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Metal ion speciation: Understanding its role in wine development and generating a tool to minimise wine spoilage



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Project NWG1401:

Metal ion speciation: Understanding its role in wine development and generating a tool to minimise wine spoilage.

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Abbreviations

BCA	Bicinchoninic acid
CMLD	Carboxymethine-Linked Dimer
DMS	Dimethyl Sulfide
FAAS	Flame Atomic Absorption Spectroscopy
GC-SCD	Gas Chromatography with Sulfur Chemiluminescence Detector
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
HPLC	High Performance Liquid Chromatography
ICPOES	Inductively Coupled Plasma with Optical Emission Spectrometry
LMWSC	Low Molecular Weight Sulfur Compounds
ME CCSP	Medium Exchange Constant Current Stripping Potentiometry
PES	Polyethersulfone
UHPLC	Ultrahigh Pressure Liquid Chromatography
SPCE	Screen Printed Carbon Electrode
SPE	Solid Phase Extraction

1 ABSTRACT

Iron and/or copper can influence the rate of oxidation reactions and accumulation of reductive flavour compounds in wine. This project investigated the main binding agents for these metals and assessed how binding impacted the metals' mediation of oxidative and reductive development. The results showed that copper mainly existed in a sulfide-bound form, but the non-sulfide bound form was more efficient for mediating oxidation reactions when ascorbic acid was present. The non-sulfide bound form of copper could also readily sequester sulfide from precursors during reductive development. Unlike copper, iron binding in wine was not dominated by a single binding agent and had minimal influence on oxidation in the presence of ascorbic acid. A colorimetric method was developed to allow colorimetric determination of copper concentration in wine.

2 EXECUTIVE SUMMARY

Copper and iron are known to participate in oxidative and reductive reactions in wine. However, despite an abundance of scientific literature detailing methods to measure different forms of the metals in wine (termed metal speciation), little was known on the main wine components binding the metals. Furthermore, there was no application of the scientific measures of metal forms to understand the oxidative and/or reductive processes in wine, or to develop industry orientated measures to prevent metal induced spoilage of wine.

This project assessed the viability of numerous published measures of Cu and Fe speciation in wine, and selected those methods with appropriate sensitivity and reproducibility to conduct a survey of Australian wines. In the process, several methods were improved and a novel electrochemical method developed. A survey of Australian wines showed that Cu predominantly existed in a sulfide-bound form (i.e., copper(I) sulfide), although in some wines there was a significant portion of Cu not bound to sulfide. The copper(I) sulfide form was dispersed throughout the wine, did not settle, and was not visible to the eye. It was found that the Cu could undergo transition from a sulfide-bound to a sulfide-free form quite easily, with oxidising and/or aerating conditions favouring conversion to a non-sulfide bound form, while low oxygen conditions favoured the sulfide-bound form of Cu. The results also demonstrated that during bottle aging of wine, the natural progression of non-sulfide bound Cu at bottling was to sequester sulfide from precursors in the wine and form copper(I) sulfide. Furthermore, the vast majority of wines appeared to have sufficient sulfide precursors to ensure that most the Cu was bound to sulfide upon bottle aging.

The electrochemical measure of non-sulfide bound Cu showed that free hydrogen sulfide only accumulated in the wine when the non-sulfide bound Cu dropped below a concentration of 0.02 mg/L. This may provide winemakers a threshold concentration of non-sulfide bound Cu to aim for when performing Cu fining trials and thereby minimise the addition rate of Cu to wines. However, as the non-sulfide bound Cu readily sequesters further sulfide during wine aging the stability will only be short-term. Further research is also required on whether copper(I) sulfide has any activity in inducing reductive characters in wine, through conversion of sulfide precursors to hydrogen sulfide, or whether apparent hydrogen sulfide production by Cu only occurs until all Cu is bound by sulfide. The link between methanethiol production and Cu speciation also requires further investigation.

In terms of the mediation of oxidation reactions, the Cu species behaved quite differently depending on whether ascorbic acid was present in wine. For white wines with ascorbic acid the non-sulfide form of Cu was the key catalyst with copper(I) sulfide having little catalytic activity. Conversely, in white wines without ascorbic acid and in red wines, the non-sulfide bound form of Cu showed little relationship to oxygen consumption rates.

Regards the removal of Cu from wine during production, bentonite treatment and filtration were both investigated in white wines. In different batches of protein-unstable Chardonnay that had post-fermentation Cu concentrations of ranging from 0.08-0.35 mg/L, bentonite treatment lowered the Cu concentrations by 50-85 %. More research is required to assess whether the type and concentration of protein in the wine is critical to the efficiency of Cu removal by bentonite. Particle size measurements showed that copper(I) sulfide present in wine was

generally smaller than the pore size of typical membrane filters used in wineries (i.e. < 0.20-0.45 μm) but slightly larger than the strict definition of nanoparticles (<0.10 μm). However, membrane filters could still remove copper(I) sulfide via adsorption and polyethersulfone (PES) and nylon membranes provided the best adsorption of the different membrane media studied. White wine polysaccharides and proteins were found to hinder the adsorption process, particularly for cellulose-based membranes. Further filtration experiments, including investigations of cross flow filtration, are required on a large-scale volume and the removal of copper(I) sulfide from red wine via filtration or low addition rates of bentonite should also be explored.

A colorimetric test for total Cu concentration in wine was developed. This method utilises an addition of silver(I) to wine in order to effectively release copper from sulfide and allow its determination by a colorimetric reagent. The test will allow winemakers to assess the amount of Cu in their wine after fermentation, after bentonite fining and/or after Cu fining. The technique is being further refined to allow measurement of non-sulfide bound fractions of Cu.

In contrast to Cu, Fe showed a distribution among different forms in wines that depended more on the pH of the wine and the phenolic concentration. In low pH white wines, organic acid complexes were favoured, whilst in higher pH red wines, phenolic-Fe interactions were dominant. Although less focus in this project was on Fe than Cu, it was observed that oxygen consumption in red wine was linked more to Fe(II) concentrations added to the wine than Cu speciation. Fe and its speciation showed little impact on the evolution of hydrogen sulfide and methanethiol in a range of Chardonnay wines after 1-year of bottle aging.

Significant progress has been made during this project in understanding the different forms of metals in wine and how they can be measured. Furthermore, a variety of links between the metal forms and reduction and oxidation processes in wine have been established. An industry applicable measure of Cu in white wine has been developed to provide rapid assessment of Cu in white wines by most wineries. Further research is required to refine the measure to allow measurement of total Cu in red wine, and measurement of non-sulfide bound Cu in white and red wines. Additional work is required on establishing further links between Cu speciation and the oxidative and reductive aging of wine.

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The National Wine and Grape Industry Centre is a research centre at Charles Sturt University created as an alliance with the New South Wales Department of Primary Industry and the New South Wales Wine Industry Association. Input and support from the external partners of the NWGIC is gratefully acknowledged.

The Australian Wine Research Institute was a partner organisation in this research project. One full-time equivalent post-doctorate researcher was located at the NWGIC and a half-time equivalent post-doctorate researcher was located at the AWRI.

The research visit to the NWGIC by Dr. Anque Guo was funded by the China Scholarship Council and complements the research of the Key Agricultural Technology Program of Shaanxi Province, China (2015NY131).

The wines analysed in Section 6.2 of the report were donated by wine companies.

3 BACKGROUND

Oxidative, reductive and colloidal phenomena are recognised as major contributors to wine faults. Iron and/or copper can influence all these areas, impacting the rate of oxidation reactions, the concentrations of certain sulfur compounds, and contributing to haze formation. Both these metal ions exist in a variety of different forms including different redox forms, and are bound by wine components at different binding strengths. The agents responsible for the binding of metal in wine, especially in relation to techniques that enabled measurement of different forms of metals in wine, were not known prior to this project.

The maximum total concentration of metals in wine recommended to avoid metal-induced spoilage is ambiguous. Generally, recommendations of broad ranges (0.2-0.6 mg/L for Cu) rather than specific concentrations are provided (Kean and Marsh 1956, Amerine and Ough 1980, Peynaud 1984, Shields 1986, Rankine 2004, Cowey 2008, Iland et al. 2012). The recommendations also vary from author to author, due to the difficulty in linking an absolute total concentration to specific metal-induced effects in wine. This situation is not unique to wine, 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.

From a survey of key scientific literature, prior to the commencement of this project, it was apparent that:

- Research tools existed for the measurement of different forms of metals (i.e., termed *metal speciation*) in wine.
- Linking these measures to the rates of relevant metal-induced effects including oxygen consumption rate, and hydrogen sulfide removal had not been investigated in wine.
- Binding agents for metal ions in wine, capable of influencing the classification of metals via speciation measures, had not been identified.
- Published metal speciation measurement techniques were not practical for routine usage in the wine industry, and required development into a routine test.

Based on this, it was clear that a knowledge gap existed in the potential use of the speciation measures to predict metal-induced processes. Brief mention will be made on recent research of particular relevance to the measurement of metal speciation in wine. Our earlier research (Clark and Scollary 2000) showed that white wines require acidification to pH 0 to convert copper from bound forms into ‘free’ forms, with the latter being measurable by electrochemical methods (the ‘*free*’ forms were termed ‘*labile*’ copper within the studies). Acidification to pH 1 was not sufficient, indicating the strength by which certain wine compounds can bind copper in

wine. Furthermore, in a survey of red and white wines, the fraction of labile copper in untreated wines ranged from 10-90 % of the total. Several studies have showed similar ranges of copper speciation when measured by different techniques in wine (Wiese and Schwedt 1997, Pohl and Sergiel 2009). In addition, speciation procedures have also been developed for iron in wine and/or model wine systems (Tašev et al. 2006, Pohl and Prusisz 2009, Danilewicz 2013). Research has been conducted in model systems by Danilewicz (2013), comparing the rates of Fe(II) oxidation and Fe(III) reduction in the presence of different ligands (i.e., malic, tartaric and acetic acids). The results demonstrated the role of coordinating ligands in influencing the favoured redox form of iron (Fe(II) vs Fe(III)) and hence oxygen consumption rates.

Wine production is commonly conducted with additions of copper to minimise sulfidic-off odours and/or allow further expression of fruit characters. It is proposed that a metal speciation measure accessible to winemakers will allow assessment of the amount of 'active' copper present in the wine, thereby providing more informed additions of copper to wine. The metal speciation tool may identify a wine that is particularly prone to oxygen exposure and/or development of reductive characters due to high levels of reactive copper and/or iron. The metal speciation tool will be applicable to all winemakers and potentially lead to wines with increased shelf life and improved aroma characteristics.

4 PROJECT AIMS AND PERFORMANCE TARGETS

The overall aim of this project was to develop knowledge regarding the metal speciation in wine, wine binding agents and also to produce a routine test applicable to winery use that will allow winemakers to assess the impact of residual copper and/or iron in wine on spoilage and beneficial reactions.

The objectives of this project were to:

1. Survey a range of white and red wines for copper and iron speciation.
2. Identify the components of wine that influence the speciation of copper and iron.
3. Relate the metal speciation measures of copper and iron to the rate of wine oxidation, and the formation of volatile sulfur compounds in reductive conditions.
4. Relate the metal speciation measures to existing colorimetric assays of metal reactivity and modify the assays as required to reflect the ability of metals to affect spoilage processes.

The outputs and activities that have been completed during this project are listed below in Table 1. The outputs and activities underwent several changes throughout the lifetime of the project but the final approved outputs/activities are those shown in Table 1. The Outputs (a) and (b) for Year 4, were partly met with the development of the colorimetric measure of total Cu in wine, and corresponding 'How To Guide', rather than the development of a colorimetric Cu speciation measure. Preliminary work was performed for the colorimetric speciation measure but further work is required for its development.

Table 1. Project Outputs and Activities.

Year 1	Output	Section/s of Report	Activities
a	Knowledge regarding main 'coarse' speciation measures most appropriate to use in wine survey.	6.1	At least three published coarse speciation measures will be applied to at least five wines to assess repeatability and reproducibility. Those measures that provide adequate responses will be selected for usage in the subsequent phase of the project. (NWGIC)
b	Knowledge regarding main 'fine' speciation measures most appropriate to use in wine survey.	6.1	Published fine speciation measures (distinguishing Cu(I)/Cu(II)/Fe(II)/Fe(III)) will be applied to at least five wines to assess repeatability and reproducibility. Those that provide adequate responses will be selected for usage in the subsequent phase of the project. (AWRI: Cu/ NWGIC: Fe)
c	Data from a range of wines for metal ion speciation profiles of copper and iron.	6.2	At least 50 wines will be measured for metal speciation by the measures identified as having suitable repeatability and reproducibility. This will include both red and white wines. (AWRI: Cu(I),Cu(II)/ NWGIC: coarse speciation, Fe(II), Fe(III))
d	Key grape and wine components sourced for use in metal binding experiments.	6.3	Isolate total wine proteins, total wine polysaccharides, total wine polyphenols and total wine tannins from at least 1 white and 1 red wine in sufficient quantities to perform metal binding studies. (AWRI)
e	Knowledge on the binders of copper/iron in real wines.	6.3 6.10	Binding agents in surveyed wine will be chromatographically fractionated and characterised for metal content and composition of the material to which it is bound (using existing analytical techniques). (AWRI)

Table 1. Project Outputs and Activities (continued).

Year 2	Output	Section/s of Report	Activities
a	Data showing relationships between metal speciation and rate of wine oxidation.	6.6 6.7 6.8	Real wines (from survey) or model wines (created with metal/s bound to key binding partner/s) with known metal speciation profile assessed for impact on wine oxidation, using optimised temperature and oxygen conditions. This will include: -impact on rates of oxygen consumption -ability to catalyse relevant aldehyde-flavanol reactions. (NWGIC)
b	Data showing the relationship between copper speciation and precipitation of hydrogen sulfide.	6.5	Add hydrogen sulfide to real wine (from survey) or model wine and assess extent of copper precipitation based on variable copper speciation profile of wines. (NWGIC)
c	Further knowledge on the specific binders of copper/iron in real wines	6.3 6.10	A variety of thiol compounds assessed for binding efficiency to Cu, and red wine polyphenol compounds for binding to Cu and Fe. The impact of pH on the binding of the metal ions established (AWRI/NWGIC)
d	Data showing relationships between metal speciation and evolution of Low Molecular Weight Sulfur compounds during aging	6.6 6.9	Real wines (from survey) or model wines (created with metal/s bound to key binding partner/s) with known metal speciation profile prepared and stored under anaerobic conditions. LMWSC's measured at 1, 3 and 6 months. (AWRI)
e	Report to AGWA on stop/go milestone.	Submitted after year 2 of the project (June 2016)	Prepare report summarising the data linking metal speciation measures to specific phenomena in wine. This report will enable assessment of the viability to complete the final phase of the project where research speciation measures are developed into industry-orientated measures.

Table 1. Project Outputs and Activities (continued).

Year 3	Output	Section/s of Report	Activities
a	Data showing relationships between metal speciation and rate of oxygen consumption in red wines and white wines (without ascorbic acid), and investigations into the mechanistic aspects of hydrogen sulfide production.	6.6 6.7	Develop assays to provide an assessment of the impact of metal species on oxygen consumption rates in red wines and white wines (without ascorbic acid). Investigate the release of hydrogen sulfide from the non-labile (electrochemical) or hydrophobic (SPE ICPOES) forms and the production of hydrogen sulfide. (NWGIC and AWRI)

Year 4	Output	Section/s of Report	Activities
a	Colorimetric assay(s) for metal speciation that best identify the reactive fraction of metal ion.	6.11	Based upon knowledge of active metal fractions of wine, survey a range of colorimetric reactions that will identify a similar metal ion fraction. This will involve applying a range of known colorimetric reactions for metal ions to wines of known metal speciation profiles and assessing which provides the most consistent measurement to the 'research orientated' measurements. (NWGIC and AWRI)
b	Metal speciation 'How to' guide for winemakers.	6.12	Develop a set of guidelines for winemakers for routine measurement of metal ion speciation in wines and information to interpret the speciation measures. (NWGIC/AWRI)
c	Teaching materials based on metal speciation measures in wine and their interpretation.	6.13	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. (NWGIC/AWRI)

5 METHODS

5.1 Speciation methods utilised in the study

Table 2 shows the types of published speciation methods trialled. Details of the main speciation techniques utilised within the study can be found within the following references: Clark et al. (2016) for labile Cu by medium exchange constant current stripping potentiometry (ME CCSP), Kontoudakis, Smith et al. (2017) for solid phase extraction and detection via inductively coupled plasma with optical emission spectroscopy (SPE ICPOES) and Kontoudakis et al. (2018) for liquid extraction and detection by graphite furnace atomic absorption spectroscopy (GFAAS). A brief description for each of the main speciation measures is provided below.

5.1.1 Medium exchange constant current stripping potentiometry (Clark et al. 2016)

In brief, this technique utilises a mercury film on a screen printed carbon electrode. Wine is passed over the electrode during which a negative potential is applied. This allows the deposition of loosely bound fraction of Cu from the wine to form a metallic copper amalgam within the mercury film. After the wine is typically passed over the electrode for 120-500s (depending on the concentration of copper in the wine), the mercury film is washed with 60 % aqueous ethanol and then 4.0 M ammonium acetate. The potential of the electrode is then allowed to increase, and at a potential characteristic of metallic copper, the deposited copper is stripped, via oxidation with a constant current, from the mercury film. As the copper is stripped from the film the potential remains constant, and the time for which the potential remains constant is measured. This time required to strip the copper can be related to the detected copper in the wine via the use of calibration standards in model wine. The detected copper in the wine is termed ‘labile Cu’. The total copper in the wine is measured by ICPOES, and the ‘non-labile Cu’ can be calculated by subtracting the labile Cu concentration from the total copper concentration. Generally, labile Cu will be loosely bound Cu while non-labile Cu will be copper that is more strongly bound.

5.1.2 Solid Phase Extraction with detection by Inductively Coupled Plasma with Optical Emission Spectroscopy (Kontoudakis, Smith et al. 2017)

Wine (15 mL) is passed over a divinylbenzene solid phase extraction (SPE) cartridge and the amount of copper retained by the cartridge determined by Inductively Coupled Plasma with Optical Emission Spectroscopy (ICPOES). This fraction of metal (Cu or Fe) was designated the ‘**hydrophobic’** metal ion based on the fact that divinylbenzene SPE material typically sequesters more non-polar and hydrophobic components from aqueous solutions. The eluent from the first divinylbenzene SPE cartridge was then taken and passed through a cation exchange SPE cartridge and the amounts of metal being retained or not by this cartridge were determined by ICPOES. The fraction of Cu retained by the cation exchange column was designated ‘**cationic**’ metal and that passing through this cartridge designated as ‘**residual’** metal. The term ‘cationic’ is merely to designate that the Cu was trapped by the cation exchange column and does not imply that this Cu is in a free Cu form. The term ‘residual’ metal refers to metal that was able to pass through both divinylbenzene and cation exchange SPE cartridges.

