



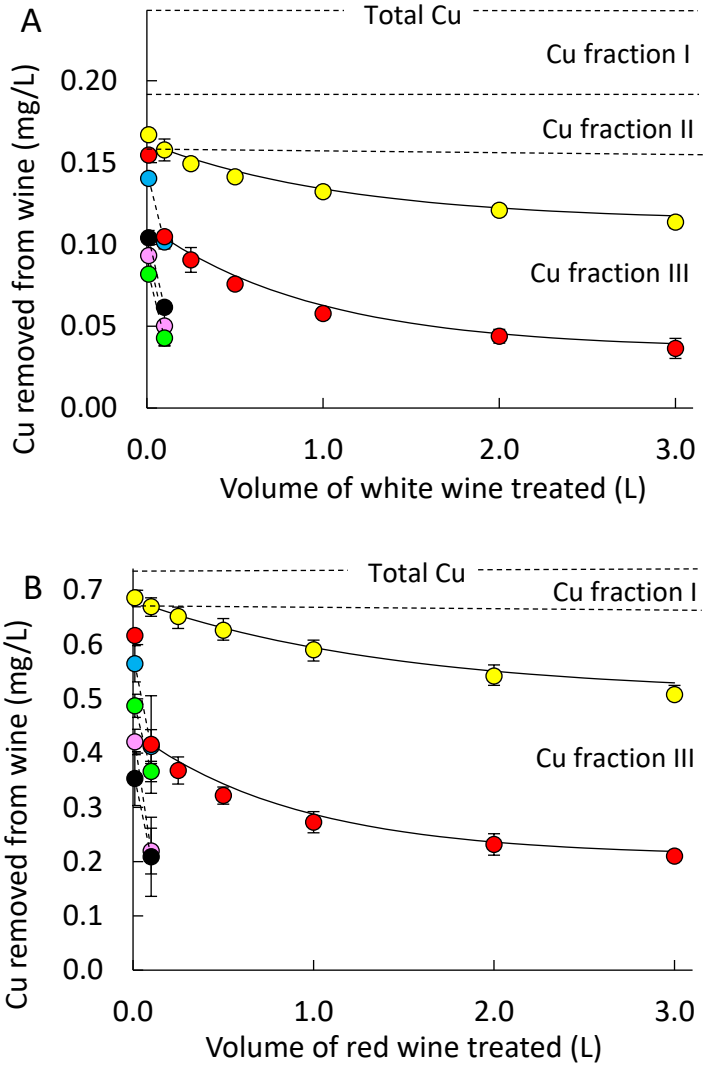
Appendix 7. The test flows for measurement of Cu fraction 1 (a) and Cu fraction 1+2 (b) by the automated analyser with the photometric Cu kit (manufacture by Steroglass supplied by CIScal) containing reagent 1 (acetate buffer, preservatives and stabilizers) and reagent 2 (3,5-Di-Br-PAESA reagent). The only difference in the methods is the incubation time after the addition of reagent 2. The incubation temperature is 37 °C.

14. Appendix 8



Appendix 8. The Cu removed (mg/L) from five white (yellow symbols) and five red (red symbols) wines with three different oxygen conditions (anaerobic, directly from wine bottle and saturated) and three different membrane filtration media: A) regenerated cellulose, B) nylon and C) polyethersulfone (PES). The symbols for the different white and red wines are: wine 1 (○), wine 2 (△), wine 3 (□), wine 4 (◇) and wine 5 (×).

14. Appendix 9



Appendix 9. Cu removed from a white wine (A) and red wine (B) using depth filters on 0.010 – 3.00 L of wine. The cellulose-based depth filters included those with diatomaceous earth, at tight (●), medium (●) and coarse (●) pore size ratings, and those without diatomaceous earth, at tight (●), medium (●) and coarse (●) pore size ratings.

14. Appendix 10.

Specifications of the 4 x 1000 L batches of Chardonnay and Shiraz wines used for depth filtration and PVI/PVP experiments.



2023 Chardonnay (current vintage at the time of filtration). The Cu concentration was after addition of copper(II) sulfate pentahydrate to achieve a pre-filtration Cu concentration of approximately 0.6 mg/L.

	Tank 1	Tank 2	Tank 3	Tank 4	Average
pH	3.39	3.34	3.31	3.25	3.32 ± 0.06
alcohol (%(v/v))	12.92	12.88	12.85	12.90	12.89 ± 0.03
titratable acidity (g/L TAE at pH 8.2)	6.54	6.59	6.65	6.87	6.7 ± 0.2
acetic acid (g/L)	0.27	0.24	0.24	0.25	0.25 ± 0.01
malic acid (g/L)	2.16	2.15	2.16	2.19	2.17 ± 0.02
glucose (g/L)	0.14	0.15	0.14	0.14	0.14 ± 0.01
fructose (g/L)	1.89	1.89	1.88	1.90	1.89 ± 0.01
free sulfur dioxide (mg/L)	31	33	34	34	33 ± 1
total sulfur dioxide (mg/L)	187	187	188	189	188 ± 1
copper (mg/L)	0.69	0.63	0.64	0.64	0.65 ± 0.03

2023 Shiraz (current vintage at the time of filtration). The Cu concentration was after addition of copper(II) sulfate pentahydrate to achieve a pre-filtration Cu concentration of approximately 0.6 mg/L.

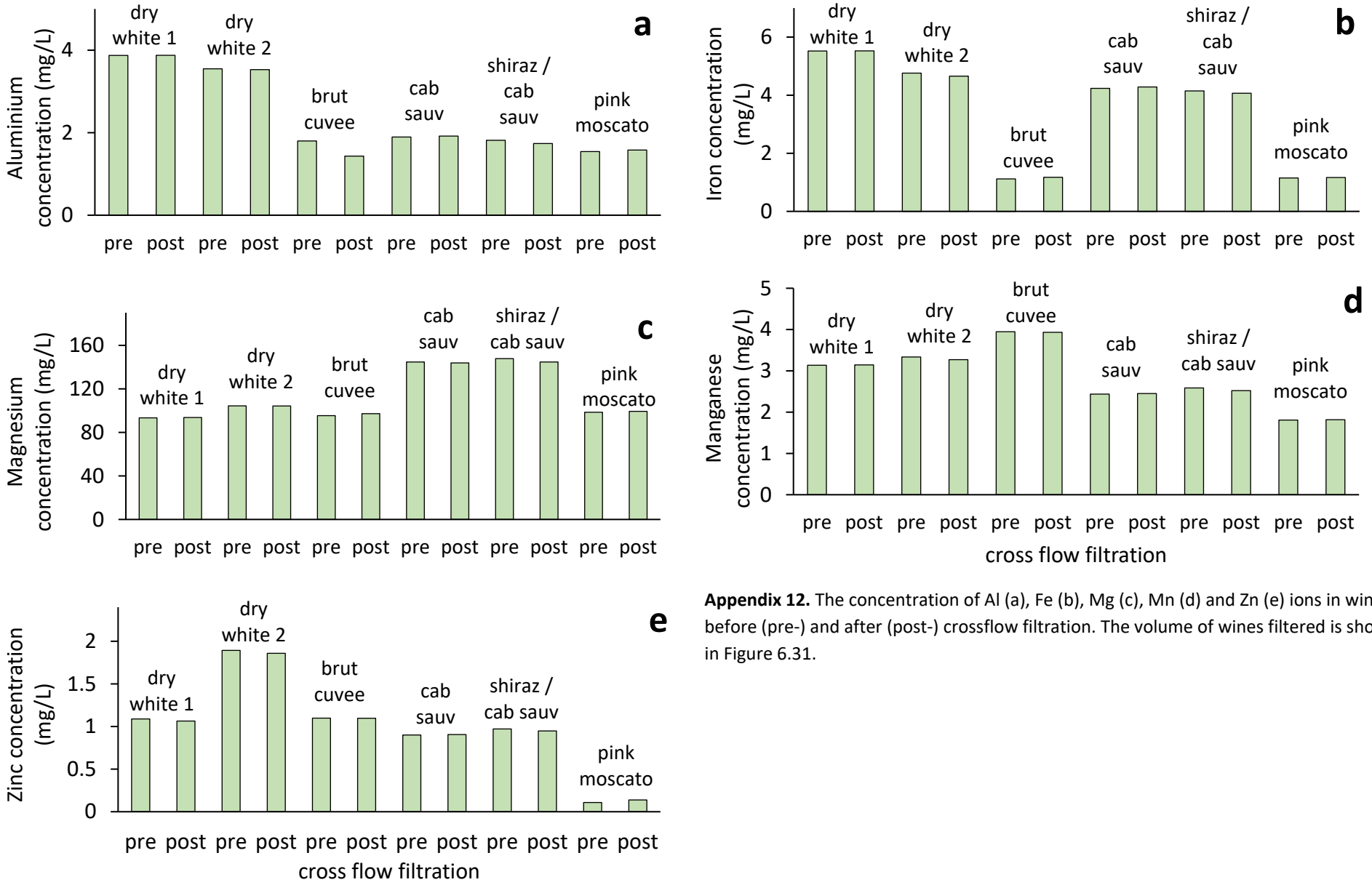
	Tank 1	Tank 2	Tank 3	Tank 4	Average
pH	3.30	3.32	3.32	3.33	3.32 ± 0.06
alcohol (%(v/v))	14.44	14.12	14.13	14.82	14.4 ± 0.3
titratable acidity (g/L TAE at pH 8.2)	6.78	6.6	6.6	6.53	6.6 ± 0.1
acetic acid (g/L)	0.46	0.44	0.43	0.43	0.44 ± 0.01
malic acid (g/L)	0.12	0.12	0.12	0.12	0.12
glucose (g/L)	0.42	0.43	0.41	0.42	0.42 ± 0.01
fructose (g/L)	0.27	0.26	0.25	0.25	0.26 ± 0.01
free sulfur dioxide (mg/L)	26	24	24	24	25 ± 1
total sulfur dioxide (mg/L)	80	77	73	72	76 ± 4
copper (mg/L)	0.63	0.64	0.65	0.67	0.65 ± 0.02

14. Appendix 11.

Appendix 11. Information of the commercial wines utilised for the PVI/PVP treatment (conducted in 2020).

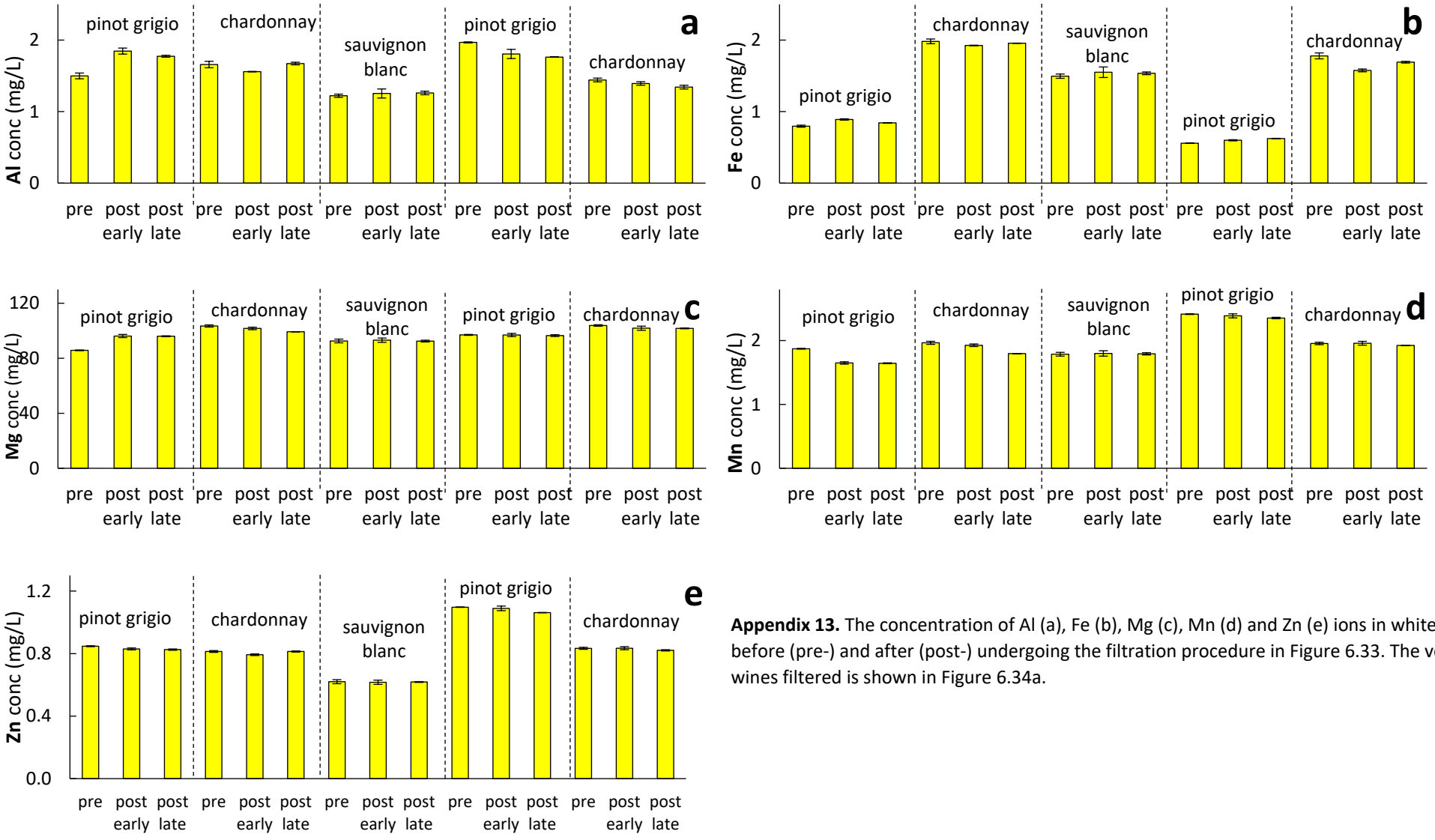
Wine	Variety	Vintage	Alcohol degree	pH
W1	Riesling	2019	11.8 %	3.13
W2	Pinot Grigio	2019	12.5 %	2.84
W3	Sauvignon Blanc	2020	11.5 %	3.25
W4	Pinot Grigio	2019	12 %	3.27
R1	Shiraz	2019	13 %	3.39
R2	Cabernet Sauvignon	2019	13.5 %	3.49
R3	Shiraz	2019	13.5 %	3.55
R4	Shiraz	2019	12.5 %	3.41

14. Appendix 12.



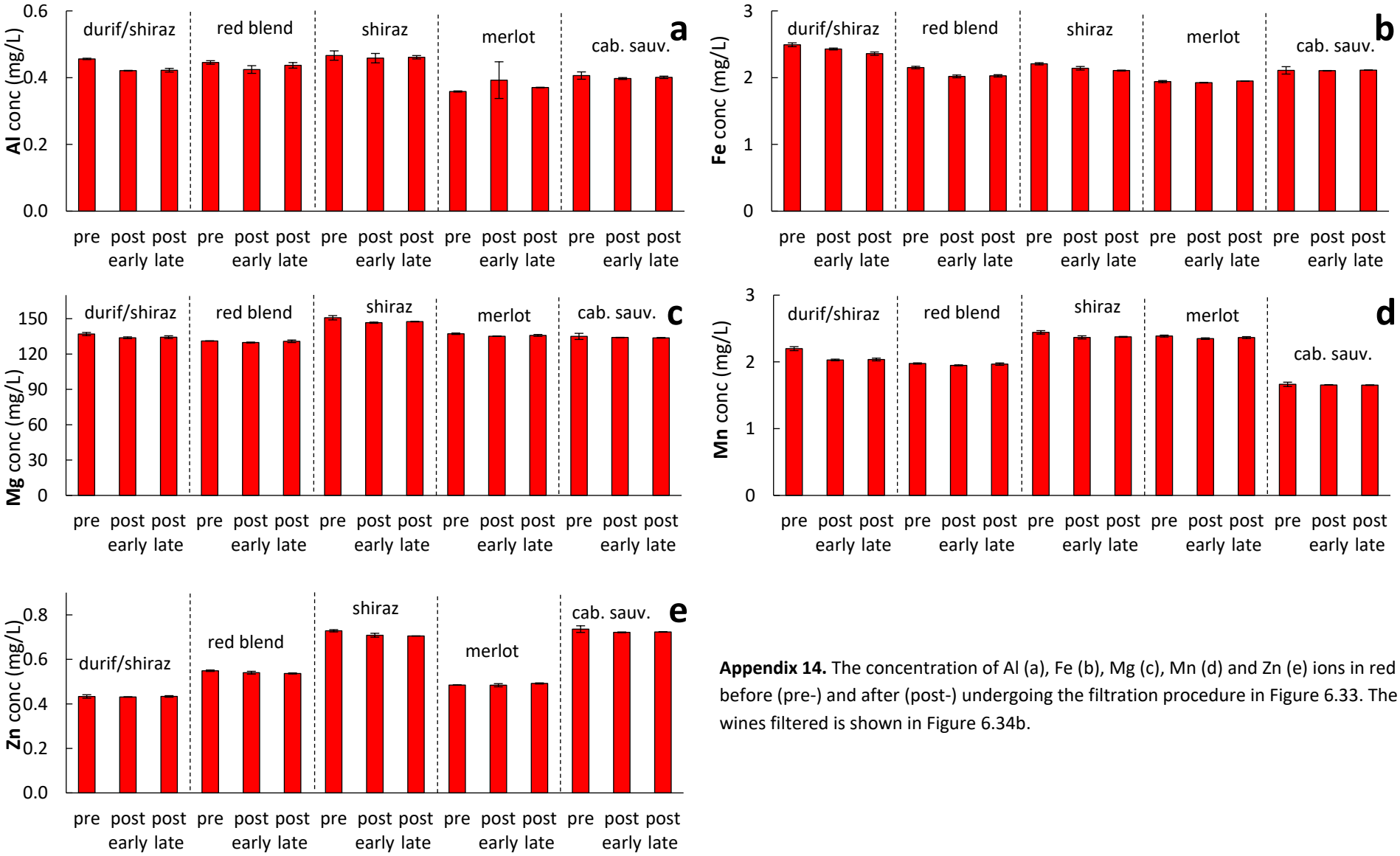
Appendix 12. The concentration of Al (a), Fe (b), Mg (c), Mn (d) and Zn (e) ions in wines before (pre-) and after (post-) crossflow filtration. The volume of wines filtered is shown in Figure 6.31.

24. Appendix 13.



Appendix 13. The concentration of Al (a), Fe (b), Mg (c), Mn (d) and Zn (e) ions in white wines before (pre-) and after (post-) undergoing the filtration procedure in Figure 6.33. The volume of wines filtered is shown in Figure 6.34a.

14. Appendix 14.



Appendix 14. The concentration of Al (a), Fe (b), Mg (c), Mn (d) and Zn (e) ions in red wines before (pre-) and after (post-) undergoing the filtration procedure in Figure 6.33. The volume of wines filtered is shown in Figure 6.34b.

14. Appendix 15A.

Appendix 15a. Details on the 50 white wines.

	Variety[a]	vintage (age)[b]	pH	free SO_2 (mg/L)	total SO_2 (mg/L)	ascorbic acid (mg/L)	glutathione (mg/L)	3-sulfanylhexanol (ng/L)	3- sulfanylhexyl acetate (ng/L)	phenylmethanethiol (ng/L)
1	Vermentino	2019 (3)	3.29 ± 0.02	24 ± 1	91 ± 1	< LOQ	0.14 ± 0.01	560 ± 80	< LOQ[c]	20 ± 0.13
2	Chardonnay	2020 (2)	3.42 ± 0.02	21 ± 1	86 ± 2	63 ± 2	0.44 ± 0.06	540 ± 20	5.3 ± 0.1	< LOQ[f]
3	Verdelho	2021 (1)	3.39 ± 01	29 ± 1	168 ± 1	53 ± 4	4.7 ± 0.6	540 ± 30	19.6 ± 0.6	7 ± 2
4	Pinot Grigio	2020 (2)	3.03 ± 0.03	25 ± 1	104 ± 6	59 ± 2	0.22 ± 0.06	750 ± 80	< LOQ[d]	13 ± 4
5	Sem., Sauv. bl.	2020 (2)	3.17 ± 0.01	25 ± 1	108 ± 2	< LOQ	0.27 ± 0.01	620 ± 20	< LOQ[e]	120 ± 60
6	Sauvignon blanc	2021 (1)	3.18 ± 0.01	20 ± 1	116 ± 2	59 ± 3	2.66 ± 0.09	990 ± 30	27 ± 1	50 ± 20
7	Pinot blanc	2021 (1)	3.20 ± 0.07	8 ± 1	53 ± 1	< LOQ	0.08 ± 0.01	290 ± 30	< LOQ[e]	200 ± 100
8	Sauv. bl., Fiano	2020 (2)	3.12 ± 0.01	23 ± 2	126 ± 1	59 ± 3	0.29 ± 0.01	1010 ± 90	4.5 ± 0.6	10 ± 1
9	Pinot Grigio	2021 (1)	3.26 ± 0.01	13 ± 1	38 ± 1	< LOQ	0.48 ± 0.04	250 ± 10	< LOQ[e]	110 ± 60
10	Verd., Chenin b.	2020 (2)	3.33 ± 0.01	20 ± 1	115 ± 1	< LOQ	0.17 ± 0.01	1180 ± 80	11 ± 2	80 ± 20
11	Picpoul de Pinet	2020 (2)	3.30 ± 0.01	13 ± 3	89 ± 8	73	0.30 ± 0.02	520 ± 30	5.7 ± 0.4	10 ± 1
12	Chardonnay	2020 (2)	3.51 ± 0.02	28 ± 2	137 ± 1	< LOQ	0.3 ± 0.2	390 ± 50	< LOQ[c]	40 ± 6
13	Pinot Gris	2020 (2)	3.32 ± 0.02	18 ± 1	69 ± 1	< LOQ	0.20 ± 0.01	430 ± 20	< LOQ[c]	21 ± 8
14	Sauvignon blanc	2020 (2)	3.12 ± 0.01	15 ± 2	98 ± 1	< LOQ	0.43 ± 0.02	1600 ± 100	23 ± 2	40 ± 9
15	Gruner Veltliner	2021 (1)	3.09 ± 0.01	19 ± 1	97 ± 2	< LOQ	1.10 ± 0.04	1160 ± 50	29.7 ± 0.7	21 ± 3
16	Pinot Gris	2021 (1)	3.12 ± 0.01	27 ± 2	97 ± 1	< LOQ	1.51 ± 0.07	480 ± 6	6.8 ± 0.3	23 ± 4
17	Chardonnay	2021 (1)	3.28 ± 0.02	15 ± 1	61 ± 2	< LOQ	0.14 ± 0.02	550 ± 10	< LOQ[e]	129 ± 9
18	Chardonnay	2020 (2)	3.18 ± 0.01	20 ± 1	99 ± 2	< LOQ	0.27 ± 0.01	1030 ± 40	< LOQ[d]	60 ± 20
19	Chardonnay	2021 (1)	3.32 ± 0.01	20 ± 1	93 ± 2	< LOQ	0.41 ± 0.08	480 ± 50	< LOQ[e]	140 ± 70
20	Viognier	2018 (4)	3.46 ± 0.01	21 ± 2	68 ± 2	49 ± 4	0.34 ± 0.01	230 ± 50	< LOQ[c]	8 ± 2
21	Sem., Sauv. bl.	2021 (1)	3.46 ± 0.01	4 ± 1	57 ± 1	< LOQ	Not determined	Not determined	Not determined	Not determined
22	Pinot Grigio	2021 (1)	3.33 ± 0.02	27 ± 2	133 ± 3	88 ± 4	0.84 ± 0.03	290 ± 20	12.9 ± 0.9	< LOQ[f]
23	Chardonnay	2021 (1)	3.32 ± 0.02	21 ± 2	109 ± 1	< LOQ	0.49 ± 0.18	600 ± 100	< LOQ[d]	24 ± 7
24	Sauvignon blanc	2021 (1)	3.26 ± 0.05	22 ± 1	106 ± 3	40 ± 10	0.91 ± 0.05	1200 ± 400	24 ± 6	< LOQ[g]
25	Sauvignon blanc	2021 (1)	3.30 ± 0.02	20 ± 6	135 ± 2	14 ± 2	0.80 ± 0.01	4500 ± 100	300 ± 70	60 ± 40
26	Pinot Grigio	2021 (1)	3.22 ± 0.01	18 ± 1	108 ± 1	61 ± 3	0.60 ± 0.01	478 ± 8	2.3 ± 0.1	6 ± 1
27	Chardonnay	2021 (1)	3.38 ± 0.02	30 ± 1	115 ± 1	44 ± 2	1.34 ± 0.02	333 ± 5	5.1 ± 0.1	13 ± 2
28	Sem., Sauv. bl.	2021 (1)	3.13 ± 0.01	22 ± 1	119 ± 2	< LOQ	0.22 ± 0.01	680 ± 20	2.5 ± 0.3	26 ± 4
29	Chardonnay	2021 (1)	3.42 ± 0.05	21 ± 1	149 ± 2	< LOQ	1.4 ± 0.1	102 ± 5	< LOQ[e]	74 ± 7
30	Sauvignon blanc	2021 (1)	3.15 ± 0.02	23 ± 1	126 ± 1	< LOQ	0.28 ± 0.03	912 ± 5	< LOQ[e]	67 ± 6
31	Chardonnay	2021 (1)	3.38 ± 0.01	24 ± 1	112 ± 1	42 ± 1	0.98 ± 0.07	447 ± 3	6.3 ± 0.3	18 ± 1
32	Riesling	2021 (1)	2.92 ± 0.01	20 ± 2	106 ± 6	54 ± 2	1.1 ± 0.2	160 ± 20	< LOQ[e]	28 ± 1
33	Chardonnay	2021 (1)	3.40 ± 0.01	22 ± 2	137 ± 9	< LOQ	1.28 ± 0.06	230 ± 70	16 ± 4	40 ± 20
34	Chardonnay	2021 (1)	3.40 ± 0.01	23 ± 1	102 ± 1	49 ± 1	1.07 ± 0.03	440 ± 30	9.3 ± 0.7	11 ± 1
35	Riesling	2020 (2)	3.37 ± 0.01	20 ± 1	107 ± 4	60 ± 10	0.23 ± 0.01	110 ± 20	< LOQ[e]	40