5.1.3 Liquid extraction with detection via Graphite Furnace Atomic Absorption Spectroscopy (Kontoudakis et al. 2018)

Wine (3 mL) is agitated with 3 mL of 8 M of ammonium thiocyanate for 5 minutes at room temperature to induce the more reactive fraction of copper in the wine to form a copper thiocyanate complex. A 3 mL volume of methyl isobutyl ketone is then added to the solution and the mixture agitated for 5 minutes at room temperature to allow extraction of the copper thiocyanate complex from the aqueous phase to the organic phase. After allowing separation of the liquid phases, the organic phase was taken and the copper concentration measured by GFAAS. The copper able to be extracted as the thiocyanate complex into the organic phase was termed '**labile Cu**' while that not extracted was termed '**non-labile Cu**'. As for the electrochemical method (5.1.1), the labile Cu was more likely to be loosely bound Cu while the non-labile Cu as more strongly bound Cu.

5.2 The survey of 50 wines by metal speciation measures

A total of 50 wines were donated by the Australian wine industry, and a further two were purchased, in order to compare a range of metal speciation measures with each other and also to relate the measures to wine composition. Of the 52 wines, 20 were white wines, 29 were red and the final 3 were miscellaneous (rose, sparkling and sweet). Upon opening the wines, the sulfur dioxide (free and bound via flow injection analysis) and hydrogen sulfide (free and bound) measurements were conducted immediately, while the electrochemical (ME CCSP) and SPE ICPOES methods were conducted within 30-60 minutes of opening the bottle. The liquid extraction GFAAS method, in combination with several others listed in Table 2, were conducted within 120 minutes of opening the bottle. The bound hydrogen sulfide was determined by measuring the total hydrogen sulfide in wines with gas chromatography with sulfur chemiluminescence detection (GC-SCD) (wines+salt+heating, as reported in Kontoudakis, Smith et al. (2017) and adapted from Siebert et al. (2010)), and subtracting the free hydrogen sulfide (wine+heating, as reported in Kontoudakis et al. (2017) and adapted from Franco-Luesma and Ferreira (2014)).

5.3 Survey of Cu and Fe binding components within wine

The details for the survey of binding components for Cu and Fe in wine can be found within Kontoudakis, Smith et al. (2017). All potential binding agents were added a concentrations typically found in white and red wines. The exception were the organic acids which were compared to each other on an equimolar concentration (0.018 M).

5.4 Details of wine trials

The details of the Chardonnay wine trials based on juice with different Cu and Fe concentrations and subsequent treatment of wine with and without bentonite are described within Rousseva et al. (2016) and also within Kontoudakis, Guo et al. (2017). The results of this trial are described within Sections 6.4, 6.5.2, 6.7.1.2 and 6.9.2. The details of the additional wine trials and studies are provided within the report.

6 RESULTS AND DISCUSSION

6.1 Assessment of a range of speciation measures for Cu and Fe in wine

Table 2 shows the methods assayed and those selected for inclusion in the later stage of the project. Many methods assayed were not sufficiently robust to provide the required repeatability, reproducibility and sensitivity, especially when applied to both red and white wines. Furthermore, certain techniques required comprehensive pre-treatment of the wine and hence were likely to perturb the forms of metal within the wine during the measurement itself. These techniques were not pursued.

Table 2. Metal speciation techniques assessed for suitability to use in later stages of the project.

Technique	Ref	Description	Trialled in wine survey
Solid phase extraction-ICPOES	1	Fractionation of Cu and Fe as hydrophobic, cationic and residual. Modification of original method.	✓ (Cu) ✓ (Fe)
CCSP (non-medium exchange)	2	Electrochemical detection of Cu as labile and non-labile. Extensive perturbation of sample during measurement.	× (Cu)
Hg film SPCE-medium exchange-CCSP	3	Electrochemical detection of Cu as labile and non-labile.	✓ (Cu)
Kinetic/Colorimetric - Azide	4	Kinetic colorimetric determination of Cu as labile and non-labile. Not sufficiently sensitive or applicable to red wine.	× (Cu)
Colorimetric – Ferene	5	Colorimetric detection of Fe(II). Insufficient level of accuracy and not applicable to red wine.	× (Fe)
padap-HPLC	6	Colorimetric detection of Fe(II), and Fe(III). Modification of original method. Insufficient sensitivity for Cu.	× (Cu) ✓ (Fe)
HPLC – iron(III) tartrate		Colorimetric detection of Fe(III). This method was developed during the project.	✓ (Fe)
Thiocyanate-phenanthroline-GFAAS	7	GFAAS determination of labile Cu(II). Modification of original method. Background contamination too severe for Fe.	✓(Cu) × (Fe)
Colorimetric - bicinchonic acid	8	Colorimetric determination of total Cu and Cu(I). Modification of original method.	✓ (Cu)

[1] Rousseva et al. Food Chem (2016) 203, 440. [2] Misiego et al. J Agric Food Chem (2004) 52, 5316. [3] Clark et al., Talanta (2016) 154, 431. [4] Wiese et al. Fresenius J Anal Chem (1997) 358, 718. [5] Ferreira et al. Anal Chim Acta (2007) 602, 89; Hennessy et al. Can J Chem (1984) 62, 721. [6] Oszwaldowski and Pikus, Talanta (2002) 58, 773. [7] Tasev et al. Food Control (2006) 17, 484. [8] do Nascimento Rocha et al, Spectrochimica Acta Part A (2008) 71, 1414; Brenner and Harris, Analytical Biochemistry (1995) 226, 80; Sekine and Onishi, Analytical Letters 1974, 7, 187; Yamamoto and Motomizu, Analyst 1987, 112, 1011; and Maity et al. Organic Letters 2012, 14, 6008.

6.1.1 Validation of a novel electrochemical technique for the measurement of copper speciation in wine

As part of this project, a novel method was devised for the measurement of copper speciation in wine utilising screen printed carbon electrodes with mercury film in conjunction with medium exchange, constant current stripping potentiometry (see 'Hg film SPCE-medium exchange-CCSP' in Table 2). The technique was validated and published (Clark et al. 2016). Although not suited to a winery environment, this technique had some obvious benefits for studying the measurement of copper speciation in wine that included minimal perturbation of the sample matrix during the measurement, low sensitivity and excellent repeatability. Previously, the most relevant technique published (Clark and Scollary 2006) required combined electrodes that are no longer manufactured and the methodology of the 2006 method was more labour intensive and less accurate for determination of the labile Cu than the one devised as part of this project.

6.2 A survey of 52 wines for their Cu and Fe speciation

The analysis of 52 wines with the techniques outlined in Table 2 (those with 'Trialled in wine survey' = ✓) was conducted, and included 29 red wines and 20 white wines as well as one of each of the following; rosé, sweet and sparkling. The data shown in Figure 1 shows the results corresponding to the 29 red wines and 20 white wines.

During the analysis of wines, it was discovered that two of the colorimetric methods adopted (bicinchonic and HPLC-iron(III) tartrate methods) were unduly impacted by the variability of wine matrices. Although they were sufficiently unaffected during the method assay stage of the project, their application to a larger sample set enabled the identification of substantial matrix interferences. Both methods have the potential to overcome these deficiencies but this was deemed beyond the scope of the current project to develop them further.

6.2.1 The measurement of Cu speciation in 49 wines

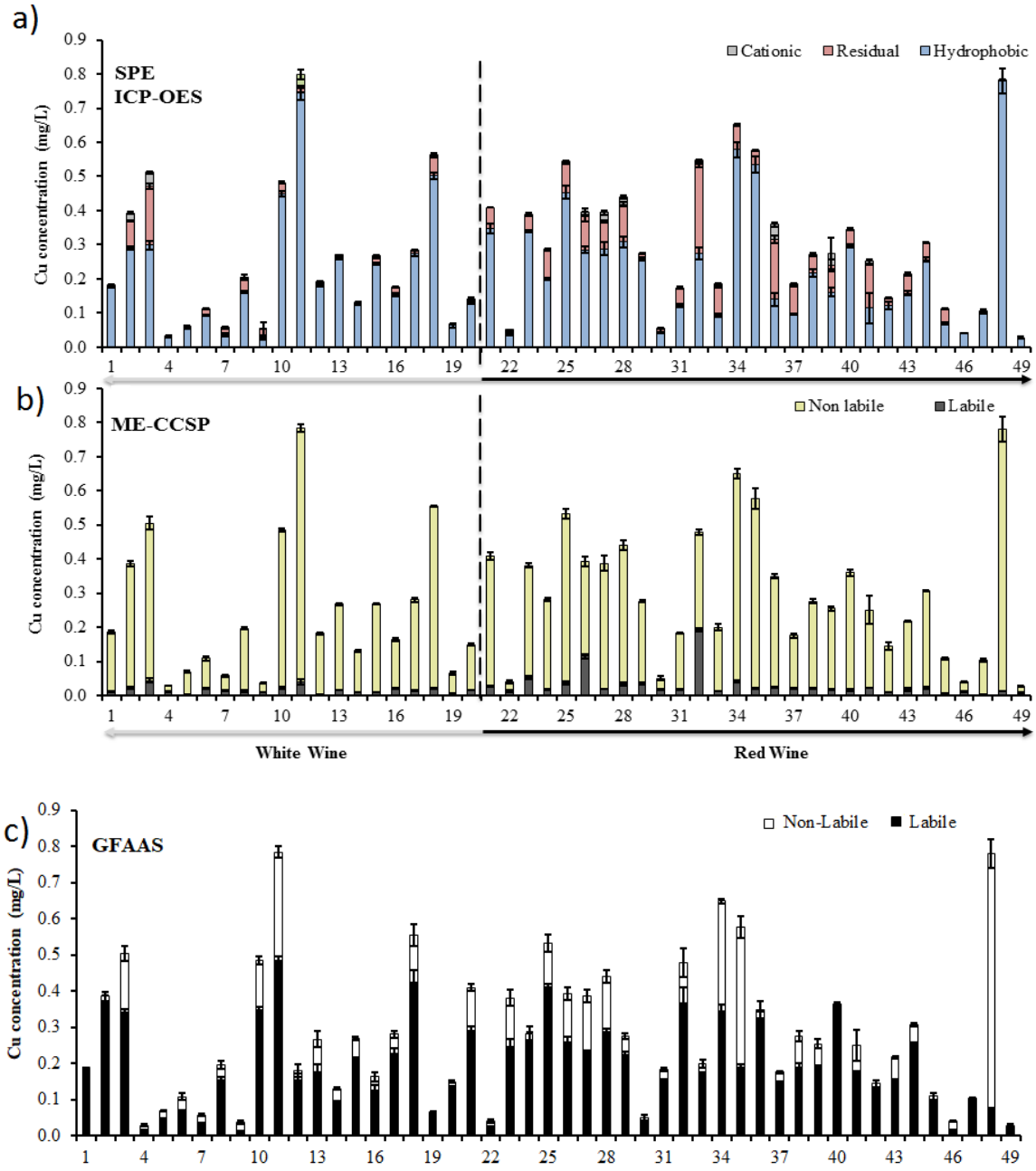
Across the 49 wines, the average total Cu concentration was 0.28 mg/L with a range from 0.028 to 0.78 mg/L. For the white wines, the average total concentration of Cu was 0.24 mg/L with a range from 0.030 to 0.78 mg/L, while for red wines the average was slightly higher at 0.30 mg/L with a range similar to that of white wine (0.028 to 0.78 mg/L).

The electrochemical technique and the SPE ICPOES techniques each suggested that a single form of Cu dominated the wines, which was the hydrophobic form for the SPE ICPOES technique (Figure 1a), and the non-labile form for the electrochemical technique (Figure 1b). In the red wines, the residual form of Cu (i.e., as measured by the SPE ICPOES technique, Figure 1a), was consistently higher than in white wines, and the reason for this will be discussed later (see Section 6.3.1.2). Both the major forms of Cu as measured by the different techniques correlated well with the total concentration of Cu in the wines and with each other (Kontoudakis et al. 2018).

For the thiocyanate-GFAAS method (Figure 1c), the labile copper concentrations were relatively high compared to alternate techniques, suggesting that the method itself cause significant perturbation of the sample especially after the addition of thiocyanate and a high concentration of salt (ammonium sulfate) to the wine (required for the method). This meant that this

technique was more suitable for identifying wines with particularly inert forms of Cu (or those strongly bound), but it also had limitations with accuracy in some instances.

Figure 1. The Cu speciation in 49 white and red wines as determined by a) solid phase extraction with detection by inductively coupled plasma with optical emission spectroscopy (SPE ICPOES), b) medium exchange constant current stripping potentiometry (ME CCSP) and c) liquid extraction with detection by graphite furnace atomic absorption spectroscopy (GFAAS).



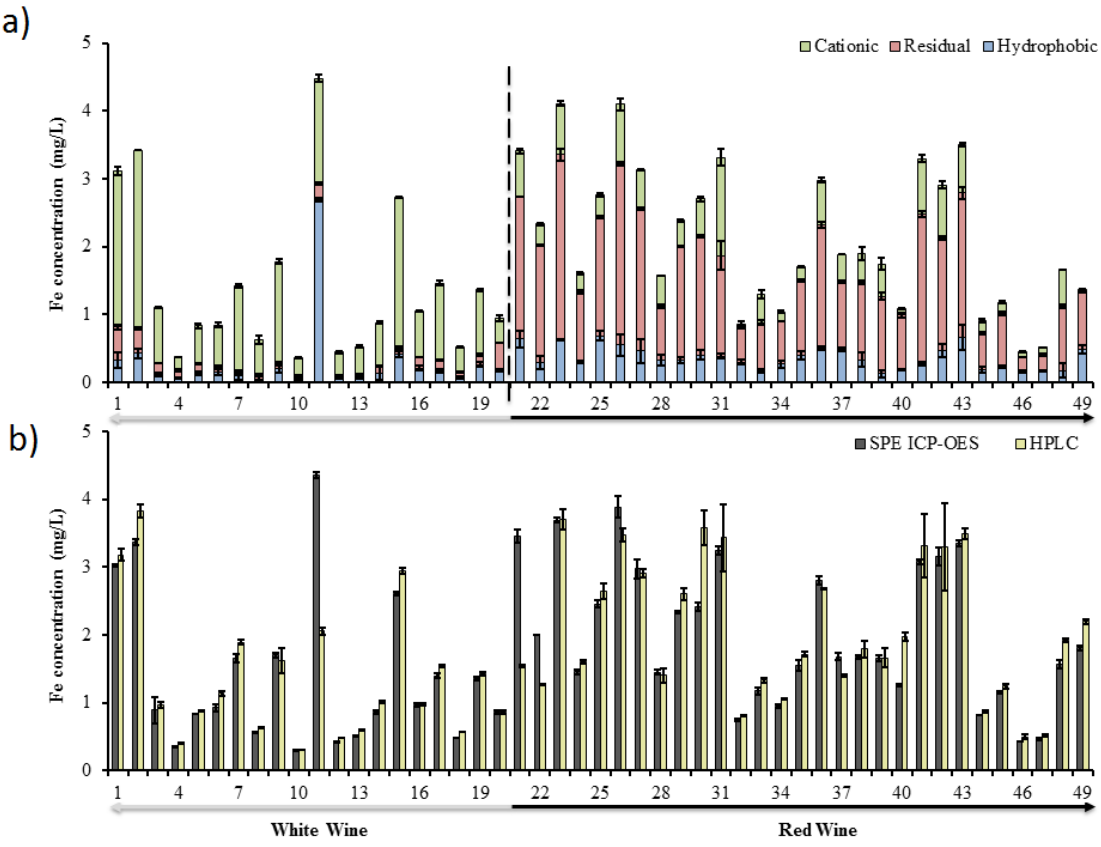
6.2.2 The measurement of Fe speciation in 52 wines

Figure 2a and 2b show the SPE ICPOES and padap-HPLC data for Fe in the 49 wines. The average Fe concentration in the wines was 1.76 mg/L with a range from 0.29 to 3.89 mg/L. The average concentration of Fe in red wine (2.04 mg/L) appeared significantly higher than that in white wine (1.37 mg/L), although the range of concentrations were similar in white and red wines (i.e., 0.29 – 4.36 mg/L and 0.42 – 3.89 mg/L, respectively).

The padap-HPLC technique, compared to direct spectrophotometric measurement of wines samples with padap added, allowed the advantage that both Fe(III) and Fe(II) could be measured simultaneously, and also measured in red wine. However, this technique suffered from significant conversion of Fe(III) to Fe(II) during the measurement, regardless of how soon samples were injected on the HPLC after mixing with the padap reagent (data not shown). Consequently, the technique was only used to provide a measure of total Fe and achieved reasonable agreement compared to the ICPOES total values (Figure 2b).

The SPE ICPOES analysis of the 49 wines showed that Fe was distributed between the three categories of hydrophobic, cationic and residual, more so than found for Cu (Figure 2a). In white wine, the cationic fraction of Fe dominated, while in red wine the residual form was more dominant. The wine components contributing to the different fractions of Fe in the wines are discussed later (see Section 6.3.2).

Figure 2. The a) Fe speciation in 49 white and red wines as determined by solid phase extraction with detection by inductively coupled plasma with optical emission spectroscopy (SPE ICPOES), and b) the total Fe concentration determined by padap-HPLC and ICPOES.



6.3 Identification of wine components contributing to the classification of metal speciation in wine

6.3.1. Wine components contributing to Cu speciation

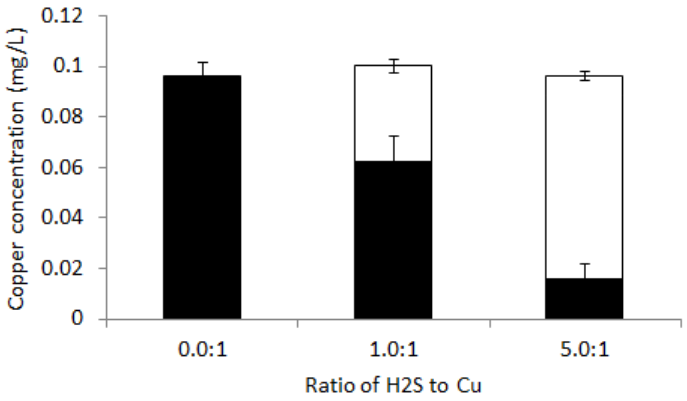
6.3.1.1 Wine components contributing to the electrochemical Cu speciation measure (ME CCSP)

The results from the electrochemical analysis of copper speciation in model wine with various wine components at pH 3.25 and 4.00 are shown in Table 3. From the results, it is evident that only the addition of hydrogen sulfide to the model wine system afforded a significant change in the classification of Cu by the electrochemical technique. For all other model wine systems the predominant form of Cu was labile Cu, identical to the classification of Cu in the model wine without any binding component added, that is, with Cu present as Cu(II) tartrate. However, in the case of hydrogen sulfide, all the Cu was classified as non-labile, regardless of the sample pH. In parallel to this study, research by Kreitman et al. (2016) showed that the complexed formed by Cu(II) and hydrogen sulfide in wine was most likely copper(I) sulfide. Therefore this copper(I) sulfide was responsible for the non-labile Cu classified by the electrochemical system. As further confirmation, Figure 3 shows that a white wine with all its Cu in a labile (i.e., unbound) form allowed conversion of the Cu to a non-labile form with addition of hydrogen sulfide to the wine.