36	Pinot Grigio	2021 (1)	3.27 ± 0.01	21 ± 1	101 ± 1	40 ± 2	0.41 ± 0.03	340 ± 20	< LOQ[d]	10
37	Sauvignon blanc	2021 (1)	3.26 ± 0.02	28 ± 1	129 ± 1	77 ± 3	1.33 ± 0.03	1770 ± 50	50 ± 2	7 ± 1
38	Chenin dom.	2021 (1)	3.19 ± 0.02	20 ± 3	131 ± 2	72 ± 2	1.68 ± 0.05	380 ± 20	23 ± 2	< LOQ[f]
39	White blend	2021 (1)	3.20 ± 0.02	20 ± 2	102 ± 1	69 ± 2	2.05 ± 0.03	630 ± 30	29.5 ± 0.7	5 ± 1
40	Chardonnay	2021 (1)	3.31 ± 0.01	18 ± 1	116 ± 1	66 ± 1	1.62 ± 0.07	604 ± 7	12.1 ± 0.3	6 ± 1
41	Chardonnay	2021 (1)	3.27 ± 0.01	18 ± 3	115 ± 3	83 ± 1	1.4 ± 0.1	470 ± 30	8.9 ± 0.1	40 ± 10
42	Sauvignon blanc	2021 (1)	3.04 ± 0.02	15 ± 1	106 ± 3	80 ± 1	2.2 ± 0.2	1200 ± 30	50 ± 3	< LOQ[g]
43	Sauvignon blanc	2021 (1)	3.27 ± 0.01	17 ± 2	116 ± 2	67 ± 5	2.8 ± 0.4	1000 ± 40	12.2 ± 0.5	7 ± 2
44	Sem., Sauv. bl.	2021 (1)	3.14 ± 0.01	18 ± 2	106 ± 2	72 ± 5	1.02 ± 0.04	730 ± 10	13.5 ± 0.1	5 ± 1
45	Pinot Grigio	2021 (1)	3.13 ± 0.01	17 ± 2	95 ± 2	79 ± 8	0.8 ± 0.2	500 ± 20	6.5 ± 0.1	4 ± 1
46	Pinot Gris	2021 (1)	3.31 ± 0.01	18 ± 2	90 ± 1	49 ± 5	0.78 ± 0.01	259 ± 6	4.5 ± 0.2	11
47	Riesling	2021 (1)	3.01 ± 0.02	19 ± 2	103 ± 6	62 ± 6	1.00 ± 0.08	200 ± 10	4.4 ± 0.1	13 ± 2
48	Riesling	2021 (1)	3.02 ± 0.01	11 ± 2	127 ± 1	< LOQ	1.01 ± 0.07	204 ± 8	4.8 ± 0.6	23 ± 6
49	Riesling	2021 (1)	2.96 ± 0.01	16 ± 1	94 ± 1	78 ± 3	0.89 ± 0.02	300 ± 100	4.9	17
50	Chardonnay	2021 (1)	3.17 ± 0.01	13 ± 1	104 ± 1	39 ± 1	0.70 ± 0.01	454 ± 9	5.7 ± 0.1	21 ± 4

[a] 'bl.' = blanc

[b] The age indicated in parentheses is in units of years.

< LOQ: ascorbic acid less than 5 mg/L;

<LOQ[c] 3-sulfanylhexyl acetate (3SHA) less than 1.70 ng/L, <LOQ[d] 3SHA less than 3.68 ng/L, <LOQ[e] 3SHA less than 7.89 ng/L; and <LOQ[f] PMT less than 8.64 ng/L, <LOQ[g] PMT less than 26.99 ng/L.

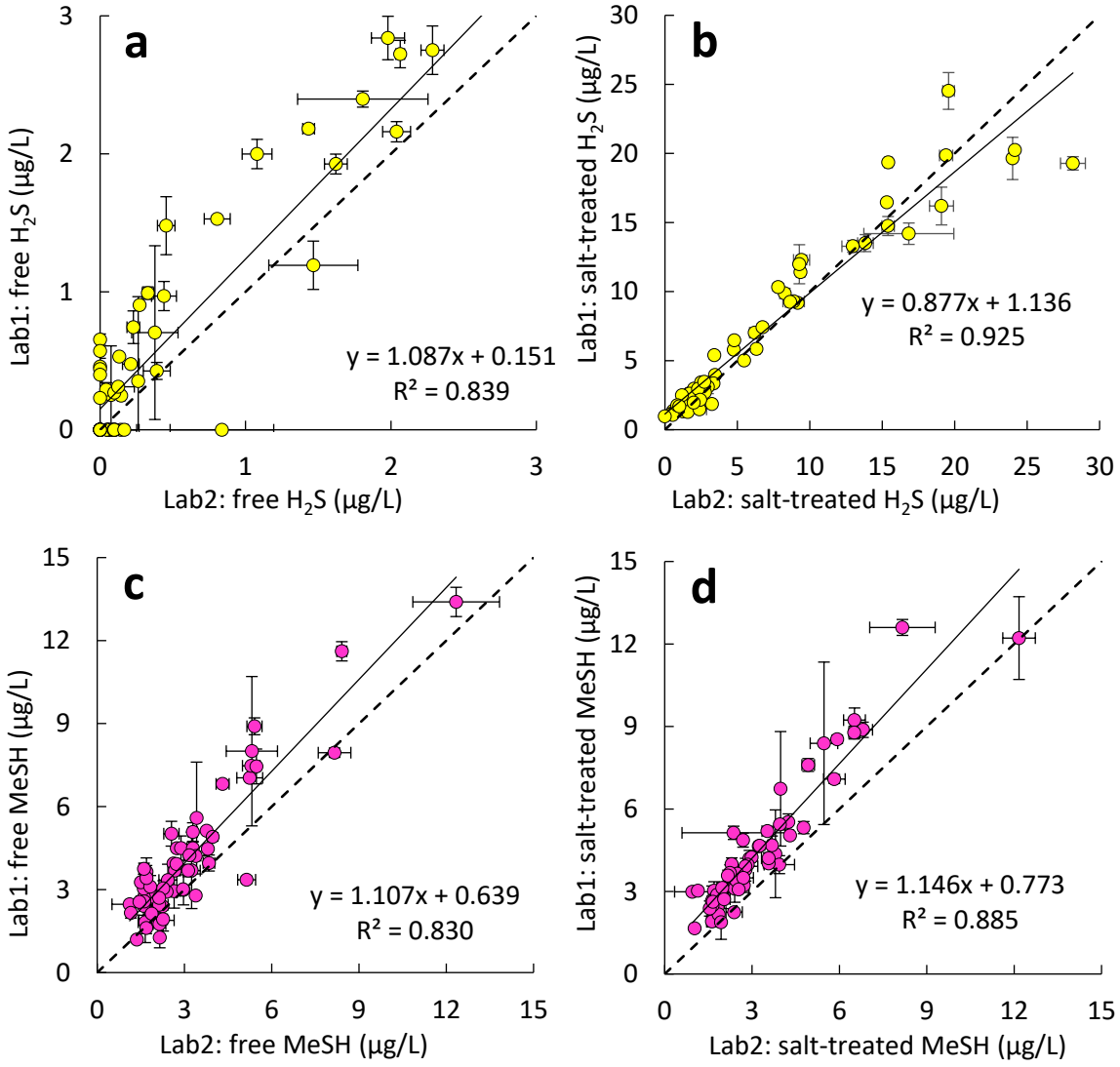
14. Appendix 15B.

Appendix 15b. Metal concentrations in the 50 wines as determined colorimetry and/or ICP-OES.

wine	# of bottle codes	*analysis by colorimetry*				*analysis by ICP-OES*					
		Cu fraction I (mg/L)	Cu fraction II (mg/L)	Cu fraction III (mg/L)	total Cu (mg/L)	total Cu (mg/L)	total Fe (mg/L)	total Al (mg/L)	total Mg (mg/L)	total Mn (mg/L)	total Zn (mg/L)
1	2	0.003 ± 0.002	0	0.030 ± 0.004	0.032 ± 0.002	0.032 ± 0.003	1.275 ± 0.005	1.90 ± 0.01	75.1 ± 0.1	0.769 ± 0.002	0.644 ± 0.004
2	2	0	0.001 ± 0.001	0.015 ± 0.004	0.017 ± 0.003	0.019 ± 0.004	1.09 ± 0.02	1.95 ± 0.07	74 ± 2	0.97 ± 0.02	0.73 ± 0.01
3	1	0.01 ± 0.01	0.002 ± 0.001	0.04 ± 0.02	0.051 ± 0.002	0.050 ± 0.003	0.620± 0.005	0.60 ± 0.02	97.1 ± 0.6	1.256 ± 0.008	1.202 ± 0.005
4	2	0.001 ± 0.001	0.002 ± 0.001	0.035 ± 0.001	0.038 ± 0.001	0.06 ± 0.03	0.6 ± 0.1	2.6 ± 0.2	42 ± 2	0.49 ± 0.05	0.53 ± 0.02
5	1	0.004 ± 0.001	0.006 ± 0.005	0.033 ± 0.005	0.042 ± 0.004	0.049 ± 0.004	2.26 ± 0.01	2.52 ± 0.02	97.1 ± 0.5	2.03 ± 0.01	0.652 ± 0.005
6	1	0.000 ± 0.001	0.002 ± 0.004	0.007 ± 0.004	0.009 ± 0.001	0.002 ± 0.001	0.365 ± 0.001	0.724 ± 0.008	82.8 ± 0.5	1.75 ± 0.01	1.17 ± 0.01
7	2	0.006 ± 0.003	0	0.05 ± 0.02	0.05 ± 0.02	0.08 ± 0.05	0.89 ± 0.02	1.61 ± 0.09	69 ± 3	0.9 ± 0.2	0.3 ± 0.1
8	1	0.003 ± 0.001	0.002 ± 0.001	0.090 ± 0.001	0.095 ± 0.001	0.099 ± 0.004	3.31 ± 0.03	2.65 ± 0.04	121 ± 1	1.96 ± 0.02	1.46 ± 0.02
9	1	0.003 ± 0.002	0	0.026 ± 0.001	0.030 ± 0.001	0.025 ± 0.003	0.749 ± 0.01	1.34 ± 0.03	89 ± 1	0.84 ± 0.01	1.02 ± 0.01
10	1	0.001 ± 0.001	0.002 ± 0.004	0.026 ± 0.005	0.029 ± 0.001	0.032 ± 0.003	0.771 ± 0.003	0.43 ± 0.01	73.4 ± 0.9	0.325 ± 0.005	0.427 ± 0.003
11	1	0.001 ± 0.001	0	0.02 ± 0.01	0.02 ± 0.02	0.02 ± 0.02	1.7 ± 0.4	2.16 ± 0.05	85 ± 5	0.83 ± 0.08	0.68 ± 0.08
12	1	0.003 ± 0.002	0.001 ± 0.001	0.077 ± 0.004	0.080 ± 0.005	0.110 ± 0.002	1.652 ± 0.005	0.69 ± 0.06	109.0 ± 0.3	0.858 ± 0.001	1.098 ± 0.005
13	1	0.001 ± 0.001	0	0.027 ± 0.002	0.029 ± 0.001	0.036 ± 0.003	0.923 ± 0.001	0.63 ± 0.02	83 ± 1	0.605 ± 0.003	1.57 ± 0.01
14	1	0.003 ± 0.002	0	0.027 ± 0.002	0.030 ± 0.001	0.025 ± 0.001	3.74 ± 0.04	1.07 ± 0.01	80.2 ± 0.9	0.495 ± 0.005	0.749 ± 0.007
15	1	0.003 ± 0.002	0	0.029 ± 0.002	0.032 ± 0.003	0.029 ± 0.001	0.86 ± 0.01	0.92 ± 0.02	119 ± 2	0.499 ± 0.008	0.593 ± 0.009
16	1	0.010 ± 0.002	0.026 ± 0.001	0.098 ± 0.004	0.134 ± 0.001	0.134 ± 0.003	0.350 ± 0.002	0.60 ± 0.02	94.1	0.654 ± 0.004	0.769 ± 0.006
17	2	0.003 ± 0.001	0	0.007 ± 0.001	0.010 ± 0.002	0.004 ± 0.007	0.90 ± 0.04	1.03 ± 0.03	66.0 ± 0.4	0.675 ± 0.007	0.82 ± 0.01
18	1	0.003 ± 0.002	0	0.017 ± 0.001	0.020 ± 0.002	0.017 ± 0.004	1.10 ± 0.01	1.43 ± 0.02	109 ± 1	0.93 ± 0.01	0.651 ± 0.009
19	1	0.025 ± 0.003	0.051 ± 0.001	0.162 ± 0.004	0.238 ± 0.008	0.255 ± 0.007	1.478 ± 0.008	0.50 ± 0.07	87.7 ± 0.1	2.296 ± 0.005	1.545 ± 0.003
20	1	0.009 ± 0.001	0.028 ± 0.005	0.61 ± 0.02	0.64 ± 0.02	0.558 ± 0.004	1.85 ± 0.01	0.87 ± 0.04	102.5 ± 0.6	0.817 ± 0.004	0.679 ± 0.004
21	1	0.001 ± 0.001	0	0.008 ± 0.002	0.008 ± 0.003	0.099 ± 0.004	3.31 ± 0.03	2.65 ± 0.04	121 ± 1	1.96 ± 0.02	1.46 ± 0.02
22	1	0.018 ± 0.004	0.08 ± 0.02	0.21 ± 0.03	0.31 ± 0.05	0.31 ± 0.05	1.51 ± 0.05	3.7 ± 0.3	90.3 ± 0.6	2.35 ± 0.02	0.84 ± 0.01
23	2	0.02 ± 0.01	0.02 ± 0.02	0.1 ± 0.1	0.1 ± 0.2	0.2 ± 0.2	2.19 ± 0.03	2.12 ± 0.04	114 ± 1	2.23 ± 0.02	0.961 ± 0.005
24	2	0.011 ± 0.003	0.029 ± 0.007	0.35 ± 0.05	0.39 ± 0.06	0.41 ± 0.06	1.33 ± 0.02	1.27 ± 0.09	88 ± 2	1.80 ± 0.03	0.895 ± 0.009
25	1	0.010 ± 0.002	0.019 ± 0.004	0.209 ± 0.006	0.238 ± 0.001	0.236 ± 0.005	1.059 ± 0.006	2.21 ± 0.03	84.0 ± 0.9	1.0 ± 0.01	0.76 ± 0.01
26	1	0.007 ± 0.001	0.026 ± 0.003	0.208 ± 0.001	0.241 ± 0.004	0.251 ± 0.008	1.29 ± 0.01	1.383 ± 0.009	82.3 ± 0.5	1.48 ± 0.01	0.683 ± 0.008
27	1	0.001 ± 0.001	0.001 ± 0.001	0.033 ± 0.001	0.036 ± 0.001	0.032 ± 0.007	0.981 ± 0.008	1.13 ± 0.02	104.5 ± 0.7	2.17 ± 0.02	1.017 ± 0.007
28	1	0.015 ± 0.004	0.016 ± 0.003	0.086 ± 0.001	0.117 ± 0.007	0.138 ± 0.008	2.45 ± 0.02	3.68 ± 0.04	104.2 ± 0.9	2.18 ± 0.02	0.648 ± 0.008
29	2	0.005 ± 0.002	0	0.012 ± 0.001	0.016 ± 0.002	0.009 ± 0.001	0.9 ± 0.2	1.2 ± 0.1	101 ± 3	0.83 ± 0.03	0.3 ± 0.5
30	1	0.031 ± 0.007	0.020 ± 0.009	0.076 ± 0.003	0.127 ± 0.001	0.125 ± 0.005	1.63 ± 0.01	2.91 ± 0.03	91.6 ± 0.5	1.87 ± 0.01	0.575 ± 0.002
31	1	0.003 ± 0.001	0.008 ± 0.006	0.030 ± 0.007	0.041 ± 0.001	0.042 ± 0.002	1.172 ± 0.004	1.17 ± 0.03	100.6 ± 0.5	1.778 ± 0.009	0.868 ± 0.006
32	2	0.003 ± 0.003	0.003 ± 0.003	0.010 ± 0.003	0.016 ± 0.003	0.019 ± 0.002	0.41 ± 0.01	0.61 ± 0.02	88 ± 2	0.85 ± 0.02	0.92 ± 0.02
33	2	0.035 ± 0.009	0.12 ± 0.03	0.115 ± 0.009	0.27 ± 0.03	0.28 ± 0.02	0.47 ± 0.05	1.02 ± 0.01	96 ± 6	0.9 ± 0.5	0.78 ± 0.07
34	1	0.010 ± 0.005	0.008 ± 0.003	0.118 ± 0.003	0.135 ± 0.001	0.134 ± 0.008	1.85 ± 0.02	1.30 ± 0.06	110 ± 1	2.87 ± 0.03	1.27 ± 0.01

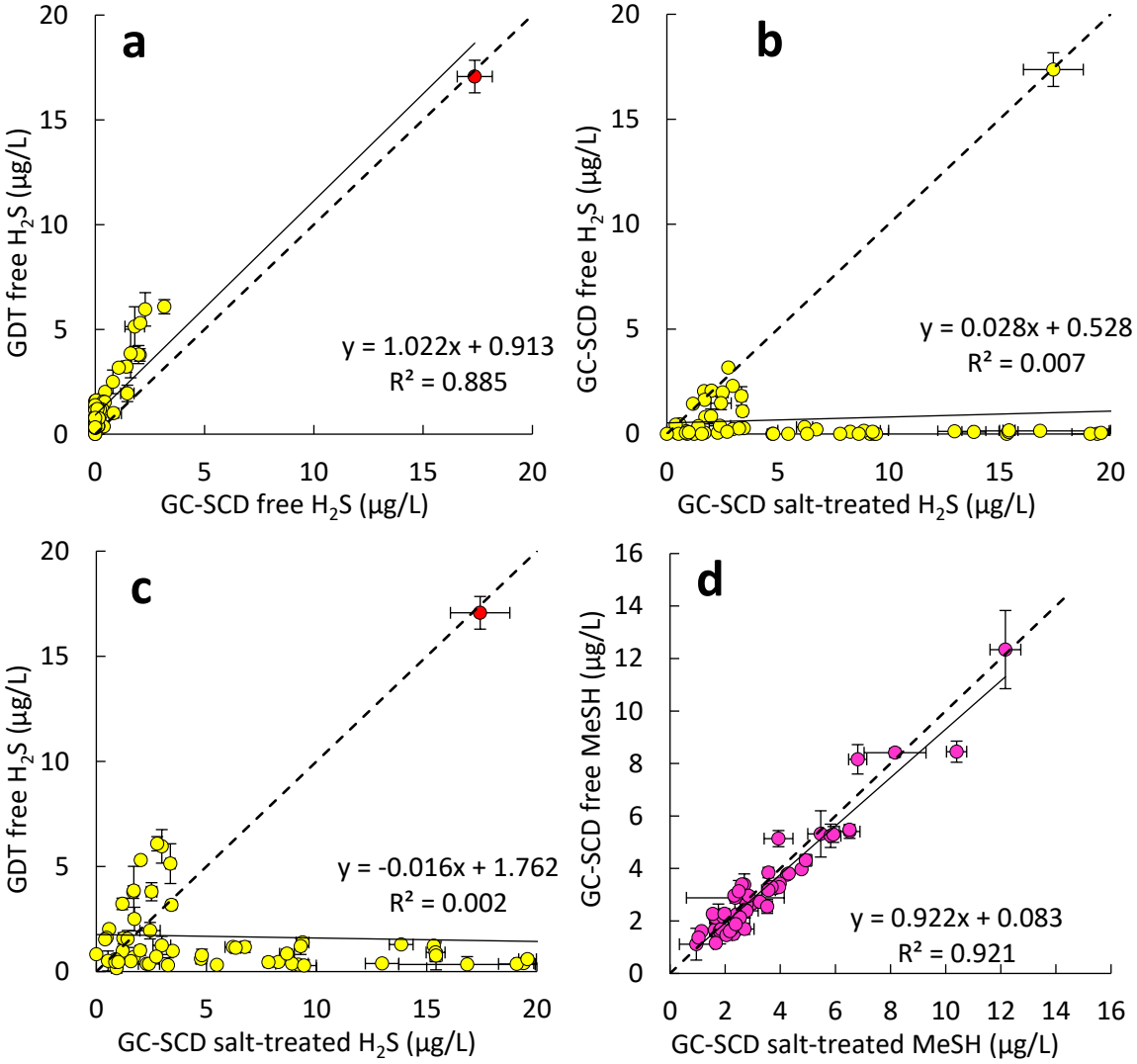
35	2	0.015 ± 0.006	0.09 ± 0.05	0.25 ± 0.04	0.4 ± 0.1	0.4 ± 0.1	0.37 ± 0.02	0.62 ± 0.04	106 ± 4	0.77 ± 0.04	0.95 ± 0.03
36	1	0.016 ± 0.002	0.026 ± 0.006	0.194 ± 0.005	0.236 ± 0.008	0.238 ± 0.009	2.32 ± 0.02	3.8 ± 0.1	88.8 ± 0.1	2.133 ± 0.004	0.585 ± 0.004
37	1	0.003 ± 0.003	0.004 ± 0.002	0.080 ± 0.005	0.087 ± 0.004	0.088 ± 0.003	0.96 ± 0.01	1.38 ± 0.04	85.7 ± 0.7	2.25 ± 0.02	1.26 ± 0.01
38	1	0.01 ± 0.01	0.05 ± 0.04	0.21 ± 0.05	0.274 ± 0.006	0.277 ± 0.003	0.864 ± 0.002	1.26 ± 0.01	94.2 ± 0.4	0.965 ± 0.005	0.795 ± 0.005
39	1	0.001 ± 0.001	0.004 ± 0.002	0.125 ± 0.002	0.130 ± 0.001	0.123 ± 0.002	0.474 ± 0.005	0.56 ± 0.01	90.0 ± 0.3	1.326 ± 0.008	1.158 ± 0.005
40	1	0.002 ± 0.002	0.011 ± 0.001	0.154 ± 0.008	0.167 ± 0.006	0.194 ± 0.007	0.882 ± 0.009	1.01 ± 0.02	107 ± 2	0.96 ± 0.02	0.70 ± 0.01
41	1	0.003 ± 0.001	0.012 ± 0.001	0.129 ± 0.001	0.144 ± 0.002	0.137 ± 0.006	0.542 ± 0.006	0.670 ± 0.005	100.4 ± 0.2	1.789 ± 0.007	1.256 ± 0.006
42	1	0.010 ± 0.001	0.028 ± 0.009	0.146 ± 0.009	0.184 ± 0.003	0.182 ± 0.003	0.946 ± 0.006	0.781 ± 0.005	73.6 ± 0.4	0.805 ± 0.004	0.826 ± 0.004
43	1	0.004 ± 0.001	0.008 ± 0.001	0.178 ± 0.001	0.190 ± 0.002	0.172 ± 0.010	0.915 ± 0.007	1.42 ± 0.06	82.7 ± 0.3	0.711 ± 0.002	0.896 ± 0.002
44	1	0.004 ± 0.001	0.012 ± 0.001	0.233 ± 0.001	0.249 ± 0.001	0.261 ± 0.003	0.905 ± 0.01	0.657 ± 0.007	75.1 ± 0.4	0.491 ± 0.002	0.584 ± 0.005
45	1	0.003 ± 0.001	0.008 ± 0.001	0.135 ± 0.001	0.146 ± 0.001	0.152 ± 0.008	0.899 ± 0.005	1.022 ± 0.005	86.3 ± 0.3	2.0 ± 0.01	1.176 ± 0.007
46	1	0.001 ± 0.001	0.004 ± 0.001	0.067 ± 0.002	0.072 ± 0.001	0.072 ± 0.007	0.492 ± 0.001	1.27 ± 0.01	86.1 ± 0.3	0.608 ± 0.003	0.892 ± 0.002
47	1	0.003 ± 0.001	0.003 ± 0.001	0.078 ± 0.003	0.085 ± 0.002	0.005 ± 0.006	0.452 ± 0.002	1.08 ± 0.02	94.3 ± 0.8	0.508 ± 0.005	0.645 ± 0.008
48	1	0.001 ± 0.001	0	0.024 ± 0.003	0.025 ± 0.002	0.021 ± 0.005	0.419 ± 0.001	1.89 ± 0.02	98 ± 2	0.841 ± 0.007	0.545 ± 0.003
49	1	0.002 ± 0.001	0.003 ± 0.001	0.047 ± 0.001	0.052 ± 0.001	0.051 ± 0.003	0.342 ± 0.004	1.5 ± 0.1	114 ± 2	0.950 ± 0.008	0.764 ± 0.005
50	1	0	0	0.010 ± 0.001	0.010 ± 0.001	0.08 ± 0.01	0.55 ± 0.02	0.55 ± 0.03	108 ± 5	0.72 ± 0.03	0.94 ± 0.04