Table 3. The impact of wine components within a model wine system, at pH 4.00 and/or 3.25, on the Cu speciation as determined by ME CCSP.

	Treatment	Total	Labile	Non-labile
pH 4.00	Control	0.39 ± 0.01	0.40 ± 0.01	n.d.
	Red Wine Polyphenol	0.41 ± 0.01	0.42 ± 0.01	n.d.
	Red Wine Tannin	0.45 ± 0.02	0.38 ± 0.01	0.07 ± 0.01
	Epicatechin	0.41 ± 0.01	0.42 ± 0.01	n.d.
	Gallic acid	0.39 ± 0.01	0.41 ± 0.01	n.d.
	Caffeic acid	0.38 ± 0.01	0.43 ± 0.01	n.d.
	Quercetin 3-glucoside	0.39 ± 0.01	0.45 ± 0.01	n.d.
	Ellagic acid	0.43 ± 0.01	0.41 ± 0.01	0.03 ± 0.01
	H_2S	**0.43 ± 0.02**	**0.01 ± 0.01**	**0.42 ± 0.03**
	MeSH	0.39 ± 0.01	0.45 ± 0.01	n.d.
	EtSH	0.40 ± 0.01	0.43 ± 0.01	n.d.
	CS_2	0.42 ± 0.01	0.39 ± 0.01	0.03 ± 0.01
	DMS	0.41 ± 0.01	0.38 ± 0.01	0.03 ± 0.01
	Cysteine	0.39 ± 0.01	0.39 ± 0.01	n.d.
	Glutathione	0.37 ± 0.01	0.45 ± 0.01	n.d.
	2-FMT	0.39 ± 0.01	0.41 ± 0.01	n.d.
	4-MMP	0.30 ± 0.01	0.46 ± 0.01	n.d.
pH 3.25	Control	0.39 ± 0.01	0.43 ± 0.01	n.d.
	White wine protein	0.38 ± 0.01	0.38 ± 0.01	n.d.
	White wine polysacch.	0.39 ± 0.01	0.40 ± 0.01	n.d.
	White wine polyphenol	0.42 ± 0.01	0.39 ± 0.01	0.03 ± 0.01
	Red wine polyphenol	0.41 ± 0.0.1	0.44 ± 0.01	n.d.
	Red wine tannin	0.40 ± 0.01	0.38 ± 0.01	0.02 ± 0.01
	H_2S	**0.40 ± 0.01**	**0.01 ± 0.01**	**0.39 ± 0.01**

Figure 3. Labile copper in a white wine with increasing mole ratio of hydrogen sulfide to copper. The closed bars correspond to labile copper and open to non-labile. The error bars are the st. dev. (n=3).



Such a result demonstrates that the electrochemical technique is relatively specific towards the binding of Cu by hydrogen sulfide based on the wine components assayed in this study. Furthermore, given the results from the wine analysis (Figure 2b) it is likely that the majority of Cu in wine is in the form of Cu(I) sulfide, regardless of the wine being white or red.

6.3.1.2 Wine components contributing to the SPE ICPOES Cu speciation measure

SPE ICPOES was used to study the interaction of Cu in the model wine system (pH 3.25 and 4.00) with the same wine components as listed in Table 3 (Figure 4 and Supplementary Figure 1). The only component that provided a significant change in the measurement of Cu speciation was hydrogen sulfide (Figure 4b and 4c), as was the case for the electrochemical technique. In the presence of hydrogen sulfide the hydrophobic fraction of Cu dominated, whilst for all other components the cationic fraction of Cu was the dominant form. This was consistent with the outcome from the electrochemical study, that the main form of Cu in the wines (Figure 2a) was copper(I) sulfide, which was measured as hydrophobic Cu. The only exception were minor amounts of residual Cu in model wine at pH 4 with high concentrations of citric acid (3.5 g/L), and model wine with excessively high concentrations of oxalic acid (1.6 g/L, Supplementary Figure 1).

However, for red wines it was found that the high phenolic concentration was lowering the efficiency of the divinylbenzene cartridge to trap copper(I) sulfide, the form of Cu identified as being responsible for the hydrophobic fraction. As a consequence, the copper(I) sulfide eluting through the divinylbenzene cartridge was surviving the subsequent cation exchange cartridge and consequently being measured as residual Cu.

Consequently, it appeared that for red wine the SPE ICPOES technique was less specific for copper(I) sulfide than the electrochemical method, as it distributed copper(I) sulfide amongst two Cu fractions (hydrophobic and residual). However, due to no other wine components contributing to either hydrophobic or residual Cu in red wine, it appeared that the combined fractions were still a good indicator of copper(I) sulfide in red wine. The exception may be oxidised red wine, as oxidised wine has shown an increase in the residual fraction of Cu that was attributed to organic acid-Cu complexes (see Section 6.9.2.2).

Figure 4. The concentrations of hydrophobic, residual and cationic forms of Cu in model wine systems at pH 4.00 with a) extracted red wine macromolecules and monomeric phenolic compounds, and b) sulfur-containing compounds, and c) the model wine at pH 3.25 with extracted white and red wine macromolecules and hydrogen sulfide. The error bars are the st.dev. (n=3).

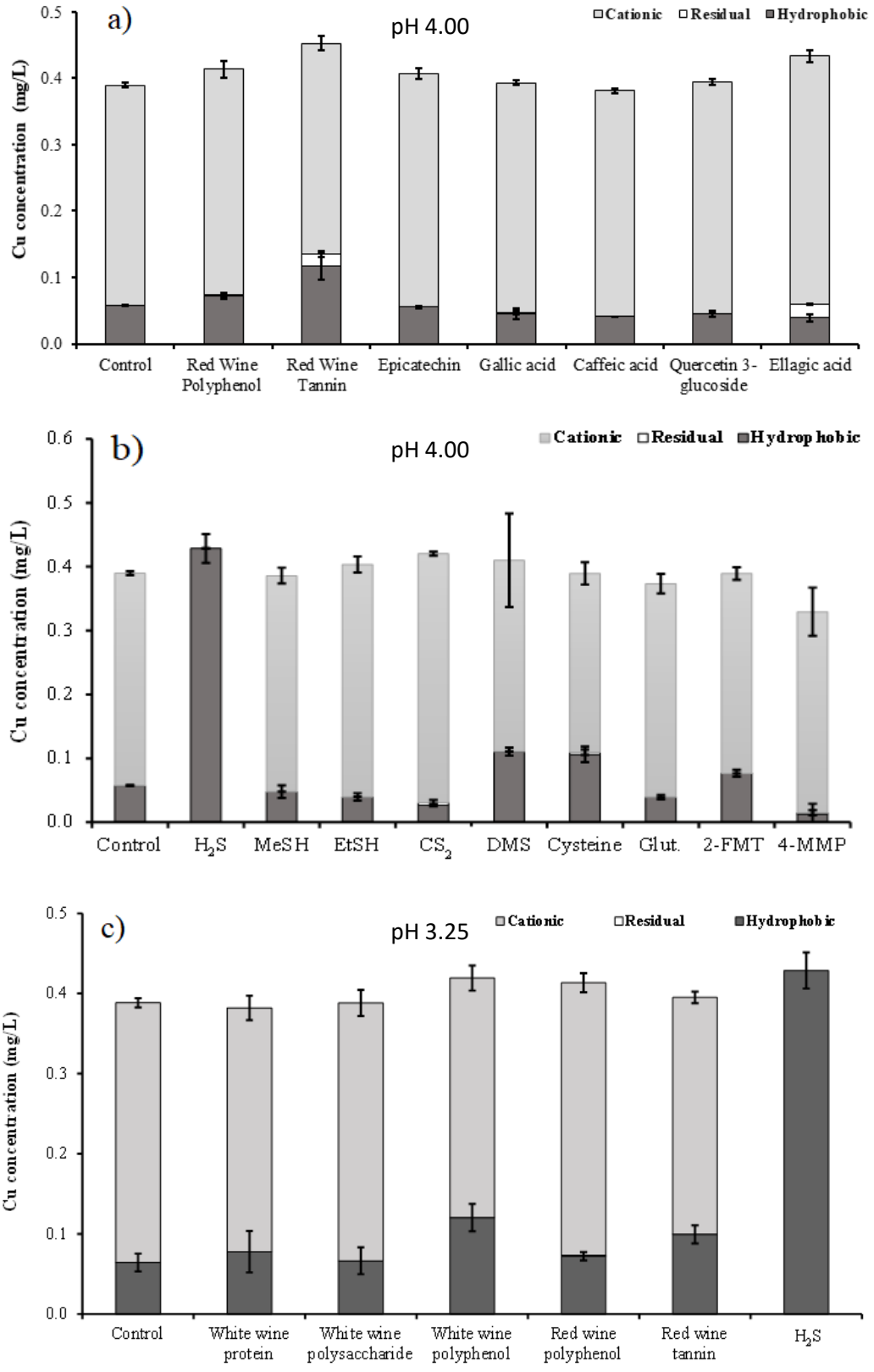


Table 4. White wine correlation coefficient (r) table for compositional parameters versus metal analyses. Positive correlation is shaded in red and negative correlation in blue.

	Cu	Cu Hyd	Cu Res	Cu Cat	LabCu GF	NonLabCu GF	LabCu Elec	NonLabCu Elec	Fe	Fe Hyd	Fe Res	Fe Cat	FePadapHPLC	Mn	Al	Zn	Mg
Free SO_2	-0.1196	-0.1126	-0.0871	-0.1098	-0.0525	-0.2289	-0.1480	-0.1163	0.3864	-0.0325	0.2962	0.5517	0.5565	0.0874	0.3626	-0.2620	0.3033
Total SO_2	0.0633	0.0095	0.2501	0.1034	0.1355	-0.0923	0.1221	0.0592	-0.1483	-0.1880	0.1892	-0.1281	-0.1070	-0.2092	-0.0047	-0.1205	0.1794
pH	0.0750	-0.0066	0.3949	0.1606	0.1163	-0.0224	0.2410	0.0641	0.2228	-0.0158	-0.0833	0.4336	0.3517	-0.0422	0.1311	-0.3599	-0.1481
TA	0.1091	0.1317	-0.1278	0.2196	0.0636	0.1774	0.1362	0.1060	-0.0288	0.1948	0.1849	-0.2605	-0.2158	0.1674	-0.0489	0.5395	0.0898
Ethanol	0.0429	-0.0333	0.3216	0.1505	0.0778	-0.0359	0.1746	0.0345	0.2136	0.0140	0.0617	0.2833	0.2987	-0.3414	0.1160	-0.5069	0.3473
Total H_2S	0.9065	0.9314	0.2010	0.4983	0.8307	0.8726	0.5715	0.9100	0.2650	0.6176	-0.0047	-0.0213	-0.0775	0.4331	0.0062	0.4871	-0.2356
Free H_2S	-0.3813	-0.3456	-0.1929	-0.3287	-0.4155	-0.2374	-0.2173	-0.3838	0.0264	-0.0912	-0.2239	0.1607	0.1253	-0.1481	-0.0223	-0.3247	-0.0015
Bound H_2S	0.9055	0.9259	0.2112	0.5093	0.8361	0.8591	0.5686	0.9090	0.2514	0.6007	0.0171	-0.0360	-0.0864	0.4292	0.0081	0.4981	-0.2256
Total MeSH	-0.3974	-0.3981	-0.0980	-0.2924	-0.4068	-0.2986	-0.2320	-0.3999	-0.0263	-0.1889	-0.1871	0.1034	0.1219	-0.3550	-0.1799	-0.3763	0.1531
Free MeSH	-0.3359	-0.3119	-0.1813	-0.3073	-0.3379	-0.2642	-0.3108	-0.3316	0.0251	-0.1237	-0.1248	0.1326	0.1508	-0.2903	-0.1413	-0.3526	0.1521
Total DMS	0.0065	-0.0540	0.3104	0.0555	0.0307	-0.0421	0.1671	-0.0025	0.0418	-0.1383	-0.1404	0.2588	0.1373	0.1165	0.0877	-0.1565	-0.1821
Total CS_2	0.0273	0.0387	-0.0506	-0.0742	0.0490	-0.0223	-0.1922	0.0389	0.2864	0.0582	-0.0832	0.4328	0.4119	0.2669	0.1657	-0.1057	-0.0054
Citric acid	0.2140	0.2406	-0.0601	0.3657	0.1072	0.3822	0.3172	0.2046	0.4854	0.5955	0.1641	0.2589	0.2025	0.3146	0.2496	0.2044	0.0577
Glucose	-0.1812	-0.1363	-0.2077	-0.1727	-0.2084	-0.0890	-0.2234	-0.1754	-0.3287	-0.1545	-0.2661	-0.3187	-0.3527	0.2585	-0.2264	0.4815	-0.4093
Fructose	-0.2174	-0.1739	-0.2256	-0.1913	-0.2465	-0.1143	-0.2340	-0.2125	-0.3715	-0.1756	-0.2408	-0.3864	-0.3966	0.1559	-0.2715	0.3992	-0.3449
Succinic acid	-0.1038	-0.1357	0.0718	0.0287	-0.0515	-0.1872	-0.0375	-0.1063	0.3528	0.0475	0.0921	0.4289	0.5078	-0.2855	0.1079	-0.4862	0.4011
Lactic acid	-0.1572	-0.1592	-0.0439	-0.1188	-0.1426	-0.1551	-0.2077	-0.1518	0.1572	-0.0334	-0.1546	0.3138	0.2962	-0.0234	-0.0553	-0.2769	0.0478
Glycerol	-0.0888	-0.1438	0.1462	0.1085	-0.0164	-0.2155	0.0173	-0.0935	0.3612	0.0329	0.2493	0.4576	0.5194	-0.3727	0.0873	-0.5014	0.4620
Acetic acid	0.0113	0.0998	-0.3497	-0.1543	-0.0355	0.1005	-0.0605	0.0155	-0.0839	0.1036	-0.1047	-0.1582	-0.1587	0.0434	-0.5543	-0.0190	-0.0632
Tartaric acid	-0.2604	-0.1977	-0.4245	-0.1580	-0.3159	-0.0975	-0.2574	-0.2560	-0.2846	-0.0430	0.0265	-0.5041	-0.3728	-0.1061	-0.2632	0.3243	0.1982
Malic acid	0.2193	0.1999	0.1523	0.2445	0.1872	0.2392	0.3835	0.2064	-0.0428	0.1111	0.1619	-0.1439	-0.1942	0.1496	0.1094	0.3685	-0.2535

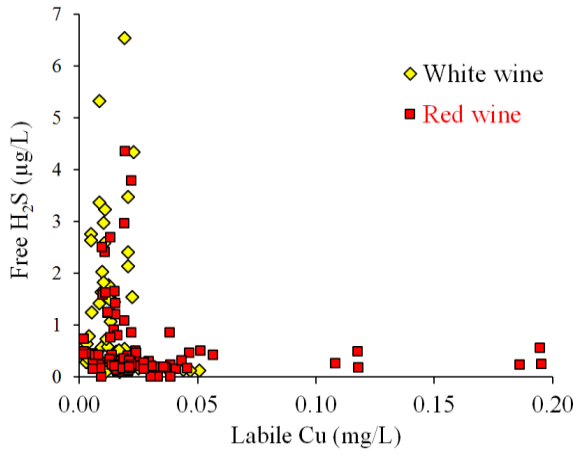
6.3.1.3 Comparison of wine compositional measures to Cu speciation measures

A range of wine compositional parameters were compared to the various metal speciation/fractionation measures (Table 4, Supplementary Table 2). Of note was the high positive correlation (r = 0.91 to 0.93) in white wine for total and bound hydrogen sulfide with total Cu, hydrophobic Cu (SPE ICPOES) or non-labile Cu (ME CCSP), and to a lesser extent with the non-labile GFAAS Cu (Table 4). However, the equivalent correlations for red wine were poor (r = -0.05 to 0.26), due to lower total hydrogen sulfide concentrations being measured for a given amount of total, hydrophobic (SPE ICPOES) or non-labile Cu (ME CCSP) than was the case for white wine (Supplementary Table 2B). Even if the hydrophobic and residual forms of Cu were summed in red wine the correlation with bound hydrogen sulfide was still poor (i.e., 0.29). Consequently, the data for combined white and red wines also had relatively weak correlations (r = 0.17 – 0.52) for total Cu, hydrophobic Cu or non-labile Cu (ME CCSP) with total or bound hydrogen sulfide (Supplementary Table 2A). This implied that either some other component of red wine was responsible for the binding of a portion of Cu, or that some copper(I) sulfide existed in a form that could not be easily released by either salting and/or dilution with brine (the method employed for measurement of bound hydrogen sulfide by GC, which was adapted from Franco-Luesma and Ferriera (2014)). Further discussion is made on the ratio of bound Cu to bound hydrogen sulfide for red and white wines in Section 6.10.

The relationship between labile Cu (ME CCSP) and free hydrogen sulfide shows that only with labile Cu concentrations below 0.02 mg/L does free hydrogen sulfide concentration in the wines accumulate above the typical aroma threshold (Figure 5). This is consistent with low solubility constant for Cu(II) in the presence of sulfide (Clark et al. 2015), and provides a threshold measure of labile Cu for which free hydrogen sulfide would then be below the aroma threshold for most consumers of wine.

No other wine compositional measure showed strong correlations with the metal speciation measures, including correlation with other metals such as Al, Zn, Mn and Mg (Table 4, Supplementary Table 2).

Figure 5. The free hydrogen sulfide versus labile Cu (ME CCSP) in red and white wines.



6.3.2 Wine components contribution to Fe speciation

The wine pH, in combination with organic acids and phenolic compounds, had a significant influence on Fe fractionation by SPE ICPOES (Figure 6 and Supplementary Figure 2). At pH 4.00, certain phenolic compounds and, to a lesser extent, organic acids induced higher concentrations of residual Fe, while at lower pH values of 3.25 the cationic fraction of Fe dominated. The exception was for citric acid which was able to maintain Fe in the residual fraction despite low pH, and this is consistent with the document efficient binding of Fe by citric acid in wine solution (Timberlake 1964). The redox form of Fe, as either Fe(II) or Fe(III), did not influence the fractionation of Fe by SPE ICPOES (Figure 6), however, other studies showed that some discrimination was possible at higher concentrations of Fe that were less relevant to the concentrations found in wine (i.e., 10 mg/L vs 1 mg/L, (Kontoudakis, Guo, et al. 2017)). Sulfur compounds, including hydrogen sulfide, methanethiol, cysteine and glutathione (Supplementary Figure 1), did not impact the speciation of Fe via SPE ICPOES. One final observation was the ability of succinic acid to induce elevated concentrations of hydrophobic Fe in wine, particularly at higher pH values (Figure 6a, see the samples labelled ‘S’). Of all the wine components tested this was the only to elevate the hydrophobic fraction of Fe to any significant extent.