14. Appendix 16.



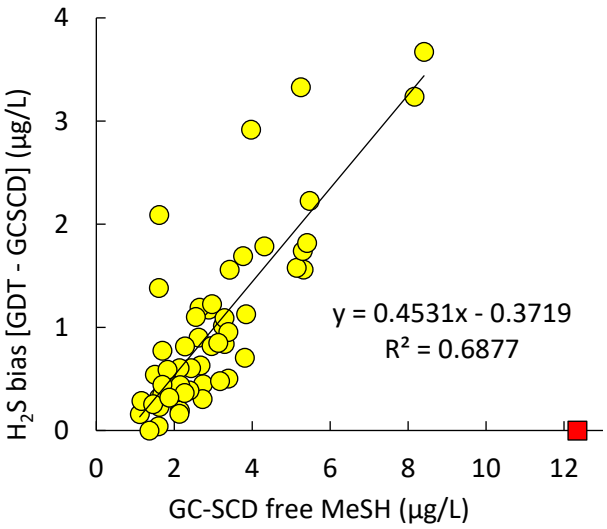
Appendix 16. Measurement of 49 white wines by GC-SCD at two different laboratories for the comparison of (a) free H_2S, (b) salt-treated H_2S, (c) free MeSH and (d) salt-treated MeSH. Wine from bottles with different bottling codes were split into separate data points. Error bars indicate the standard deviation. The solid line is the trendline for the data, and the dashed line is the ideal case (with gradient = 1.00, intercept = 0). One white wine was omitted as there was not enough bottles for analysis at both laboratories. The data from one white wine (wine 21, Appendix 11) was omitted as it could only be measured at one laboratory due to limited supply.

14. Appendix 17.



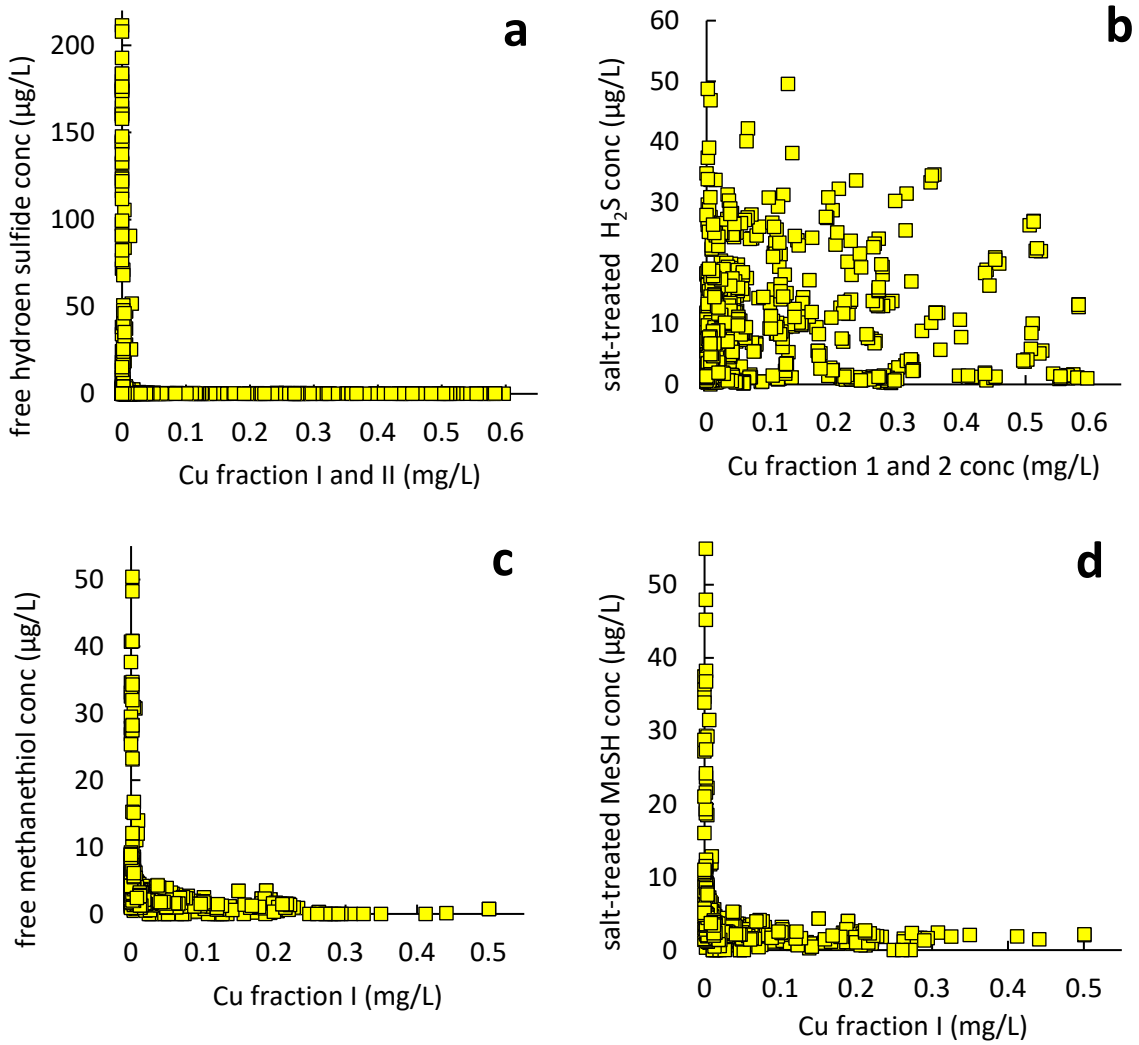
Appendix 17. The measurement of 50 wines for the comparison between (a) GDT and GC-SCD for free H_2S, (b) GC-SCD free and salt-treated H_2S, (c) GDT free H_2S and GC-SCD salt-treated H_2S, and (d) GC-SCD free and salt-treated MeSH. In panels (a) and (c), the yellow data is for wines quantified with a 4LL GDT, while the red data point is wine quantified with a 4LT GDT (less sensitive to interference from thiol compounds). Error bars indicate the standard deviation. The solid line is the trendline for the data, and the dashed line is the ideal case (with gradient = 1.00, intercept = 0).

14. Appendix 18.



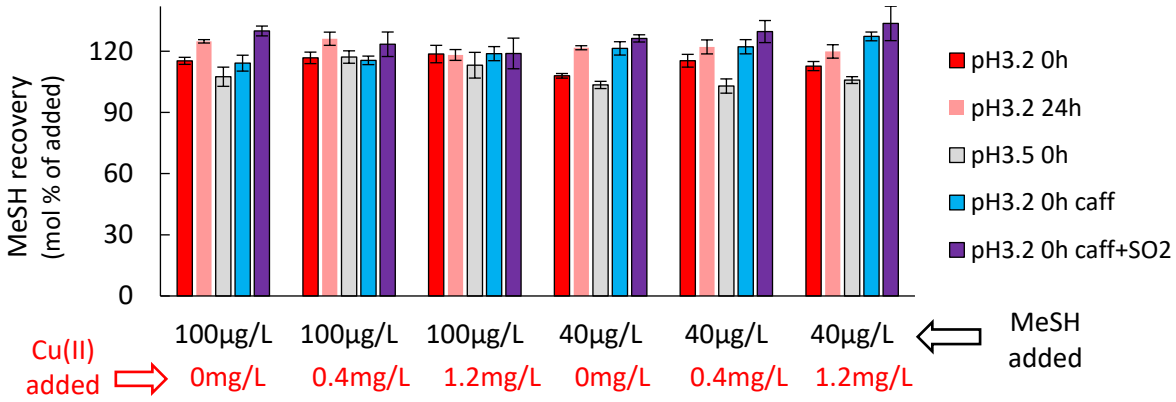
Appendix 18. The positive bias in H_2S concentration for GDT over GC-SCD versus the free MeSH concentration measured by GC-SCD. The data point highlighted in red was considered an outlier and not used in the calculation of the quoted R^2 value. Wine from bottles of different bottling codes were split into separate data points.

14. Appendix 19.



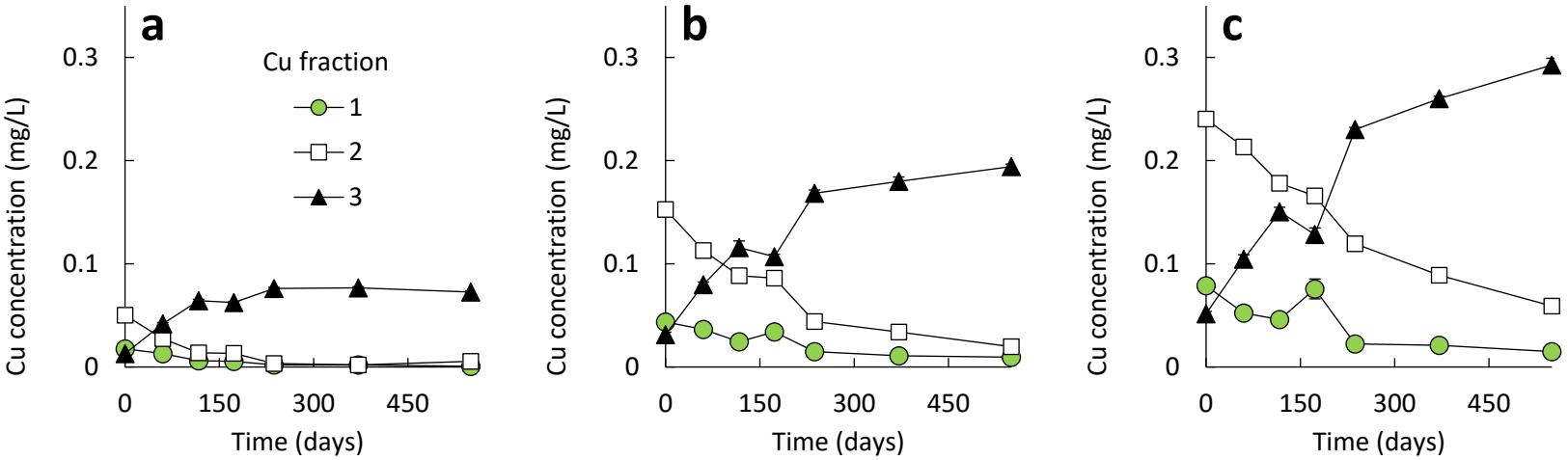
Appendix 19. The concentrations of free H_2S versus Cu fraction 1 and 2 (a), salt-treated H_2S versus Cu fraction 1 and 2 (b), free methanethiol (MeSH) versus Cu fraction 1 (c), and salt-treated MeSH versus Cu fraction 1 (d). The results for H_2S are from 957 bottles of wine, while the results for MeSH are from 893 bottles of white wine. The Cu fractions were measured by BCA colorimetry.

14. Appendix 20.



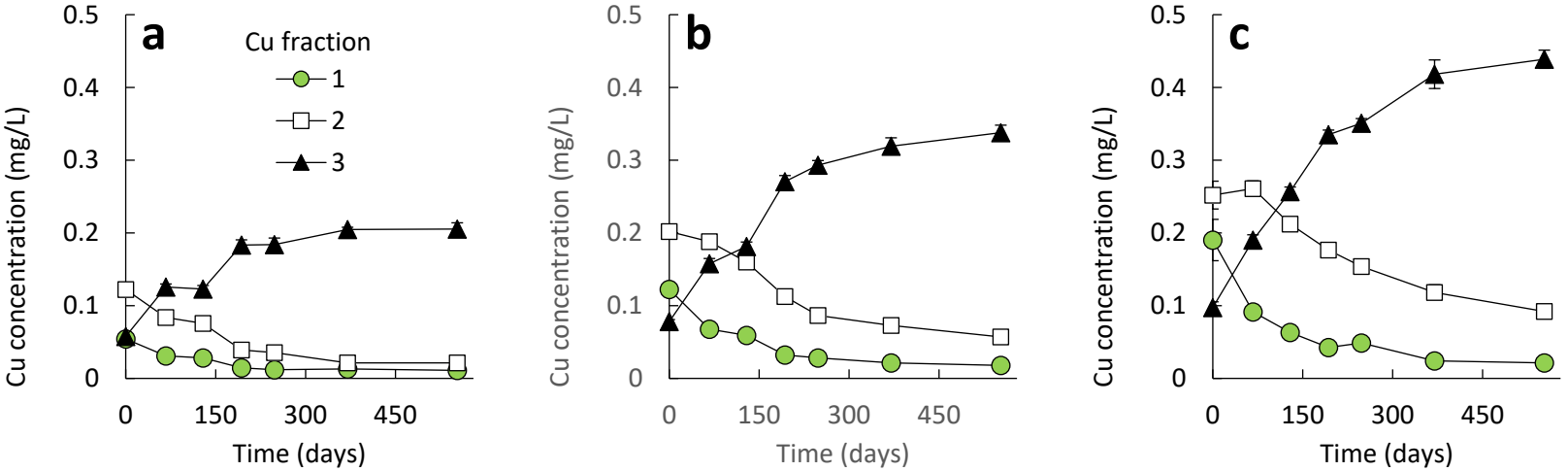
Appendix 20. The recovery of added MeSH in model wine samples. The caffeic acid and sulfur dioxide were present at concentrations of 200 and 15 mg/L, respectively. The recovery of added MeSH was calculated as: ([(mol{freeMeSH}) + (2 x mol{freeDMDS})]/mol{addedMeSH}).

14. Appendix 21.



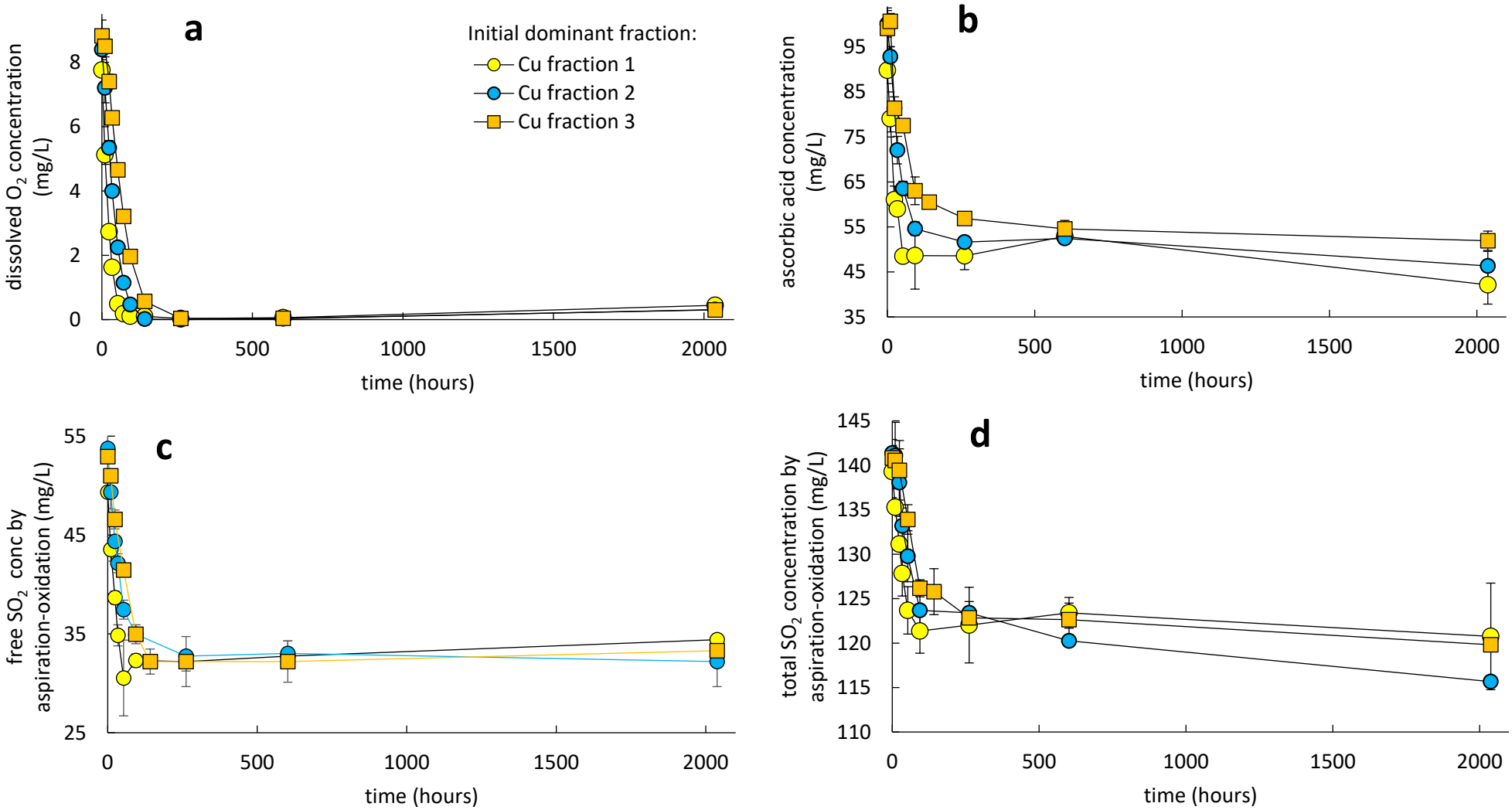
Appendix 21. Concentrations of Cu fractions in Sauvignon blanc 1 during bottling aging with 0 (a), 0.15 (b) and 0.30 (c) mg/L of Cu(II) added at bottling.

14. Appendix 22.



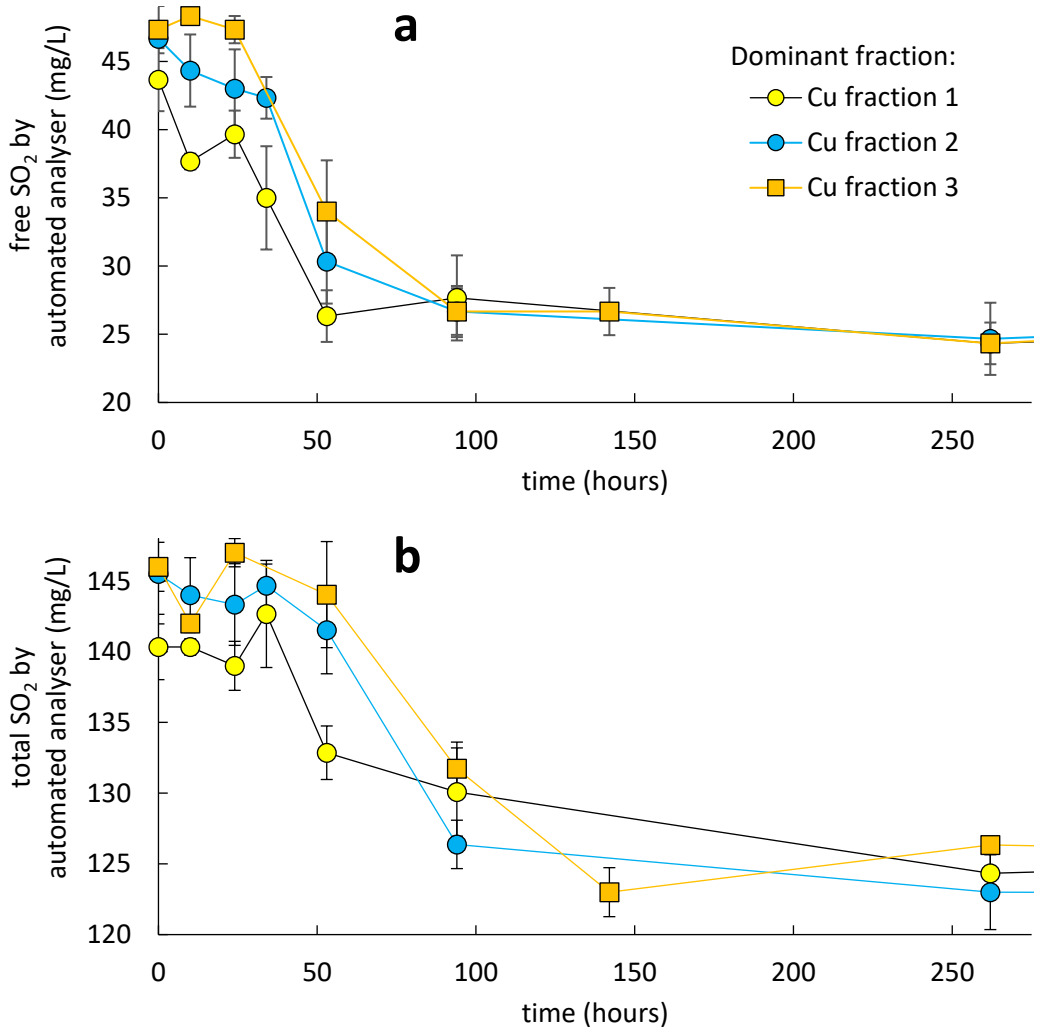
Appendix 22. Concentrations of Cu fractions in Sauvignon blanc 2 during bottling aging with 0 (a), 0.15 (b) and 0.30 (c) mg/L of Cu(II) added at bottling.