These results are largely consistent with the fractionation of Fe in the 49 wines by SPE ICPOES (Figure 2a). Red wines, with higher pH and phenolic concentration than white wines, showed higher levels of residual Fe, whilst the white wines, with lower pH, were dominated by cationic Fe. The wine compositional measures showed poor correlations ($-0.67 < r < 0.65$) with total Fe and its speciation measures (Table 4, Supplementary Table 2), demonstrating that no one component of wine dominated the classification of Fe in the different speciation forms.

Figure 6. The impact of a) pH (3.25 vs 4.00) and organic compounds, and b) phenolic compounds in model wine solutions on the speciation of 1.0 mg/L Fe as determined by SPE ICPOES. The organic acids utilised were tartaric acid (T), citric acid (C), malic acid (M), lactic acid (L), succinic acid (S) and oxalic acid (O). The phenolic compounds utilised were (-)-epicatechin (E), (+)-catechin (C), and gallic acid (G). Where indicated, Fe was added to solutions as either Fe(II) or Fe(III).

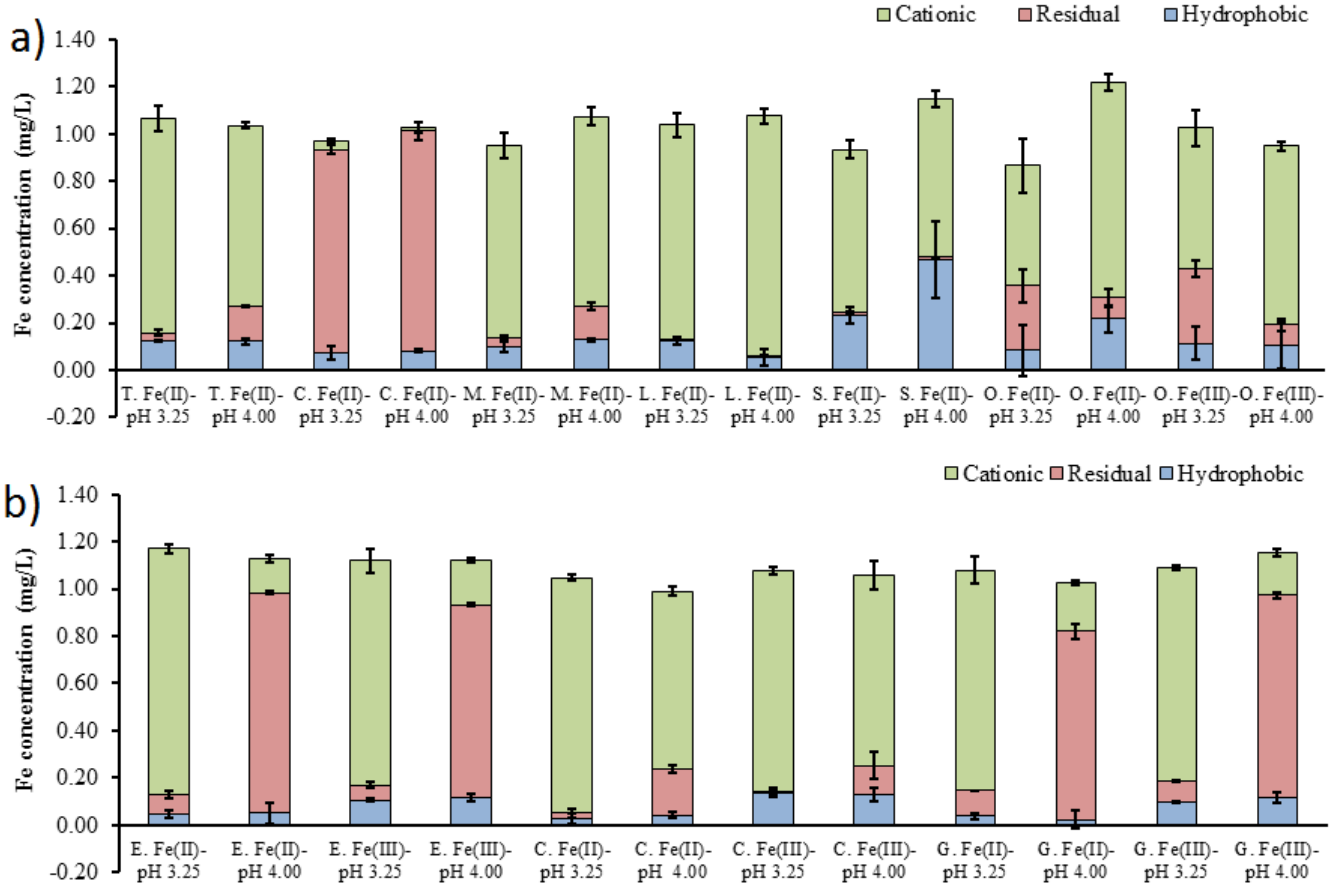


Table 5. The concentration of residual copper (mg/L) in wines W1 to W4 and model wines (MW) with indicated mole ratios of sulfide to added copper(II).

Sample	Type of clarify-cation†	Time of clarifi-cation‡	Samples prepared with mole ratio of sulfide : copper(II)				
			0:1	0.5:1	1:1	2.5:1§	5:1§
W1 (n=3)	R	1 day	1.07±0.02	1.062±0.005	1.047±0.005	1.044±0.005	0.93±0.01
	F(0.45)		1.02±0.01	1.029±0.005	1.002±0.005	0.975±0.005	0.95±0.01
	F(0.20)						0.92±0.02
W2 (n=3)	R	1 day	1.10±0.05	1.109±0.005	1.02±0.02	1.02±0.03	0.98±0.01
	F(0.45)		1.06±0.03	1.067±0.008	0.97±0.02	0.91±0.01	0.88±0.03
	F(0.20)						0.82±0.05
W3 (n=4)	R	1 day	1.04±0.05	1.1±0.1	0.98±0.04	0.95±0.04	0.99±0.04
	F(0.45)		0.99±0.03	1.01±0.02	0.9±0.1	0.87±0.01	0.91±0.02
	F(0.20)						0.87±0.02
W4 (n=4)	R	5 days	1.04±0.03	1.03±0.03	1.01±0.03	1.02±0.01	1.02±0.052
	F(0.45)		1.05±0.01	1.04±0.03	1.05±0.03	1.04±0.02	1.04±0.03
W1+ Kupzit® (n=3)	R	1 day	1.090±0.005	1.064±0.005	0.95±0.04	0.703±0.005	0.681±0.009
	F(0.45)		1.069±0.001	1.023±0.009	0.90±0.04	0.46±0.01	0.45±0.01
	F(0.20)						0.43±0.03
MW (n=4)	R	1 day	0.97±0.02	1.009±0.009	0.93±0.02	0.87±0.04	0.89±0.01
	F(0.45)		0.97±0.02	0.80±0.02	0.43±0.01	0.14±0.03	0.17±0.01
	F(0.20)						0.013±0.005

All uncertainty values are given as the standard deviation.

† Clarification type: R = racking, F(0.45µm) = filtration with pore size 0.45 µm, and F(0.20µm) = filtration with pore size 0.20 µm.

‡ Time after copper (II) sulfate addition before clarification was undertaken.

§ Hydrogen sulfide was detected in all 2.5:1 and 5:1 samples (by sensory assessment).

6.4 Impact of juice metal concentrations on speciation of metals in wine

The concentration of Cu and Fe in juice can be in the order of mg/L concentrations due to the use of various metal-containing sprays in the vineyard and due to general contamination. The impact of the juice metal concentration on the final wine metal speciation was not certain and is the basis of investigations reported in this section of the report.

Chardonnay juice was adjusted to the concentrations of Cu and Fe shown in Table 6, whereby Cu or Fe were at increasing concentration, and a sample also existed with high concentrations of both Cu and Fe. After bottling the wine, without protein stabilisation (i.e., no bentonite treatment), it was evident that most of the Cu had been removed during fermentation by yeast activity, but generally the wines made from juice with high Cu concentrations had higher concentrations of Cu in the wine than if made from juice with low Cu concentrations. For iron, the wine production process had minimal impact on its concentration. The subsequent sections describe the impact of the juice metal concentration on the corresponding metal speciation, as measured by SPE ICPOES, in the corresponding wines. As this experiment was conducted at the beginning of the overall metal speciation project, the SPE ICPOES protocol utilised C18 cartridges rather than divinylbenzene cartridges to classify 'hydrophobic' Cu. However, later work showed minimal differences in measured hydrophobic Cu when using the different cartridges for this wine (data not shown).

Chardonnay wines prepared from juice at different metal concentrations.



Table 6. The Cu and Fe concentrations in the juice and corresponding wine samples.

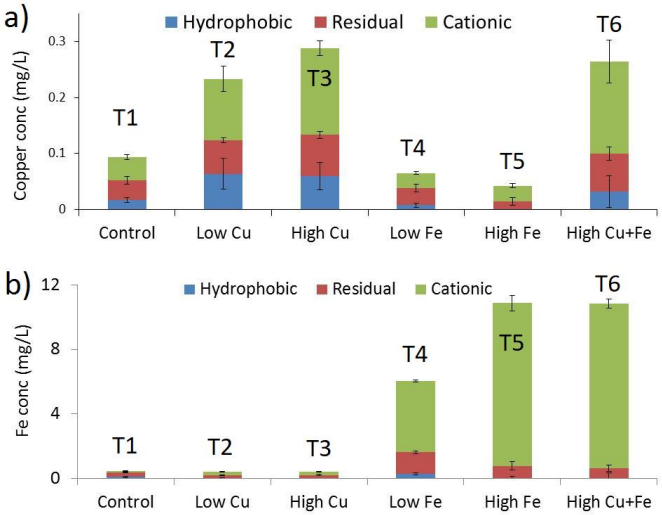
	juice Cu total (mg/L)	juice Fe total (mg/L)	wine Cu total (mg/L)	wine Fe total (mg/L)
T1	0.77±0.03^{a,b}	0.37±0.01^{a}	0.083±0.008^{a}	0.423±0.006^{a}
T2	3.61±0.09^{c}	0.38±0.02^{a}	0.233±0.005^{c}	0.425±0.009^{a}
T3	6.35±0.03^{d}	0.31±0.02^{a}	0.293±0.006^{d}	0.421±0.006^{a}
T4	0.69±0.02^{a}	6.0±0.1^{b}	0.068±0.014^{ab}	6.20±0.05^{b}
T5	0.80±0.03^{b}	11.1±0.1^{c}	0.052±0.011^{b,e}	11.7±0.1^{c}
T6	6.52±0.06^{d}	11.3±0.1^{c}	0.288±0.007^{d}	11.8±0.1^{c}

The uncertainty indicated is the standard deviation. Concentrations of a given metal with the same superscript letters in a given column are not significantly different at p=0.05.

Figure 7a shows the concentration of hydrophobic, residual and cationic fractionated forms of Cu and Fe. As a reminder, the hydrophobic metal is that metal that is trapped by the divinylbenzene SPE cartridge as the wine is passed through it, the cationic fraction is metal eluting through the first SPE cartridge but being trapped on a cation exchange SPE cartridge. Finally, the residual fraction is metal that passes through both SPE cartridges. In terms of specific fractions, the increase of Cu alone in the juice led to significantly higher concentrations of Cu in all fractions, with the largest magnitude of increase in the cationic fraction. It is of interest that the hydrophobic form of Cu, attributed to copper(I) sulfide, does not dominate the form of Cu in these wines. This suggests that much of the Cu that survived fermentation in this wine was either not in the form of copper(I) sulfide, or otherwise evolved to the other forms after fermentation and prior to the speciation measure. Indeed, the production of wine in the 20 L volumes utilised for this experiment is more prone to oxygen uptake that would favour conversion of the hydrophobic form of Cu to the other forms.

Figure 7b shows that the increase in Fe alone in the juice led to a major increase in the cationic content of Fe in the wine, to the extent that over 95 % of the Fe in the wine was in this form for high Fe treatments. Although some increase in the other fractions were observed, they were only minor in comparison to that of the cationic fraction. This preference for a specific metal fraction is in clear contrast to the results observed for Cu additions.

Figure 7. The fraction of hydrophobic, residual and cationic forms of a) Cu and b) Fe in wines T1-T6 from Table 6.

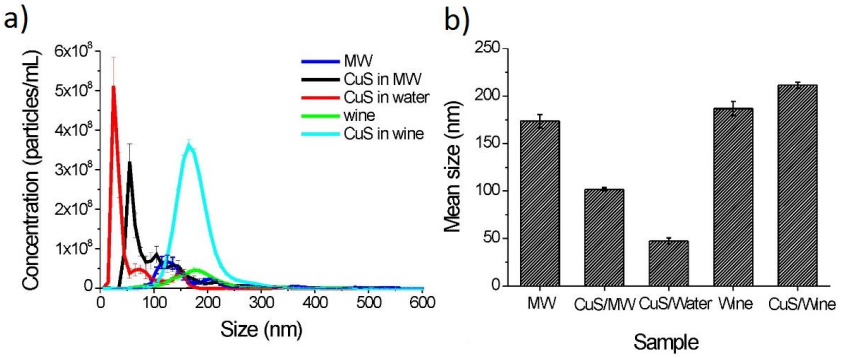


6.5 Copper(I) sulfide removal from wine via filtration or bentonite treatment

6.5.1 Removal of copper(I) sulfide by filtration

Preliminary studies (Table 5), published prior to the commencement of this project, showed that copper(I) sulfide could be significantly reduced in concentration by the membrane filtration of model wine solutions (12 % aqueous ethanol, buffered to pH 3.25 with tartaric acid) but the same was not the case in white wines (Clark et al., 2015). A cellulose-based membrane filter was utilised that is of composition more similar to pad filters used within wineries (Bowyer et al. 2013). A similar result was observed for a further ten white wines (Supplementary Table 1).

Figure 8. The a) particle size measurements for copper(I) sulfide prepared by addition of 4.8 mg/L hydrogen sulfide and 2.0 mg/L copper(II) to a Viognier wine, model wine or water (all measured 10 minutes after sample preparation), and the b) mean sizes of samples mentioned above.



Originally it was suspected that the copper(I) sulfide particles forming in the model wine solutions were larger than the particles formed in white wine, and this was impacting the comparative filterability of copper(I) sulfide in the different solutions. Complexing agents for Cu are well known to influence the size of copper(I) sulfide particles that form in aqueous solutions (Ku and Peters 1988). However, particle size measurement (Figures 8a and b) showed the opposite result to expectations, that the copper(I) sulfide particles were larger in white wine than in the model wine (i.e., ~90 vs 200 nm). Consequently, particle size was not responsible for the different filterability observed for copper(I) sulfide in white wine compared to model wine (Table 5). Instead it appeared more likely that copper(I) sulfide was being removed from model wine by adsorption onto the cellulose-based filtration membrane rather than by size retention, and some component of white wine was preventing the adsorption.

A survey was conducted to assess those components of white wine that were impacting the filterability of copper(I) sulfide on the regenerated cellulose filtration membrane. Initially components found in white wine in g/L concentration ranges were surveyed, apart from those already present in the model wine system. The main compounds that inhibit and/or promote wine oxidation were also surveyed, including sulfur dioxide, ascorbic acid, iron(II) and caffeic acid as a model phenolic compound. In all cases, the filtration of the model wine with an added component, each containing copper(I) sulfide (at 1.0 mg/L copper), resulted in less than 10 % of the copper(I) sulfide passing through the filter (data not shown) despite the presence of the individual wine components.

Consequently, it was decided to assess white wine polysaccharide and protein macromolecules, previously extracted from white wine (Kontoudakis, Smith, et al. 2017), as well as a generalized group of white wine phenolic compounds. The addition of white wine protein or polysaccharide to the model wine with copper(I) sulfide (equivalent to 1.0 mg/L copper) allowed essentially 100 % of the copper(I) sulfide to pass through the filter, compared to 4 ± 1 % in the absence of either macromolecule confirming that these two macromolecules were able to prevent the adsorption of copper(I) sulfide on the regenerated cellulose filtration media. The mechanism is most likely due to the macromolecule preferentially adsorbing onto the filtration media in place of copper(I) sulfide and/or the copper(I) sulfide interacting with the macromolecule preferentially to the filtration media. Polysaccharides and proteins are well known to adsorb onto filtration media (Susanto and Ulbricht 2007, Ulbricht et al. 2009) as well as interacting with copper(I) sulfide particles.

The white wine polyphenol compounds allowed 31 ± 5 % of the copper(I) sulfide to pass through the regenerated cellulose membrane filter. Consequently, it was able to prevent some adsorption of copper(I) sulfide onto the filtration material but not as efficiently as the macromolecules surveyed.

A variety of different membrane types (Figure 9) were assessed for their activity in adsorbing copper(I) sulfide from white wine, the model wine, and the model wine with added white wine polysaccharides. Nylon and polyethersulfone (PES) membranes are more consistent with media used for membrane filters in wineries, cellulose is more relevant to pad filters whilst Teflon (PTFE) is not routinely used in wineries but was assessed out of interest (Bowyer et al. 2013). All membrane filters were used at a pore size of 0.45 µm, typical of that used by wineries for sterile filtration of wine, but also a pore size of 0.20 µm was used for comparison. Finally a glass fibre filter was also used with a pore size of 1.2 µm. The results (Figure 10) showed that the regenerated cellulose, nylon and PES membranes could all remove the copper(I) sulfide from the model wine (see black bars, Figure 10), regardless of the pore size utilised (i.e., 0.2 or 0.45 µm) (Figure 10a and 10b) and the concentrations of copper(II) utilised to prepare the copper(I) sulfide (1.0 or 0.3 mg/L). However, the PTFE (0.2 and 0.45 µm) and the glass fibre (1.2 µm) membrane filters had minimal impact on the model wine solutions of copper(I) sulfide prepared at high copper concentration. Both had increased activity for the sample with less added copper(II), with the PTFE and glass fibre membranes retaining 70-90 % (depending on pore size) and 30 %, respectively, of copper(I) sulfide.

On addition of the white wine polysaccharide to the model wine system (see red bars, Figure 10), the regenerated cellulose membrane could not retain any copper(I) sulfide regardless of the pore size or copper(I) sulfide concentration. PES retained 30-40 % of the copper(I) sulfide, while Nylon retained the most (~70 %), again largely independent of pore size or copper(I) sulfide concentration. On repeating the experiment in a white wine (blue and yellow bars, Figure 10), the results were similar to the model wine with polysaccharide added (see red bars). A difference between the performances of the filtration media in the two solution matrices was the ability of the PES membrane to remove more copper(I) sulfide in the white wine compared to the model wine with added polysaccharide.