14. Appendix 23.



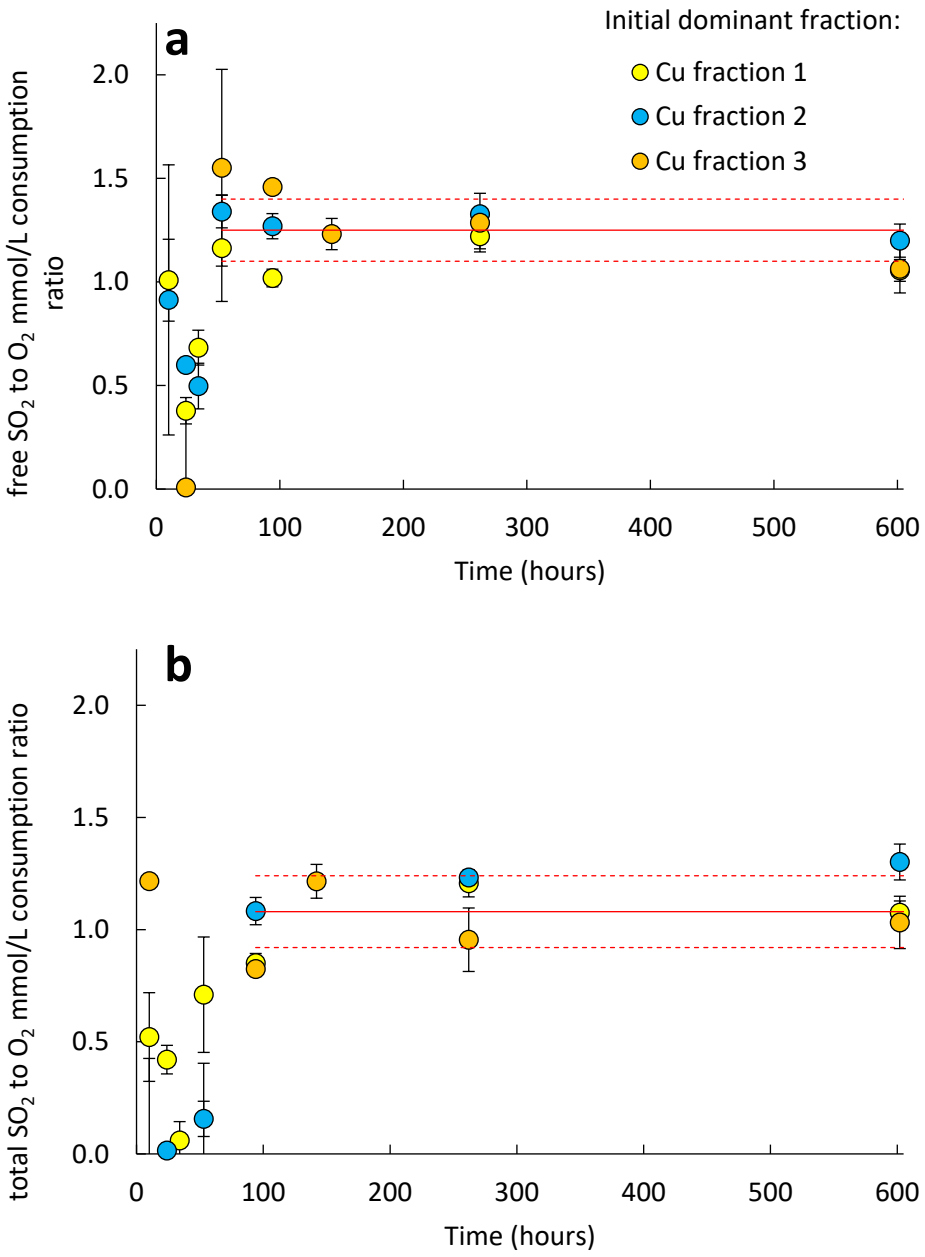
Appendix 23. The concentrations of oxygen (a), ascorbic acid (b), free sulfur dioxide (c) and total sulfur dioxide (d) in the Chardonnay samples throughout the entire storage period.

14. Appendix 24.



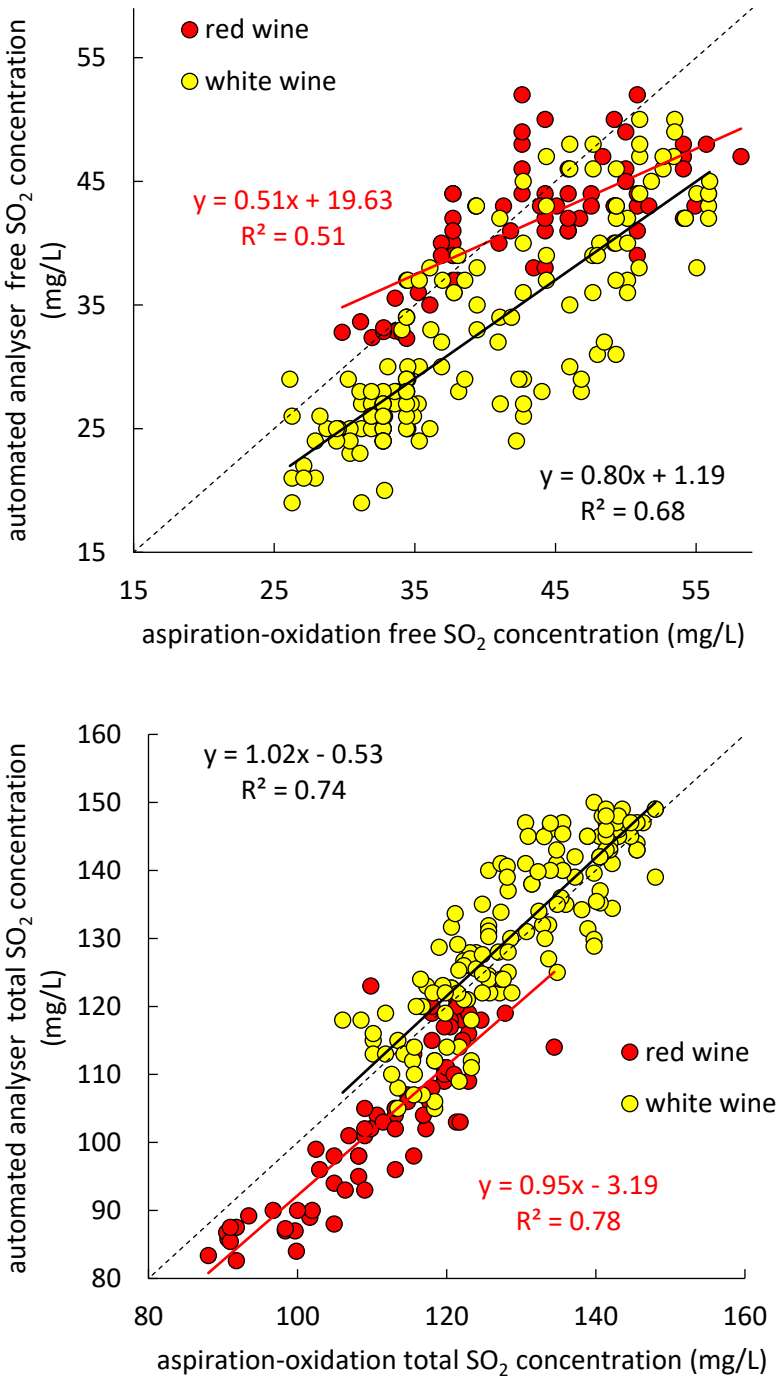
Appendix 24. The concentrations of free sulfur dioxide (a) and total sulfur dioxide (b) as determined by the automated analyser during the oxidation of a Chardonnay wine.

14. Appendix 25.



Appendix 25. The mole consumption ratios for oxygen to free sulfur dioxide (a) and oxygen to total suflur dioxide (b) when the sulfur dioxide concentrations were measured by the automated analyser. The solid red lines indicate the average consumption ratios from 100 to 600 hours, and the dashed lines are with the standard deviation added or subtracted to the average.

14. Appendix 26.



Appendix 26. Comparison of the data obtained by the automated analyser versus the aspiration-oxidation method for the measurement of free sulfur dioxide (a) and total sulfur dioxide (b) on white and red samples from Section 6.4.

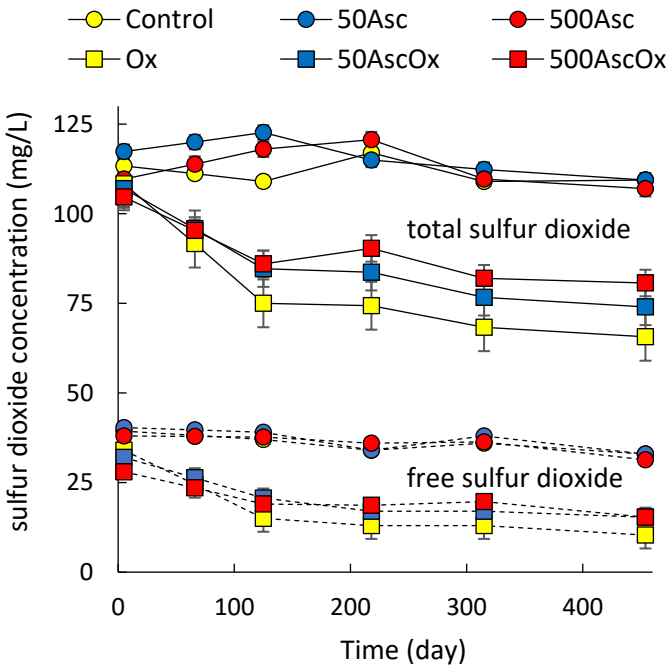
14. Appendix 27.

Appendix 27. Compositional parameters of the rosé wine samples 5-days after bottling where the uncertainty is the standard deviation (n=3).[1] Other parameters that were averaged over all treatments (n=18), due to minimal variation with treatment, were pH (3.56 ± 0.02), ethanol concentration (13.20 ± 0.04 %(v/v)), aluminium (0.71 ± 0.03 mg/L), magnesium (96 ± 3 mg/L), manganese (0.87 ± 0.02 mg/L) and zinc (0.32 ± 0.04 mg/L).

Sample	true free SO_2 (mg/L)	anthocyanin-bound SO_2 (mg/L)	free SO_2 (mg/L)	total SO_2 (mg/L)	total anthocyanin (mg/L)	total phenol (280 nm)	total Cu (mg/L)	total Fe (mg/L)
HO	17.1 ± 0.6^{cb}	17 ± 2^{abc}	34 ± 2^{ab}	108 ± 4^{ab}	26 ± 1^{a}	4.0 ± 0.2^{a}	0.264 ± 0.005^{a}	0.50 ± 0.03^{a}
LAscHO	17 ± 1^{cb}	15 ± 1^{bc}	32 ± 1^{bc}	107 ± 1^{ab}	28 ± 1^{a}	3.8 ± 0.1^{a}	0.264 ± 0.008^{a}	0.507 ± 0.009^{a}
HAscHO	15.3 ± 0.5^{c}	12.7 ± 0.5^{c}	28 ± 1^{c}	105 ± 1^{b}	27 ± 1^{a}	3.6 ± 0.1^{a}	0.251 ± 0.002^{b}	0.488 ± 0.003^{a}
LO	19 ± 2^{abc}	21 ± 3^{a}	39 ± 4^{ab}	113 ± 3^{ab}	28 ± 1^{a}	3.9 ± 0.1^{a}	0.256 ± 0.002^{ab}	0.487 ± 0.008^{a}
LAscLO	22 ± 2^{a}	18 ± 2^{ab}	40 ± 1^{a}	117 ± 4^{a}	27 ± 1^{a}	3.6 ± 0.2^{a}	0.252 ± 0.003^{ab}	0.50 ± 0.02^{a}
HAscLO	20 ± 1^{ab}	18 ± 1^{a}	38 ± 1^{a}	110 ± 4^{a}	25 ± 1^{a}	4.1 ± 0.4^{a}	0.261 ± 0.004^{ab}	0.51 ± 0.03^{a}

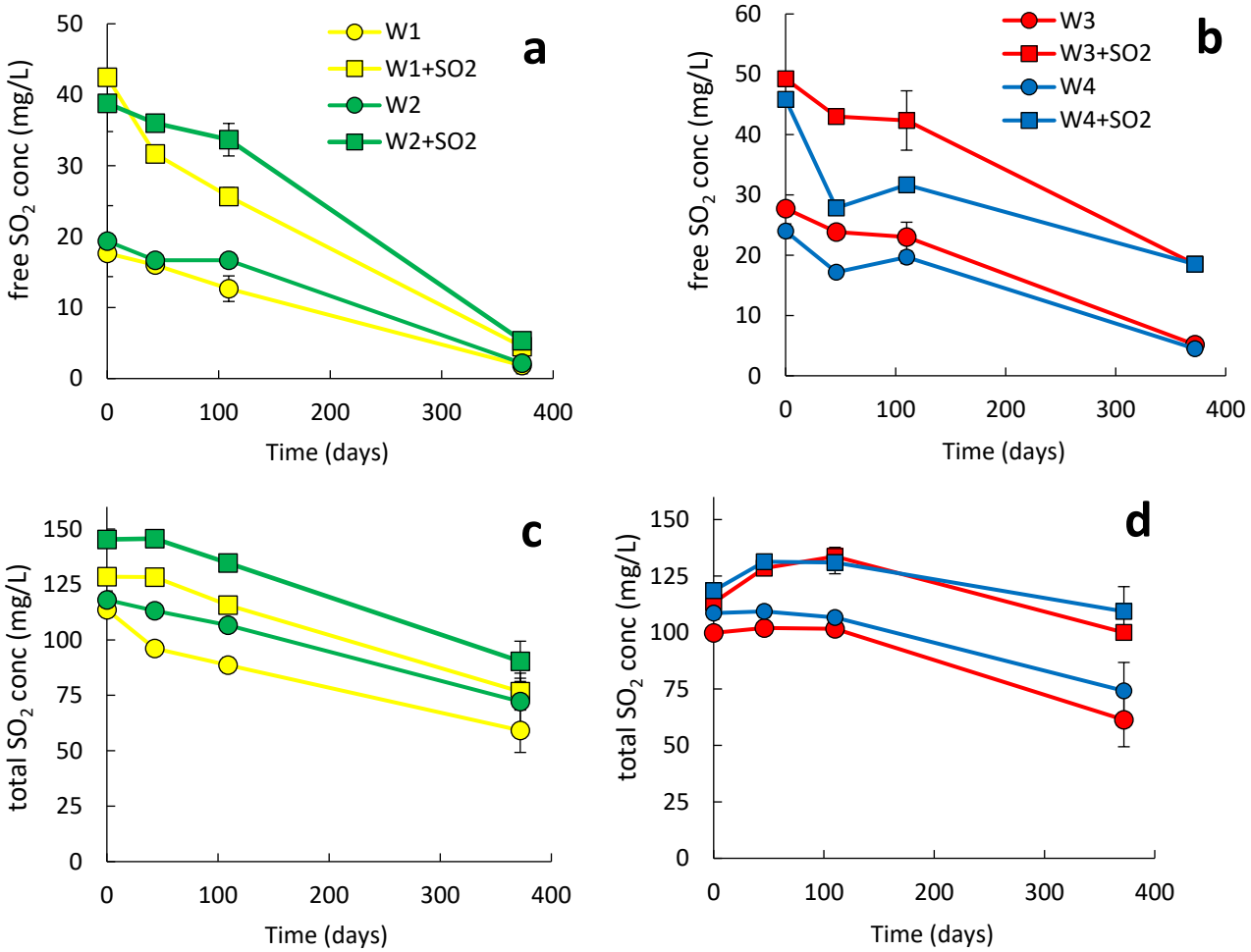
[1] Data with different superscripts within the same column indicate significant differences ($p \leq 0.05$)

14. Appendix 28.



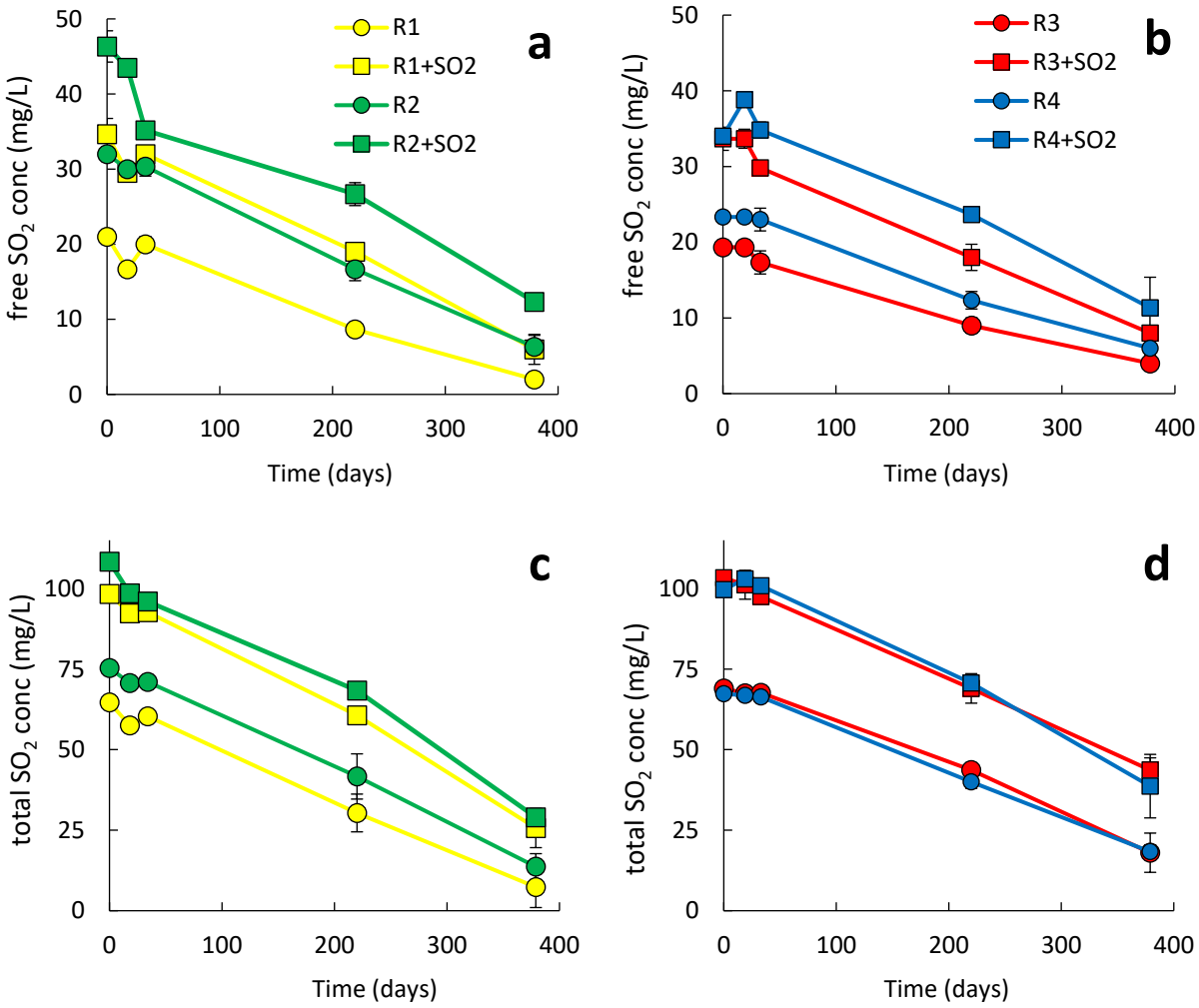
Appendix 28. The concentrations of free and total sulfur dioxide in rosé wines during bottle aging. The error bars represent the standard deviation of triplicate samples. The free sulfur dioxide concentrations for the Control sample (yellow circles) are concealed by the 50 Asc symbols (blue circles). ***Control*****:** low O2 without ascorbic acid, ***50Asc***: low oxygen + 50 mg/L ascorbic acid, ***500Asc***: low oxygen + 500 mg/L ascorbic acid, ***Ox***: high oxygen without ascorbic acid, ***50AscOx***: high oxygen + 50 mg/L ascorbic acid, and ***500AscOx***: high oxygen + 500 mg/L ascorbic acid.

14. Appendix 29.



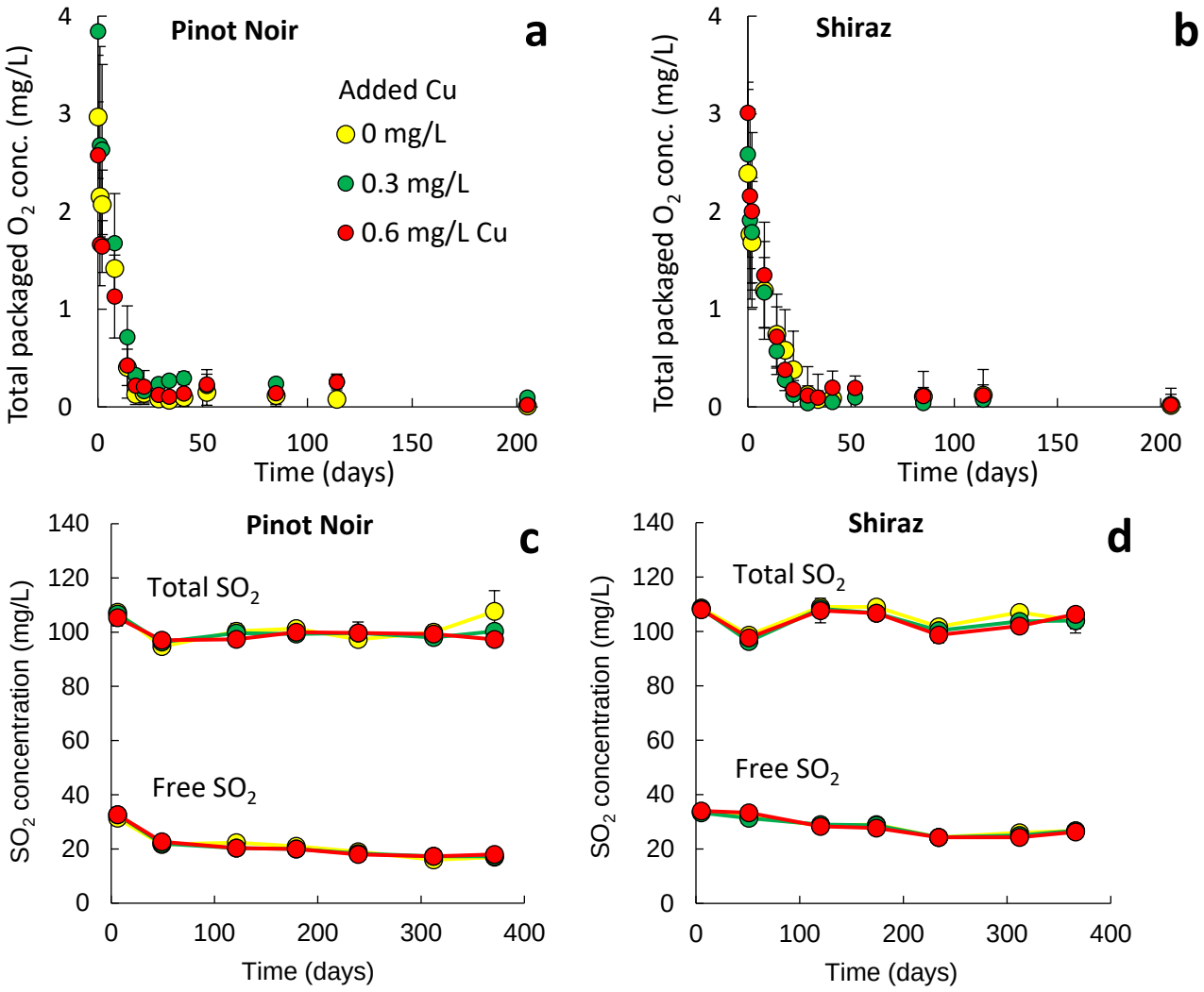
Appendix 29. The free sulfur dioxide concentrations (a,b) and total sulfur dioxide concentrations (c,d) in white wines W1 and W2 (a,c) and W3 and W4 (b,d). The wines with '+SO2' had an extra 40 mg/L SO_2 added at bottling.

14. Appendix 30.



Appendix 30. The free sulfur dioxide concentrations (a,b) and total sulfur dioxide concentrations (c,d) in red wines R1 and R2 (a,c) and R3 and R4 (b,d). The wines with '+SO2' had an extra 40 mg/L SO_2 added at bottling.

14. Appendix 31.



Appendix 31. The total packaged oxygen concentrations (a, b) and sulfur dioxide concentrations (c, d) in the Pinot noir and Shiraz wines during the storage experiment.