Figure 9. The different membrane filter media used to investigate removal of copper(I) sulfide from white wine and model wine solutions.

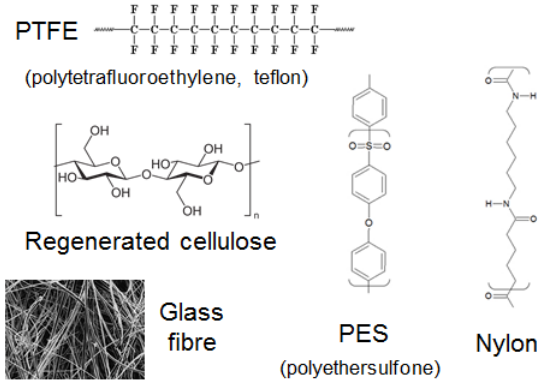
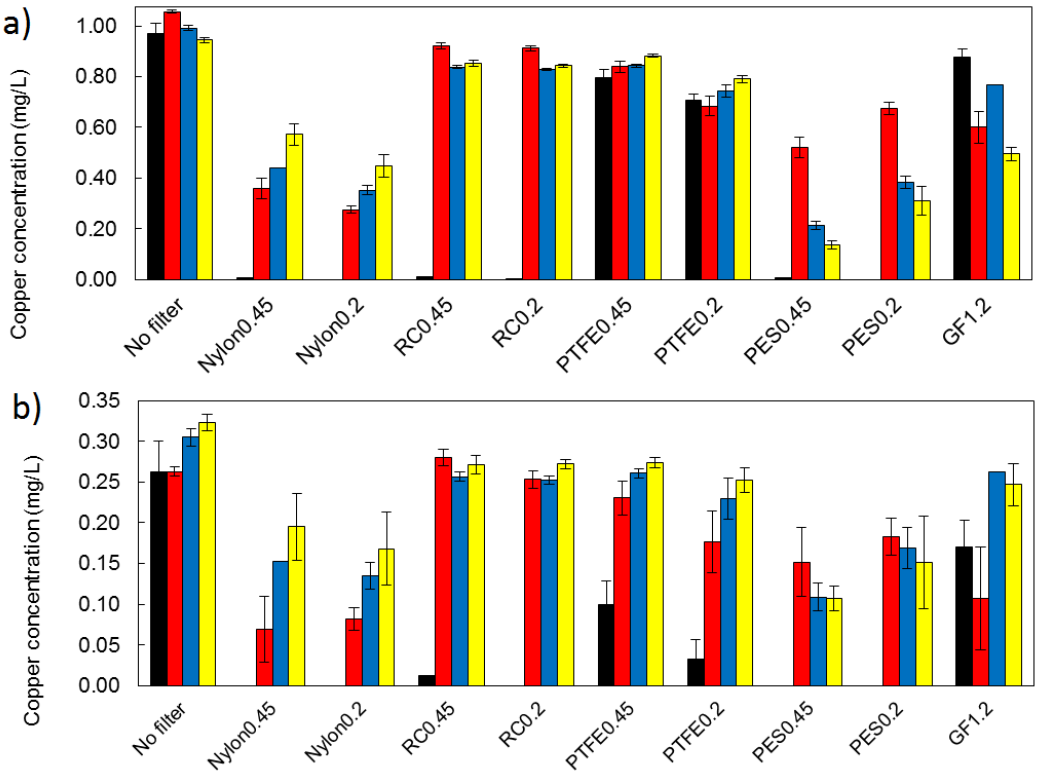


Figure 10. The impact of membrane type on the amount of copper(I) sulfide eluting through the filter at a) high (1.0 mg/L copper) and b) low (0.3 mg/L copper) concentrations of copper(I) sulfide. The copper sulfide was prepared at hydrogen sulfide to copper(II) ratios of 2:1 in the model wine (■), model wine with added polysaccharide (■) and white wine (■), as well as at 5:1 in the white wine (■).



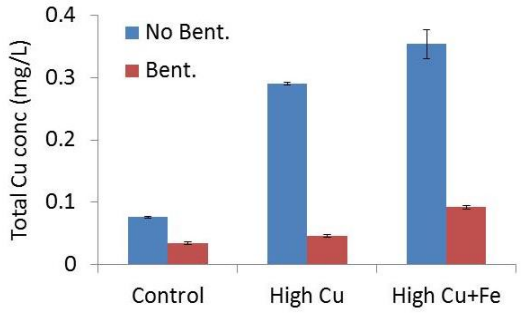
These results show that removal of copper(I) sulfide by adsorption onto membrane filtration media is hindered by white wine protein and polysaccharide macromolecules. This macromolecule interference in copper(I) sulfide removal was most apparent for regenerated cellulose media, whilst nylon and PES were able to still remove significant amounts of copper(I) sulfide. PTFE on the other hand had minimal removal of copper(I) sulfide under all conditions assayed, apart from lower metal concentrations in the model wine. It was also noted that the back pressure encountered when using the filters, based on the physical force required to depress the syringe plunger, was in the order of PTFE >> nylon > regenerated cellulose > PES > glass fibre. This was for a given pore size (0.45 or 0.2 µm), apart from the glass fibre filter which was only utilised with a higher pore size (1.2 µm). Therefore, based on this relative back pressure and efficiency to remove copper(I) sulfide in the presence of macromolecules, PES would appear to have advantages over the other membrane media.

6.5.2 Copper and iron concentrations after bentonite treatment of white wine containing grape-derived protein

Three Chardonnay white wines were produced from juice with concentrations of Cu of 0.8, 6.4 and 6.5 mg/L, as described in Section 6.4. The corresponding white wines had copper concentrations of 0.076 ± 0.001, 0.290 ± 0.002 and 0.35 ± 0.02 mg/L. The concentration of iron

in the juices was also altered, to achieve concentrations of 0.37 ± 0.01 mg/L, 0.31 ± 0.02 and 11.3 ± 0.1 mg/L such that the final wine had concentrations of 0.400 ± 0.001 mg/L, 0.41 ± 0.02 and 12.3 ± 0.1 mg/L, respectively. In contrast to Cu, it is evident that yeast are not able to remove Fe from the wine during fermentation.

Figure 11. The concentration in three Chardonnay wines, still containing grape-derived protein, before and after treatment with 1.6 g/L of a calcium bentonite.



A heat stability test on the wines showed that 1.6 g/L of a calcium bentonite was required to achieve protein stability. Upon treatment of the wines with this amount of calcium bentonite and then racking, a substantial decrease in the copper concentration was evident (Figure 11). This corresponded to a decrease in copper concentration of 55, 84 and 74 % for the wines. The decrease in Cu upon the bentonite fining of wine has been reported previously (Catarino et al. 2008). The efficiency of removal of Cu is most likely dependent on the simultaneous presence of grape-derived protein in the wine along with copper in the form of copper(I) sulfide but further work is required for confirmation. Traditional methods for the removal of suspended copper(I) sulfide in wine proposed treatment with protein (i.e., isinglass or bull's blood) (Ribéreau-Gayon 1935) to flocculate the colloidal copper sulfide, but this was typically at Cu concentrations much higher than 1 mg/L.

In contrast to the Cu, the concentrations of Fe in the wines increased by 247, 278 and 3 %, respectively, to provide concentrations of 1.39 ± 0.02 mg/L, 1.55 ± 0.02 and 12.7 ± 0.2 mg/L, respectively. This increase in Fe concentrations due to bentonite treatment has also been reported previously (Catarino et al. 2008) and was attributed to the release of exchangeable Fe from the bentonite.

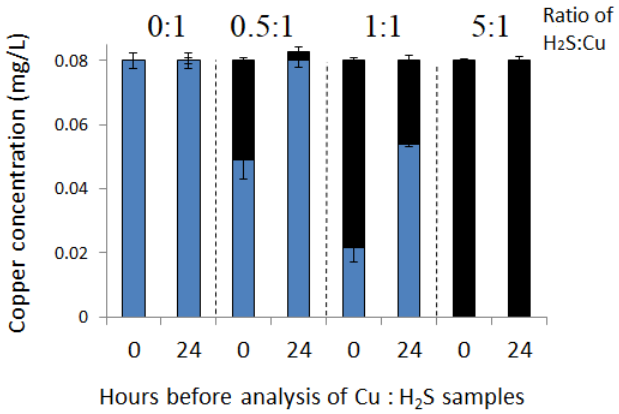
6.6 Conversion of Cu species between different forms

Given the understanding of the different Cu forms present in wine with respect to the speciation measures adopted, it was of interest to assess how easily the metals could undergo conversion between the different forms. For example, the ability of wine storage and/or oxygen availability to alter the metal speciation.

Figure 3 already showed the ability of Cu in white wine to be transformed from electrochemically labile Cu into non-labile Cu, that is, non-sulfide bound Cu into copper(I) sulfide with the addition of hydrogen sulfide. However, it was of interest to also assess whether copper(I) sulfide could be converted back to the loosely bound Cu form (hereon referred to as 'labile Cu'). Figure 12 shows that immediately after addition of hydrogen sulfide to a model wine (see '0 hours' in Figure 12), progressively less labile Cu is present as more hydrogen sulfide

is added. However, when the samples were re-analysed after storage in open containers for 24 hours at room temperature, the labile Cu increased in concentration for all samples apart from that at the highest hydrogen sulfide concentration. This suggested that with oxidative and/or volatile losses of hydrogen sulfide, the copper(I) sulfide (non-labile Cu) is gradually converted back to copper(II) (labile Cu).

Figure 12. Labile copper in a model wine with increasing mole ratio of hydrogen sulfide to copper. Analysis was conducted at 0 and 24 h after mixing of sodium sulfide and copper(II). The **blue bars** correspond to labile copper, and **black** to non-labile. The error bars are the st. dev. (n=3).



The experiment was repeated in a model wine containing a typical white wine phenolic compound (i.e., caffeic acid) in the absence or presence of ascorbic acid. In this case, the presence of copper(I) sulfide in the model wine was determined by membrane filtering the model wine with regenerated cellulose media at various time points and attributing any Cu removed by filtration as being copper(I) sulfide. As described earlier (Section 6.4.1), copper(I) sulfide in the model wine system is effectively removed by filtration with regenerated cellulose membrane filters. The model wines were all stored in conditions of excess oxygen, that is, dissolved oxygen concentrations of around 7 mg/L and a large air head-space in containers at 25 °C. The results (Figure 13) showed that in the presence of caffeic acid, at white wine-like total phenolic concentrations (0.30 g/L), the labile Cu gradually increased with storage time, as copper(I) sulfide was converted to loosely bound Cu(II), however, in the presence of ascorbic acid (0.20 g/L) this was not the case. Instead, the Cu remained in the form of copper(I) sulfide for the entirety of the experiment. This demonstrated that white wine composition is important for the stability of copper(I) sulfide. Oxidising conditions in the presence of phenolic compounds was conducive to the dissociation of copper(I) sulfide, whilst the same oxidising conditions in the presence of ascorbic acid did not impact the copper(I) sulfide. This was most likely a consequence of phenolic oxidation products binding sulfide, thereby gradually releasing Cu from copper(I) sulfide, while the oxidation products of ascorbic acid not binding sulfide.

Figure 13. Labile Cu in a model wine containing 0.30 g/L caffeic acid either without (Caff) or with (Caff/Asc) 0.20 g/L ascorbic acid. The model wine consisted of 12 % aqueous ethanol buffered to pH 3.25 with 5.0 mg/L Fe(II) and 7mg/L dissolved oxygen. Samples were stored in the darkness at 25 °C. Labile Cu was determined as the Cu in the model wine that could not be removed by filtration with a 0.20 µm regenerated cellulose membrane filter.

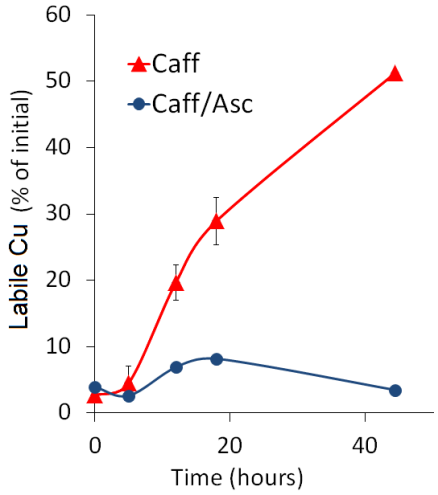
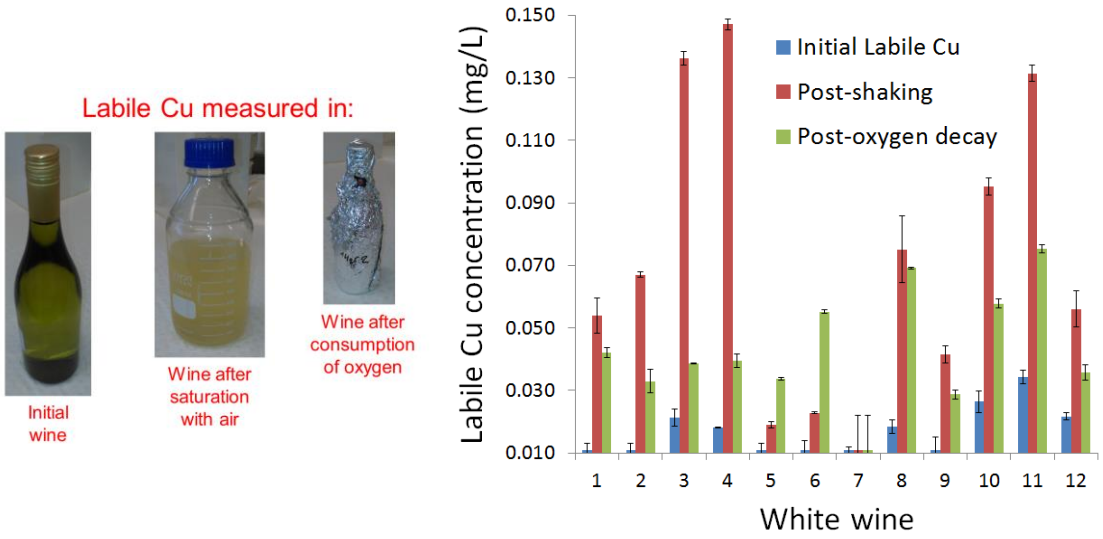


Figure 14. The labile Cu in 12 white wines immediately after opening their bottle (blue bars), after aggressive agitation of 500 mL with an air head-space of 500 mL (red bars), and after agitation with air, when all the dissolved oxygen in the wines had been consumed (green bars).



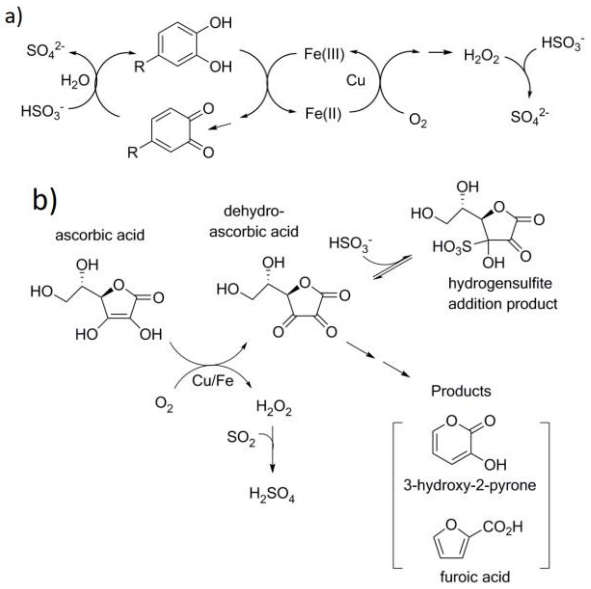
The copper speciation in wine could also be impacted by aeration of the wine and then allowing it to return to conditions of low oxygen. Figure 14 shows the original labile Cu concentration in 12 different wines (see blue bars, Figure 14). When the wines were aggressively agitated with an air head space, to saturate the wines with oxygen, the labile Cu increased dramatically (see red bars, Figure 14). Upon leaving the wines to consume the dissolved oxygen within them, which was typically around 5-8 mg/L, and reaching dissolved oxygen concentrations of less than 0.2 mg/L, the labile Cu concentrations in the wines mostly decreased (see green bars, Figure 14). These results clearly demonstrate the reversibility of labile Cu in white wine, and show that conditions conducive to aeration and oxidation favour conversion of Cu to the labile form,

whilst reductive conditions in wine favour the formation of non-labile Cu. This is consistent with the oxidative or volatile losses of hydrogen sulfide during the aggressive agitation of wine with air, and also the ability of labile Cu to sequester sulfide from an unknown source in wine in reductive conditions. Further work is required to identify the sulfur source in wine, but recent research suggests it may be linked to polysulfanes and/or polysulfides.

6.7 Cu and Fe speciation and oxygen consumption in wines

The rate of dissolved oxygen reaction in wines is a complex mechanism and known to be impacted by wine pH, temperature, phenolic compound concentration, type of phenolic compounds present, sulfur dioxide concentration, ascorbic acid concentration and metal concentrations. It is proposed within this study that the metal speciation may also impact the rate of dissolved oxygen reaction in wine. The following sections investigate this relationship of metal speciation with oxygen consumption rates in wines: white wines with ascorbic acid, white wines and red wines. The investigation into white wines with and without ascorbic acid was deemed important as the mechanism of oxidation is different in both cases, as shown in Figure 15, although both still required metal ions to catalyse the reaction.

Figure 15. Proposed oxidation mechanism for (a) phenolic compounds and (b) ascorbic acid in wine as mediated by metal ions Fe and Cu.



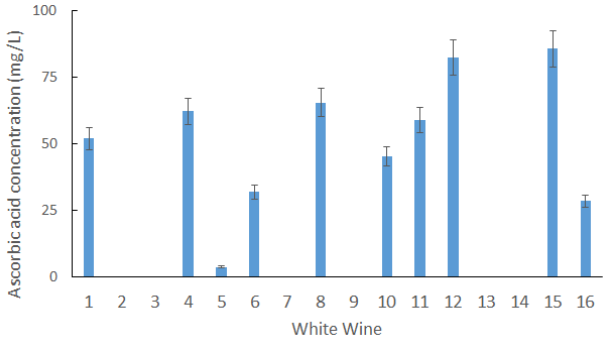
6.7.1 Cu and Fe speciation and oxygen: white wines with ascorbic acid

The impact of Cu and Fe speciation on the oxygen consumption in white wine with ascorbic acid was studied in two different sets of white wines. The first involved a selection of 12 white wines from the surveyed wines in Section 6.2 that showed diverse Cu concentrations, while the second set was based upon the Chardonnay wines produced from juice of different metal concentrations (described in Section 6.4).

6.7.1.1 Metal speciation and oxygen: 12 white wines of diverse Cu concentrations.

The first approach was to follow the oxygen consumption rates in white wines at constant temperature (25 °C), pH (3.25) and with added ascorbic acid (200 mg/L) and sulfur dioxide (145 mg/L). By using these conditions, many of the variables of wine oxidation were being controlled (including pH, temperature, ascorbic acid, sulfur dioxide), and allowed direct assessment of the effect of metals in the wine. Furthermore, the high ascorbic acid and sulfur dioxide concentrations ensured that variations in wine phenolic concentrations, and the types of phenolics present, had minimal impact on the oxygen consumption rates as they would instead be dominated by ascorbic acid. Also, a recent survey of 16 Australian white wines (at a price point < $15) showed that the majority contained ascorbic acid (Figure 16) and consequently assessing the catalytic oxidising ability of metals in the presence of ascorbic acid is of direct relevance to Australian white wines.

Figure 16. The concentration of ascorbic acid in a random selection of 16 Australian white wines.



Twelve white wines (selected from Section 6.2), as well as model wines with different concentrations of Cu(II) and Fe(II), were initially saturated with oxygen, and oxygen concentration followed by luminescence, to allow calculation of the rate of oxygen loss in the wines. SPE ICPOES and electrochemistry techniques were used to access the different metal forms present in wines, and this was conducted at the beginning and end of the oxygen decay. Figure 17a shows the typical decrease in dissolved oxygen concentration in a white wine under these conditions and allowed calculation of the first order oxygen consumption rates for a given wine (i.e., the higher this rate the faster oxygen reacted in a wine).

Figure 17b shows that the oxygen consumption rate increased with concentrations of Fe(II) or Cu(II) in the model wine with ascorbic acid, however, it was clear that at wine-like concentrations, Cu(II) was much more efficient as an oxidising catalyst than Fe(II). Fe(II) had a moderate impact even at high concentrations. This increased dependence on Cu compare to Fe is most likely consequence of the presence of ascorbic acid in this system.

The addition of hydrogen sulfide to the model wines in the presence of Cu(II) decreased the oxygen decay rates substantially (Figure 17b). The decrease was higher when the ratio of H_2S: Cu was increased. As already mentioned in this report, hydrogen sulfide can bind Cu in wine conditions to form copper(I) sulfide which does not tend to aggregate and settle in wine conditions. This copper(I) sulfide is much less efficient in catalysing wine oxidation than non-sulfide bound Cu in the ascorbic acid-containing model wine system.

Figure 17. The a) typical change in oxygen concentration during storage of a white wine, and b) the first order oxygen consumption rates in model white wine systems. All samples contained 200 mg/L ascorbic acid and 145 mg/L sulfur dioxide.

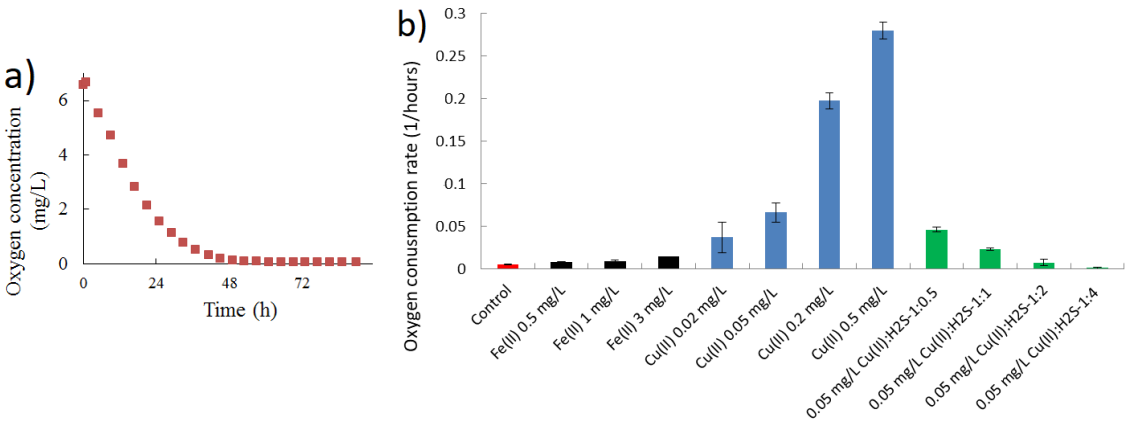
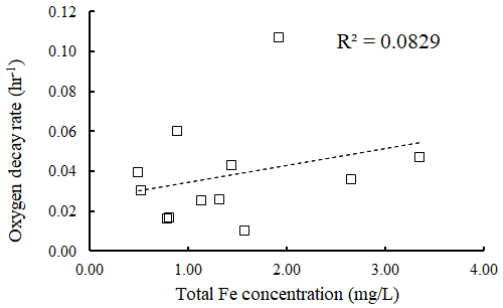


Figure 18. The poor correlation between white wine oxygen consumption rates and the total Fe concentration.



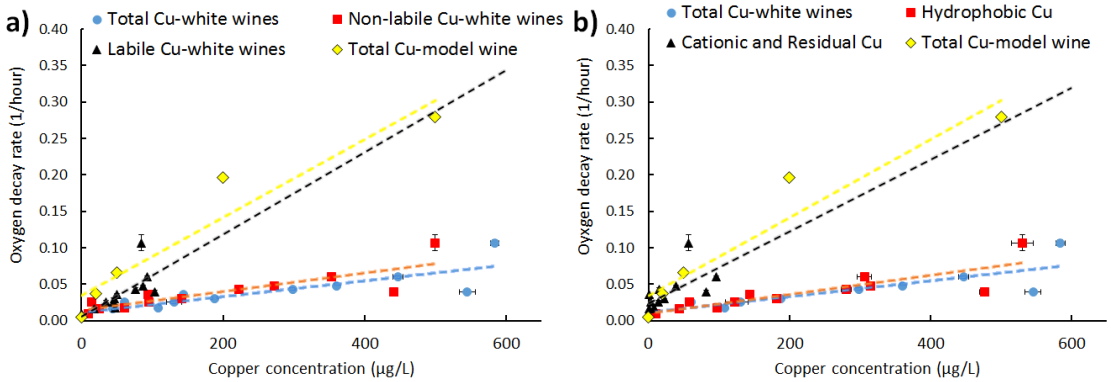
In the 12 white wines studied, the total Fe concentration correlated poorly with the oxygen consumption rates within the wines (Figure 18). This was consistent with the low catalytic efficiency of Fe in the model wine system.

In white wine, the total Cu provided good correlation with the first order oxygen consumption rates (Figure 19a, blue points). However the oxygen decay in the white wines for a given amount of Cu was much slower than in the model wine system (Figure 19a, blue vs. yellow points). This is because most of the Cu in the white wine was in the form of copper(I) sulfide which is less efficient in catalysing oxygen consumption (Figure 17b). The amount of copper(I)-sulfide in the wines is given by the electrochemical non-labile Cu concentration of the wines (Figure 19a, red points) or the hydrophobic Cu concentration in the wine (as determined by SPE ICPOES, Figure 19b, red points). Both the speciation measures (i.e., electrochemical and SPE ICPOES) were performed at the beginning and end of the oxygen consumption and the values shown in Figure 19 are the average of both. In this way, the change in copper speciation during the oxygen consumption period was accounted for to some extent.

The more reactive fractions of Cu in the wine (labile Cu for electrochemistry, Figure 19a, black points, and cationic and residual Cu for SPE ICPOES, Figure 19b, black points) provide a relationship to oxygen decay in the white wine closer to that observed for Cu in the model wine.

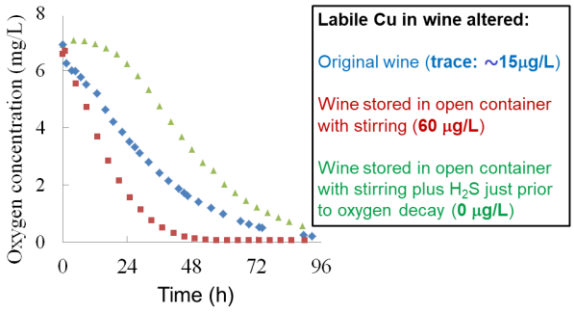
Therefore, in white wine, containing ascorbic acid, it is these forms of Cu that appear most critical in contributing to the oxygen consumption rates in the wine.

Figure 19. The relationship between various measures of Cu in white wines in relation to the oxygen consumption rates of the wine. The speciation measurements were either a) with electrochemistry, or b) with SPE ICPOES.



Further confirmation for the link between the reactive fraction of Cu with the rate of oxygen consumption in white wines can be seen from the results in Figure 20. In this case, the same white wine has had the labile Cu concentration manipulated in three samples (each prepared in triplicate) to be either 0, ~15 µg/L or 60 µg/L, while the total Cu concentration remained the same in all (160 µg/L). The original wine had a labile Cu concentration of ~15 µg/L, and by stirring it in an open container for several days the labile Cu concentration was increased to 60 µg/L, and finally by adding Na_2S to the latter wine (to form H_2S) a labile Cu concentration of 0 µg/L was obtained. Immediately prior to assessment of the oxygen consumption rates in the wines, both ascorbic acid (200 mg/L) and sulfur dioxide (145 mg/L) were added to the wines. The result (Figure 20) shows that the oxygen consumption is clearly faster in the wine with highest labile Cu and slowest in the wine with virtually no labile Cu.

Figure 20. The oxygen decay in a Riesling containing a total Cu concentration of 160 µg/L but with three different labile Cu concentrations.



It must be noted that there is a complexity in linking the initial metal speciation to the oxygen consumption rates for wines. This is because from Figure 14 (Section 6.6), it is known that the metal speciation can change during the reaction of oxygen in wine, and that the initial copper speciation at the commencement of oxygen consumption will be different to the copper speciation at the end of the oxygen consumption. However, the results do show that the rate of oxygen consumption in a wine will generally be considerably faster, in the presence of ascorbic acid, if copper is in the more reaction non-sulfide bound form (i.e., the electrochemical labile Cu form or the SPE ICPOES residual and cationic fractions) at the commencement of oxygen

consumption. Generally, most bottled wine is in the more non-reactive form of Cu in terms of catalytic ability for the reaction of oxygen in wine containing ascorbic acid.

6.7.1.2 Metal speciation and oxygen: Impact of metal in juice on wine.

Section 6.4 describes the preparation of different Chardonnay wines by treatment of the same juice with different Cu and Fe additions, and a summary of the juice and wine total metal concentrations are shown in Table 7. Section 6.4 also described the metal speciation in these wines as assessed by SPE ICPOES (Figure 7). This section describes differences in the oxygen consumption rates in these wines in comparison to their total and speciation metal concentrations. The oxygen consumption in the wine was determined after the addition of ascorbic acid and sulfur dioxide, as for the wines in the previous section (Section 6.7.1.1).

Table 7. The total Cu and Fe concentrations in the juice and corresponding wine samples, and the first order oxygen decay rates determined for each of the wines. The oxygen decay rates, provided in units of both min^{-1} and hr^{-1}, were assessed after addition of 216 mg/L ascorbic acid and 232 mg/L sulfur dioxide to the wines and incubation at 25 °C.

	juice Cu (mg/L)	juice Fe (mg/L)	wine Cu (mg/L)	wine Fe (mg/L)	wine oxygen decay (min^{-1})	(hr^{-1})
T1	$0.77\pm0.03^{a,b}$	0.37 ± 0.01^{a}	0.083 ± 0.008^{a}	0.423 ± 0.006^{a}	0.00020 ± 0.00003^{a}	0.0120
T2	3.61 ± 0.09^{c}	0.38 ± 0.02^{a}	0.233 ± 0.005^{c}	0.425 ± 0.009^{a}	$0.00045 \pm 0.00002^{b,c}$	0.0270
T3	6.35 ± 0.03^{d}	0.31 ± 0.02^{a}	0.293 ± 0.006^{d}	0.421 ± 0.006^{a}	0.00054 ± 0.00007^{b}	0.0324
T4	0.69 ± 0.02^{a}	6.0 ± 0.1^{b}	0.068 ± 0.014^{ab}	6.20 ± 0.05^{b}	0.00038 ± 0.00006^{c}	0.0228
T5	0.80 ± 0.03^{b}	11.1 ± 0.1^{c}	0.052 ± 0.011^{b}	11.7 ± 0.1^{c}	0.00038 ± 0.00005^{c}	0.0228
T6	6.52 ± 0.06^{d}	11.3 ± 0.1^{c}	0.288 ± 0.007^{d}	11.8 ± 0.1^{c}	0.00072 ± 0.00003^{d}	0.0432

The uncertainty indicated is the standard deviation. Concentrations of a given metal with the same superscript letters in a given column are not significantly different at p=0.05.

Figure 21. The change in oxygen concentration for Chardonnay with variable Cu (a) and/or Fe (b). The samples correspond to: T1 control, **T2 low Cu**, **T3 high Cu**, **T4 low Fe**, **T5 high Fe** and **T6 high Cu and Fe**.

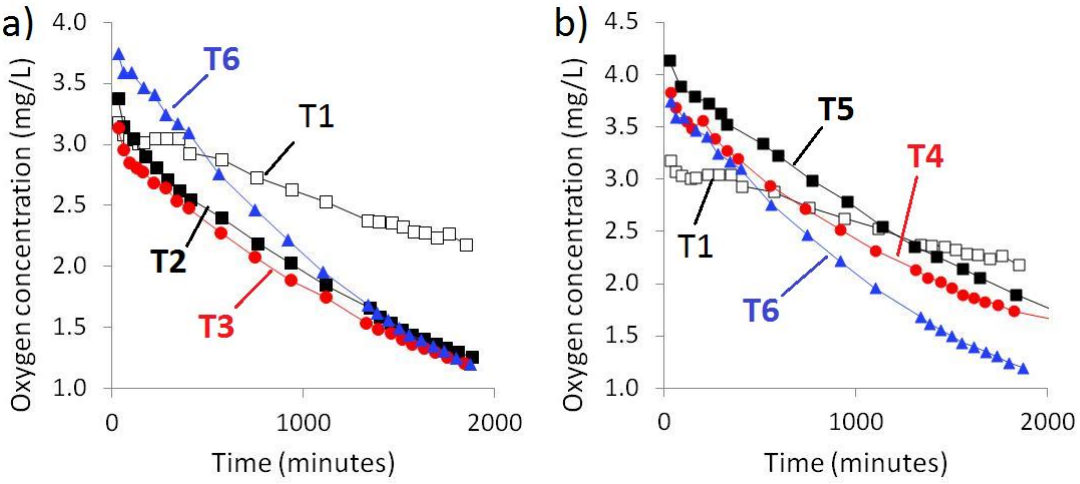


Figure 21 shows the oxygen decay rate plots for a single replicate of each wine, while Table 1 displays the overall first order oxygen decay rates for the wines (based on the oxygen decay in triplicate samples). In terms of wines with metal ions added to the juice prior to fermentation (subsequently referred to as T1-T6 as per Table 7), the addition of Cu alone to the juice led to significantly higher (125-190 %) oxygen consumption rates in the corresponding wine (Table 7,

T1 vs T2 and T3), and a trend to higher oxygen consumption with increased Cu concentrations (Table 7, T1 vs T2 vs T3). Fe addition alone to the juice also led to increased oxygen consumption (by 90 %), but no difference was evident between low and high addition rates of Fe to the juice (Table 1, T1 vs T4 and T5). Combined high additions of Cu and Fe to the juice resulted in the highest oxygen consumption rate in the corresponding wine (Table 1, T6). In terms of correlating the total metal concentrations with oxygen decay rates in the control, low and high metal treatments, Cu provided improved correlation compared to Fe (Figure 22a and 23a).

Figure 22. The correlation of (a) total Cu and (b) Cu speciation fractions (SPE ICPOES) with oxygen decay rates in the Chardonnay for wines: T1, T2 and T3.

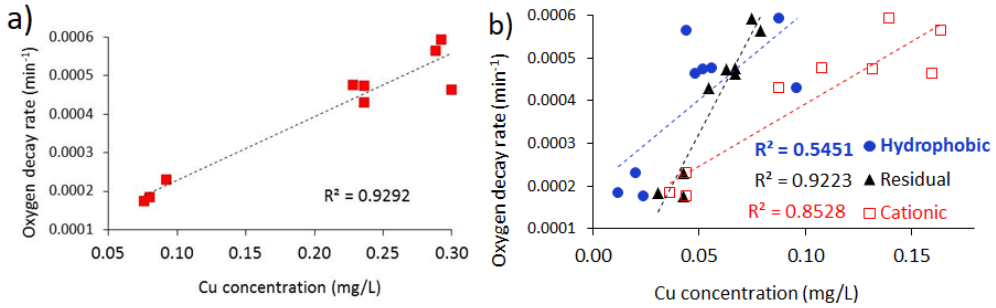
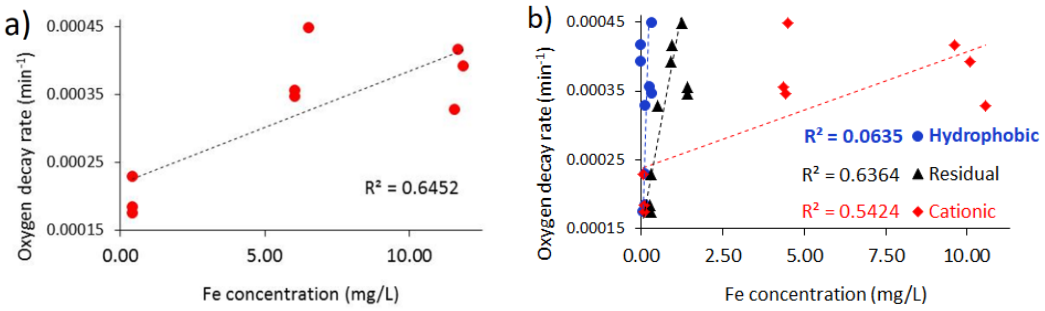


Figure 23. The correlation of (a) total Fe and (b) Fe speciation fractions (SPE ICPOES) with oxygen decay rates in the Chardonnay for wines: T1, T4 and T5.



In terms of fractions (Figure 22b), the residual form of Cu provided the best correlation with oxygen decay rate and this was of a similar correlation magnitude to that of total Cu (Figure 22a). The cationic form of Cu provided the next best correlation, whilst the hydrophobic form of Cu had the lowest correlation with oxygen decay rate. These results reinforce the previous section of this report (Section 6.7.1.1) that the cationic and residual forms of Cu, akin to labile Cu, are the most reactive for catalysing oxygen consumption in wine by ascorbic acid. For Fe, the cationic and residual fractions of Fe provided the best correlation with oxygen decay (Figure 23b), but the correlations were not as strong as observed for the respective Cu fractions. The correlation for the hydrophobic form of Fe was the only fraction that gave no statistically significant correlation (P=0.05).

Importantly, the results show that metals originating from the juice are able to impact the rate of oxygen consumption in the final wine produced. This was particularly significant for Cu whereby its total, residual and cationic forms in the wine had improved correlation with oxygen consumption compared to Fe.

6.7.2 Cu speciation and oxygen: white wines without added ascorbic acid

Given that not all white wines contain ascorbic acid, it was considered important to establish the importance of Cu speciation on the consumption of oxygen in white wines not containing ascorbic acid. The complicating factor here, in terms of experimental set-up, was normalising the effect of phenolic concentration and the different types of phenolic compounds present in white wines. Unlike the previous section, where ascorbic acid dominated the consumption of oxygen in the white wines, variable phenolic concentrations and profiles would be impacting the oxygen consumption rates in ascorbic acid-free white wines. Consequently, the approach taken was to investigate three different white wines, each with variable Cu speciation induced by the addition of hydrogen sulfide to the wines.

Three white wines without ascorbic acid present were modified to have the same pH (3.50) and free sulfur dioxide concentrations. The total concentrations of metals in the wines (i.e., Cu, Fe, Al, Mn, Mg, Zn) were all adjusted to provide the same total concentrations in the wines (i.e., 0.5, 1.5, 1.0, 1.0, 90 and 1.0 mg/L, respectively). Each wine was separated into three portions and hydrogen sulfide added to the wines to provide mole ratios of added hydrogen sulfide to added Cu of 0:1, 1:1 and 2:1. It was assumed that the native Cu present in the wines would already be in the form of copper(I) sulfide and that the added Cu would be in a non-sulfide bound form (i.e., labile Cu). The addition of hydrogen sulfide at a ratio to the added labile Cu achieved three different labile Cu concentrations in the white wines. Figure 24a (blue bars) shows the starting concentration of labile Cu (i.e., 'Initial') in the wines after saturating the wines with oxygen, while Figure 24b (white bars) show the corresponding 'Initial' non-labile Cu. As expected, the more hydrogen sulfide added to the wines, the lower the labile Cu and the higher the non-labile Cu. After oxygen consumption, the labile Cu concentration changed, as observed for the samples containing ascorbic acid (Figure 14), such that the labile Cu increased (red bars, Figure 24a) and the non-labile Cu decreased (black bars, Figure 24b).

Figure 24. The a) labile Cu and b) non-labile Cu of three different white wines at various mole ratios of hydrogen sulfide to total Cu. The copper speciation measures were performed before dissolved oxygen consumption (***Initial***) and also after dissolved oxygen consumption (***Final***). The starting dissolved oxygen concentrations were around 7-9 mg/L and the total Cu concentration in all wines was 0.50 mg/L.

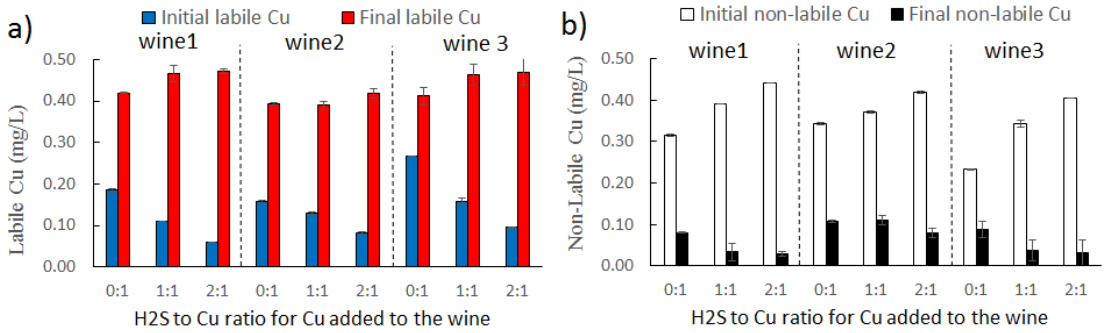


Figure 25. The initial oxygen decay rate of white wines versus the a) labile Cu concentration and b) non-labile Cu concentration prior to oxygen consumption (***Initial***), after oxygen consumption (***Final***) and the average Cu speciation of both time points (***Average***).

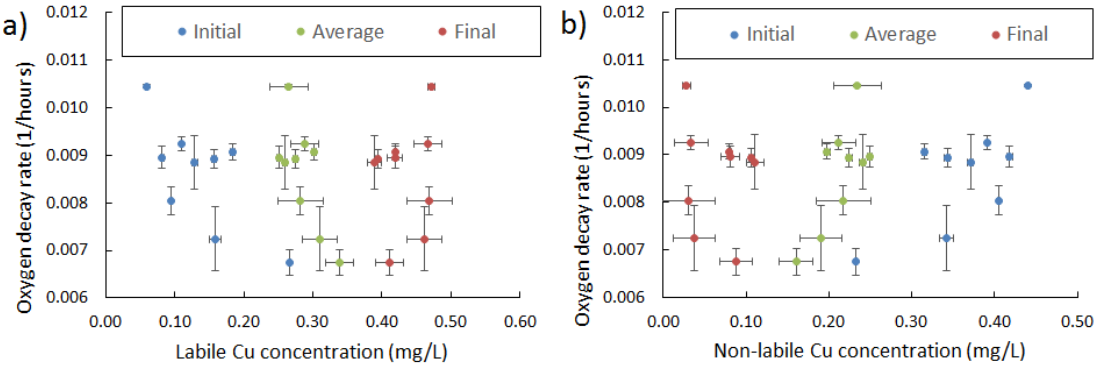


Figure 25 shows the relationship between the labile and non-labile Cu concentration with the oxygen decay rates calculated over the first 100 hours of storage of the wines. As with Figure 19, Figure 25 utilises the speciation measurements at the start of the oxygen decay (Initial), at the end (Final) and also the average between the two sets of speciation measurement (Average). From Figure 25a it is evident that there is no strong link between labile Cu in the wines and the oxygen consumption rates regardless of the timing of the labile Cu measurements. The data obtained from the overall oxygen decay rates (0->200 hours) provided a similar outcome (data not shown). The relationship between the Cu speciation measures and oxygen decay rate was still poor if the data was analysed for the individual wines at different hydrogen sulfide addition ratios (data not shown), or for the combined wines (Figure 25). If anything, there was a slight trend towards faster oxygen decay rates at low labile Cu concentrations and high non-labile (copper(I) sulfide) Cu concentrations, but even this relationship was not supported by a strong correlation within the data shown in Figure 25. Consequently, it is evident that Cu speciation has a far less impact on the oxygen consumption in wines that have no ascorbic acid compared to those with ascorbic acid.

Mechanistically, labile Cu, which is predominantly copper in the Cu(II) redox form bound to organic acid, appears to favour the mediation of the reaction between oxygen and ascorbic acid, while the same is not the case for the reaction between phenolic compounds and oxygen. If anything copper(I) sulfide may instead favour the mediation of the reaction between oxygen and white wine phenolic compounds. This is also consistent with the observation in Section 6.6 (Figure 13) of copper(I) sulfide being more stable in the model wine with ascorbic acid and caffeic acid rather than just with caffeic acid. Further work is required in this area.

6.7.3 Cu speciation and oxygen: red wines without added ascorbic acid

The link between oxygen consumption in red wines with Cu speciation was investigated in a similar manner to that of the previous section of this report (6.7.2. Cu speciation and oxygen: white wines without added ascorbic acid). In this case, five red wines were taken and adjusted to similar pH (3.50), free sulfur dioxide and total metal concentrations. As for the white wine in the previous section, the metals adjusted were Cu, Fe, Al, Mn, Mg and Zn, and their final concentrations were 0.50, 3.0, 0.80, 2.65, 160 and 1.0 mg/L. The Cu speciation in the red wines was then modified to three different concentrations of labile Cu via addition of hydrogen sulfide at a ratio to the Cu added to the wines.

Figure 26a (blue bars) shows the starting concentration of labile Cu (i.e., 'Initial') in the wines, after saturating the wines with oxygen but before its consumption of the oxygen, while Figure 26b (white bars) show the corresponding 'Initial' non-labile Cu. As expected, the more hydrogen sulfide added to the wines, the lower the labile Cu and the higher the non-labile Cu. After

oxygen consumption, the labile Cu increased (red bars, Figure 26a) and the non-labile Cu decreased (black bars, Figure 26b) as was observed for the white wines of the preceding section.

Figure 26. The a) labile Cu and b) non-labile Cu of five different red wines at various mole ratios of hydrogen sulfide to total Cu. The copper speciation measures were performed before dissolved oxygen consumption (***Initial***) and also after dissolved oxygen consumption (***Final***). The starting dissolved oxygen concentrations were around 7-9 mg/L and the total Cu concentration in all wines was 0.50 mg/L.

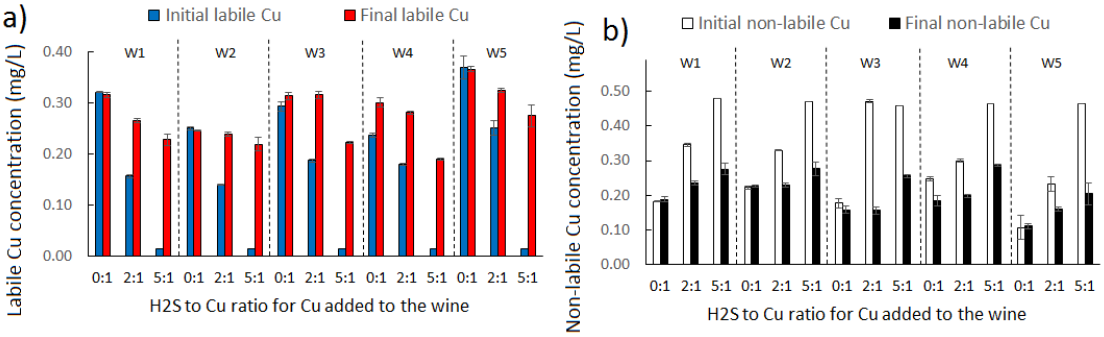


Figure 27. The initial oxygen decay rate of red wines versus the a) labile Cu concentration and b) non-labile Cu concentration prior to oxygen consumption (***Initial***), after oxygen consumption (***Final***) and the average Cu speciation of both time points (***Average***).

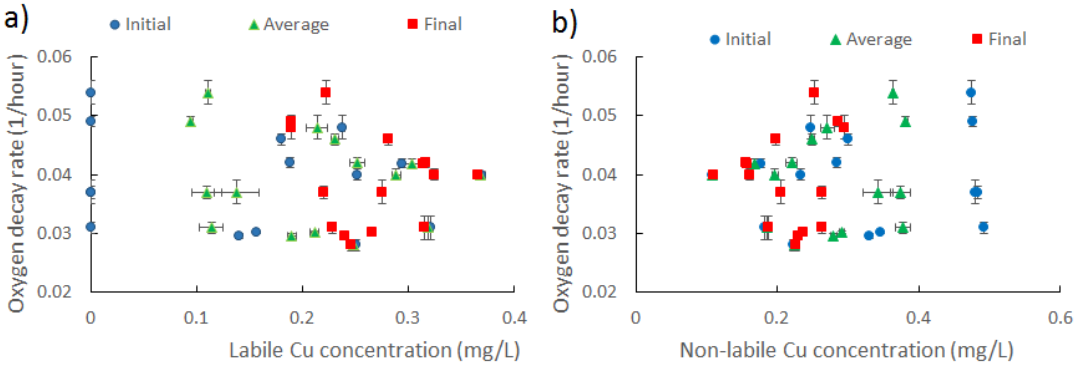
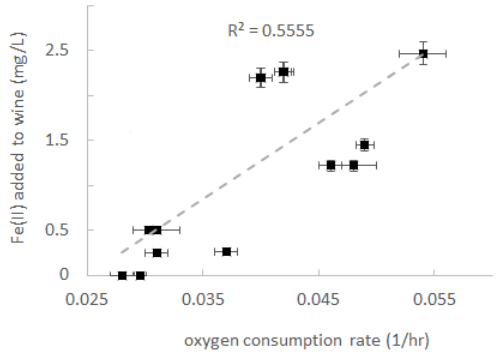


Figure 27 shows the relationship between the labile and non-labile Cu concentration with the oxygen decay rates. Figure 27 shows the speciation measurements prior to the commencement of the oxygen decay (Initial), and then again after the wine had largely depleted all the dissolved oxygen (Final). From Figure 27 is evident that there is no apparent link between labile or non-labile Cu in the wines and the oxygen consumption rates observed. The relationship between the Cu speciation measures and oxygen decay rate was still poor if the data was analysed for the individual wines at different hydrogen sulfide addition ratios (data not shown). Consequently, it is evident that Cu speciation has a far less impact on the oxygen consumption in red wines compared to white wines with ascorbic acid.

Interestingly, the best correlation with oxygen decay rates after inspection of other metal and wine compositional data was that of the added Fe(II) concentration to the wines. After initial measurement of iron in the wines, Fe(II) was added to the wines in order to achieve a uniform total metal concentration of 3.0 mg/L. Figure 28 shows the relationship between the amount of Fe(II) added to the wines and the oxygen decay rates. It is possible that through the addition of Fe(II) to the red wines, the equilibrium between Fe(II) and Fe(III) is disturbed in such a manner that enhances the conversion of Fe(II) to Fe(III) and aids consumption of oxygen in the wines. Danilewicz (2016) has shown in model wine systems that the Fe(II)/Fe(III) ratio can be important

in determining the rate of oxygen consumption. It is in contrast to the white wines with ascorbic acid present whereby Fe had poor correlation with the rate of oxygen consumed.

Figure 28. The relationship between oxygen decay rates in red wine and the amount of Fe(II) added to the wines.



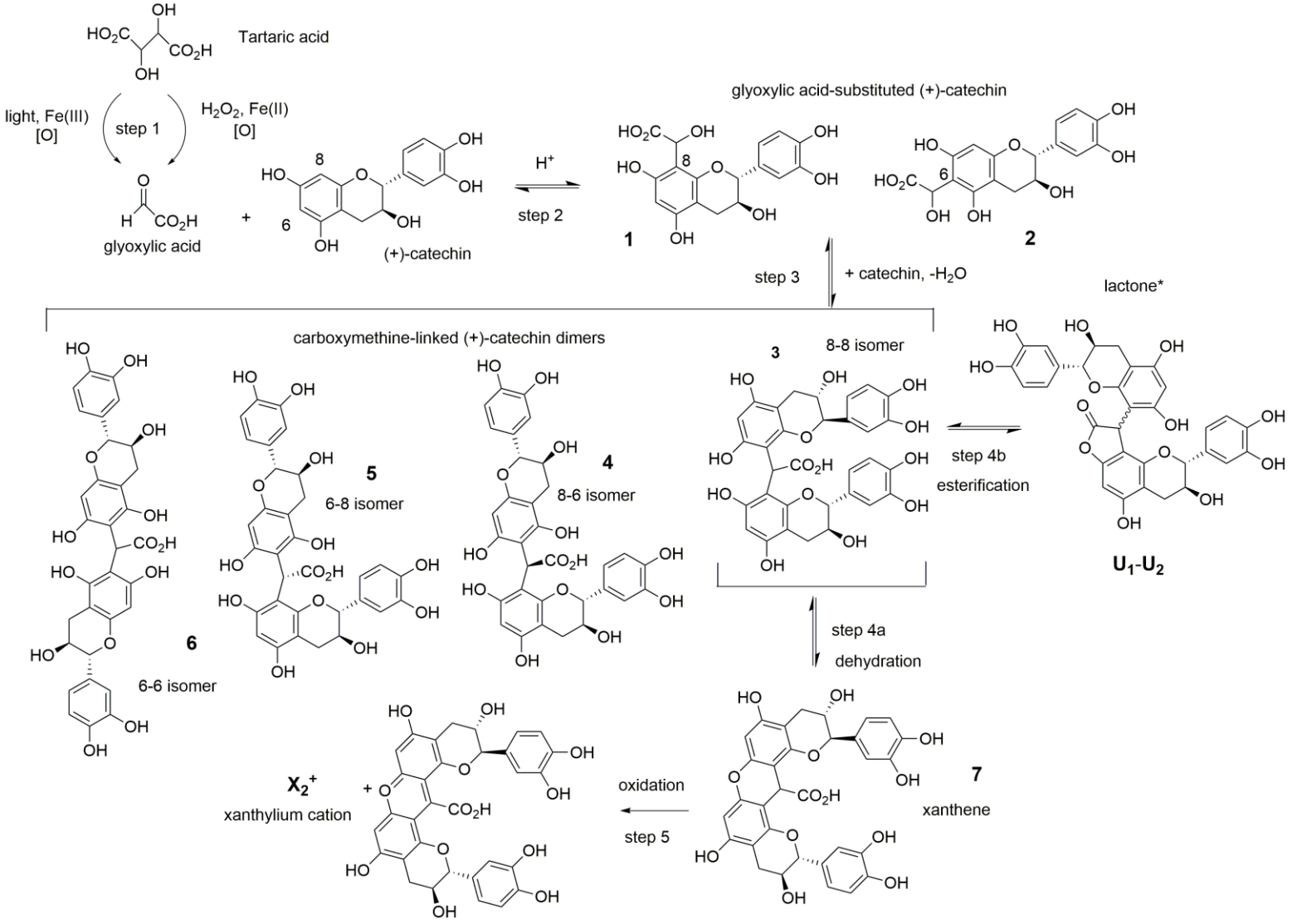
6.8 Metal speciation and catalytic polymerisation of phenolic compounds

Previous research has shown that Cu can catalyse the polymerisation of flavanols via glyoxylic acid bridging in wine conditions (Clark et al. 2003) to generate yellow pigments (see xanthylium cation, $\mathbf{X_2}^+$, Figure 29). Although the mechanism of polymerisation is complex (Figure 29), steps 2 and 3 (combined) were found to be catalysed by Cu. This reaction and the measurement of the xanthylium cation produced (i.e., absorbance at 440 nm) was utilised to assess the reactivity of Cu in model and white wine solutions (Clark et al. 2015) at 45 °C, and it was observed to largely discriminate between Cu and copper(I) sulfide in model wines but not in a white wine. However, further work was required to establish if it would be a more effective assay when used in white wine at lower temperatures (25 °C), whether other metal ions would also catalyse the reaction and if it could be used to compare the amount of reactive metal in different wines.

6.8.1 Metal catalysis of the reaction between (+)-catechin and glyoxylic acid in model wine samples

Consequently, the influence of Cu(II), Fe(II), Fe(III), Mn(II), Zn(II) and Al(III) on the reaction between glyoxylic acid and (+)-catechin was established in model wine solutions. The detailed results can be viewed in Guo et al. (2017) and a summary of the outcome of the results in model wines are provided here. As for Cu, Fe could also catalyse steps 1 and 2 (Figure 29) of the mechanism. The ability of Fe to mediate the reaction of glyoxylic acid with (+)-catechin (step 2) (Figure 29) was independent of the form in which Fe was added (Fe(III) or Fe(II)). Both forms of Fe provided bias toward the carbon-6 connected isomers (see compound **2** vs. **1**, Figure 29) compared to the samples without added metal ions or those with Cu(II). Furthermore, Fe could accelerate the oxidative conversion of the carboxymethine-linked (+)-catechin dimers to the xanthylium cation (steps 4a and 5 (combined), Figure 29), most likely by enhancing xanthene oxidation. Mn(II) could aid the conversion of carboxymethine-linked (+)-catechin dimers to the xanthylium cation in the presence of Fe (steps 4a and 5), but had little impact when alone. As already established, the role of Cu(II) would appear to be predominantly in mediating the

Figure 29. The production of xanthylium cations from tartaric acid and (+)-catechin (Guo et al. 2017).



reaction between glyoxylic acid and (+)-catechin (steps 1 and 2) (Figure 29), but for the first time it was evident that Cu enhanced carbon-8 as the preferred site of substitution on (+)-catechin (see compound **1** vs. **2**, Figure 29). Al(III) showed that it had a very minor influence on the reaction between glyoxylic acid and (+)-catechin (step 1 and/or 2), while Zn(II) had no effect. The results also allowed the identification of lactone isomers (**U_1-U_2**, Figure 29) that were reported for the first time.

In summary, Cu(II) and Fe ions are most important, and Mn(II) secondary, for enhancing the rate of production of the xanthylium cation pigments (Figure 29) from glyoxylic acid and (+)-catechin. However, the production of carboxymethine-linked (+)-catechin dimers, **3–6** (Figure 29) from added (+)-catechin and glyoxylic acid may provide a measure of Cu(II) and Fe ion activity with largely negligible impact from Mn(II), Al(III), and Zn(II).

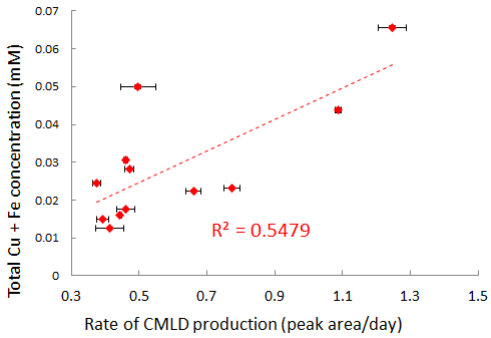
6.8.2 Metal catalysis of the reaction between (+)-catechin and glyoxylic acid in white wines vs metal speciation measurements and/or oxygen consumption

The same white wines in Section 6.7.1.1 (linking oxygen consumption rates to metal speciation measures) were taken and incubated at 25 °C with (+)-catechin and glyoxylic acid at the same concentrations utilised in the model wine system. The aim was to link the metal catalysed reaction between (+)-catechin and glyoxylic acid to the metal speciation in the wines and/or to the oxygen consumption rates in the wines. This would confirm that metal speciation is important to the catalytic ability of metals to participate in phenolic polymerisation reactions, and conversely may provide an assay to assess reactive Cu and Fe fractions in wine. In wines, compound **3** was monitored, the carbon 8-8 isomer of the carboxymethine-linked dimer (CMLD), as this was formed at the highest concentrations and could be most easily followed by UHPLC-DAD.

Prior to addition of the (+)-catechin and glyoxylic acid, acetaldehyde was also added to the wines in order to bind free sulfur dioxide present in the wines, and prevent its binding to glyoxylic acid. Cu was already known not to accelerate the reaction between (+)-catechin and acetaldehyde (Clark et al. 2003), and hence the presence of acetaldehyde was not expected to interfere with the (+)-catechin/glyoxylic acid assay.

The results showed poor correlation between the different measures of Cu speciation and total Cu and Fe concentrations in the wines with the rate of production of the CMLD. The correlations ranged from 0.1288 to 0.5479, with the best correlation being for the summed concentration of Cu and Fe in the wines (in mol/L units) against the rate of CMLD production (Figure 30). The best correlation of the CMLD production with the total metal concentrations is most likely a consequence of the copper species being converted to the free forms during the assay (i.e., over 10-15 days of storage of the wine at 25 °C with head-space oxygen present). Based upon these results it appeared that the catechin/glyoxylic acid reaction could not provide a measure of metal forms in wine but instead was a poor indicator of the combined molar total concentration of Cu and Fe in white wine.

Figure 30. The summed total Cu and Fe concentrations in white wines versus the rate of carboxymethine-linked dimer production (see compound **3** in Figure 17).

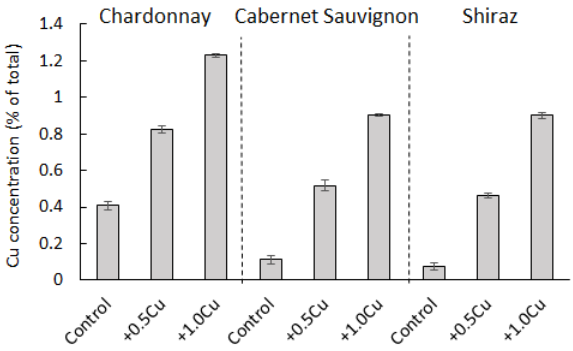


6.9 Metal speciation and the evolution of low molecular weight sulfur compounds (LMWSC)

6.9.1 Change in Cu speciation and LMWSC during bottle aging of wine

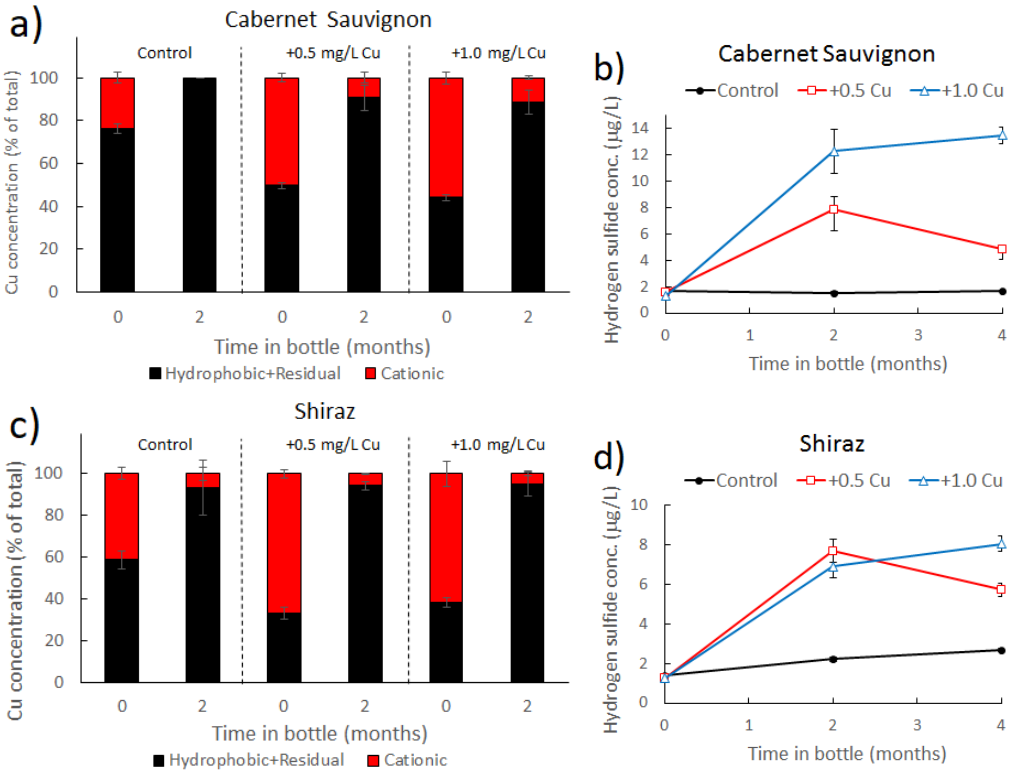
To assess the link between low molecular weight sulfur compounds and the Cu speciation of wine, three different wine varieties (Chardonnay, Cabernet Sauvignon, and Shiraz) were bottled with different additions of Cu prior to bottling. Figure 31 shows the total concentrations of Cu in the wines, and the Fe concentrations were left unaltered at values of 3.8 ± 0.1, 0.98 ± 0.01 and 1.8 ± 0.1 mg/L for Chardonnay, Cabernet Sauvignon and Shiraz, respectively.

Figure 31. The total Cu concentrations in three wine varieties after addition of 0.5 and 1.0 mg/L Cu.



The SPE ICPOES approach was utilised to assess the metal speciation during the bottle aging of the wine. However, as this experiment was conducted early in the life time of this overall project, an earlier version of the SPE ICPOES method was utilised, with a variation in the pre-treatment of the SPE cartridges in such a fashion that did not allow the hydrophobic and residual forms of Cu to be distinguished from one another. For Cu in red wine, this alternate SPE ICPOES could still distinguish copper(I) sulfide (hydrophobic and residual) from non-sulfide bound Cu (cationic). However, in white wine (Chardonnay) this wasn't the case, and the SPE ICPOES fractions are largely inconclusive in terms of relating the species to copper(I) sulfide. For this reason the SPE ICPOES data for Chardonnay are not presented.

Figure 32. The change in Cu speciation measured by SPE ICPOES after storage a) Shiraz and c) Cabernet Sauvignon for 2-months in bottle, and the corresponding hydrogen sulfide concentrations (b and d, respectively).



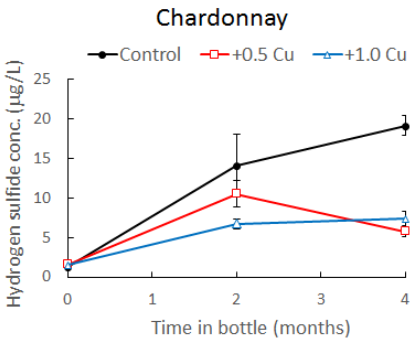
The Shiraz and Cabernet Sauvignon wines both show an increase in the proportion of cationic fraction of Cu with additions of 0.5 and 1.0 mg/L to the wines at bottling (Figure 32). This is consistent with the Cu added to the wines being initially in a non-sulfide bound form. Then as the wine is stored within the bottle for 2-months, a dramatic increase in the combined hydrophobic and residual forms of Cu is evident, to near total concentrations (Figure 32), which is consistent with the increase in copper(I) sulfide during the aging of the wines.

The measurement of hydrogen sulfide in the wines shows significant increases in concentrations for the wines with added 0.5 and 1.0 mg/L Cu. It suggests, that the wines that have significant fractions of cationic Cu can efficiently sequester hydrogen sulfide from precursors within the wine, while the control wines with minimal cationic fraction of Cu are less efficient at generating hydrogen sulfide. In the Cabernet Sauvignon wine the cationic fraction of Cu at bottling was 0.025 mg/L, 0.22 mg/L and 0.41 mg/L in the control, '+0.5' and '+1.0' samples, respectively, and the same order of samples for increasing hydrogen sulfide concentration after 2-months was evident. For the Shiraz wine the cationic fractions at bottling were 0.025, 0.23 and 0.43 mg/L in the control, '+0.5' and '+1.0' samples, respectively, and in this case the samples with added Cu were both higher in hydrogen sulfide than the control sample. Recent research has suggested that the precursor for sulfide is possibly stemming from polysulfides and/or polysulfanes (Kreitman et al. 2017).

In terms of other LMWSCs in the wines, dimethyl sulfide (DMS) showed no influence of the different Cu concentrations during aging while methanethiol showed no significant differences (Supplementary Figure 3 and 4) after two months of bottle aging.

In contrast to the red wines, the Chardonnay showed higher concentrations of hydrogen sulfide in the control wine compared to the wines with added Cu (Figure 34). However, it must be mentioned that the control wine in the Chardonnay contained a concentration of Cu that was similar to the concentration present in the '+0.5' red wine samples. Also, the Chardonnay samples with higher Cu concentration had a substantial decrease in measured total Cu concentration during bottle aging (i.e., 30-45 %). This loss of Cu may account for the lower concentrations of hydrogen sulfide observed in the samples with added Cu compared to the control, if the Cu was lost in the form of a copper(I) sulfide precipitate.

Figure 34. The hydrogen sulfide concentration in Chardonnay during 4-months of bottle aging.



This experiment has showed that in red wine, Cu that is added prior to bottling can lead to substantial amounts of non-sulfide bound Cu in the wine that then evolves relatively rapidly (i.e., within 2-months) to a sulfide-bound form, by procuring sulfide from precursors in the wine. An increase in the measured total hydrogen sulfide in the red wines is consistent with this process. It must be noted that a limitation of this experiment was the use of the non-optimum SPE ICPOES technique at the beginning of the experiment. The experiment is worthy of repeating with the updated SPE ICPOES approach along with electrochemical measurement of the Cu speciation.

6.9.2 Metal speciation in Chardonnay and LMWSC after 1-year of bottle aging

6.9.2.1 The 1-year bottle aging of non protein stabilised Chardonnay

The Chardonnay wines from Section 6.4 and 6.7.1.2, prepared with different juice metal concentrations were aged for 1-year and assessed for changes in metal speciation and also the LMWSC concentration after 1-year. This wine was not protein stable prior to bottling, and a bentonite trial established that 1.6 g/L of bentonite would have been required to ensure its stability.

For Cu, the non-protein stabilised wines (no bentonite treatment) showed a substantial decrease in Cu concentration during the 1-year of bottle aging. A deposit was evident at the bottom of the bottle after 1-year of aging. The amount of deposit was visually assessed to be similar regardless of the sample treatment. Such deposit is well known to occur in wines with

residual protein (Waters et al. 2005, Wilkes et al. 2013) and is attributed to the gradually denaturing, aggregation and settling of the grape-derived protein over time (Joslyn and Lukton 1953). In terms of total Cu, it was observed that the samples bottled with higher concentrations of Cu resulted in the largest proportional decreases in total Cu during the 1-year storage period (see T2, T3 and T6, Figure 35a). Presumably this Cu was encapsulated into the protein deposit.

In terms of proportions of fractionated Cu (Figure 35b), the general trend was towards an increase in the hydrophobic fraction of Cu and decrease in the proportion of residual and cationic fractions of Cu. The increase for hydrophobic Cu was only significant for T5 and T6. In terms of the actual concentrations, the concentrations of cationic and residual fractions of Cu decreased during the 1-year aging period (data not shown), while the hydrophobic Cu concentration remained constant (T1-T5) or increased for T6 (high Cu and high Fe). Consistent with the total Cu concentrations, the highest proportional losses in cationic and residual fractions were evident for the samples with elevated Cu concentrations (T2, T3 and T6). Such a result is consistent with the formation of hydrogen sulfide during the aging of wine in low oxygen conditions (Ugliano et al. 2011, Ugliano et al. 2012, Viviers et al. 2013). Such hydrogen sulfide formed would be able to bind with Cu(II) tartrate (i.e., cationic Cu fraction), to increase the Cu(I) sulfide concentration and hence the hydrophobic fraction of Cu. The production of hydrogen sulfide has been observed to occur in non-protein stabilised wine (Wilkes et al. 2013), and would be described as *de novo* formation of hydrogen sulfide by Franco-Luesma and Ferreira (2016).

In contrast to Cu, no loss in total Fe concentration was evident after the wine was aged for 1 year in bottle (Figure 36a). This is consistent with Fe being associated more with oxidative colloidal instability in wine rather than reductive colloidal instability. Furthermore, the concentration of Fe in the wines are similar to the concentration at bottling and also the concentrations in the juice (Figure 36a and Table 7), as fermentation is known to have little effect on the concentration of Fe, unlike the outcome for Cu (Hsia et al. 1975).

Figure 35. The total Cu concentration (a) and proportion of fractionated forms (b) in wines T1-T6 at bottling and after 1 year in low oxygen conditions. The uncertainty shown corresponds to the standard deviation (n=3).

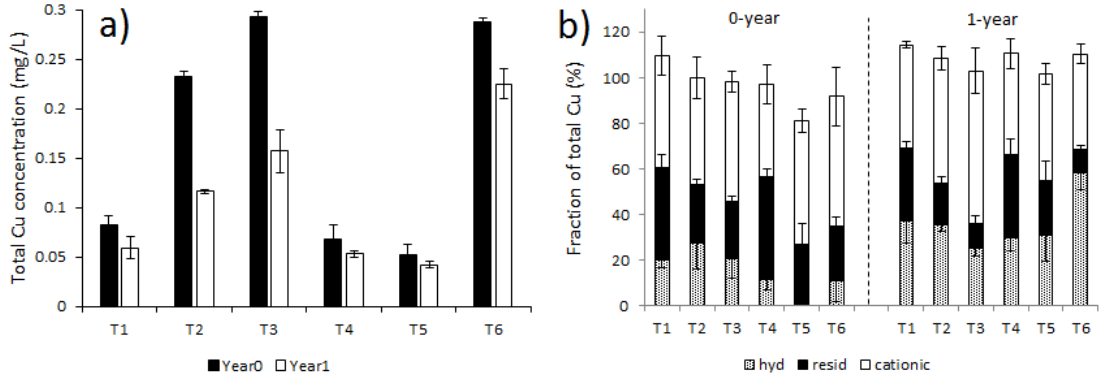
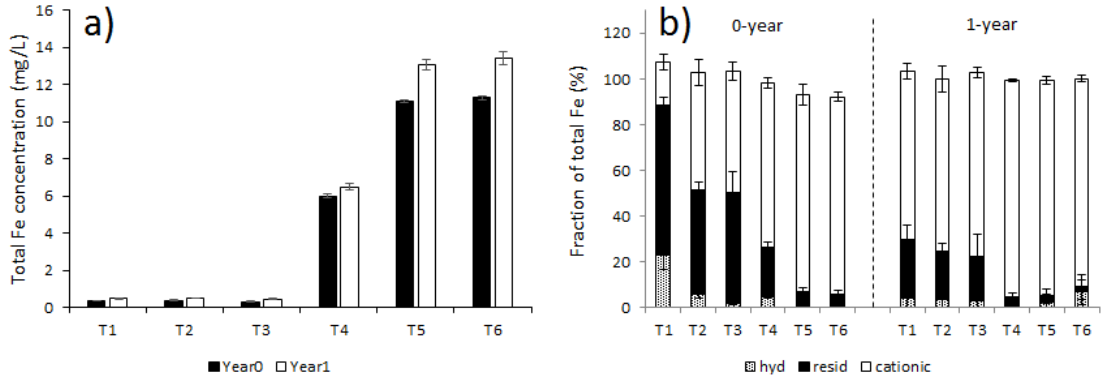


Figure 36. The total Fe concentration (a) and proportion of fractionated forms (b) in wines T1-T6 at bottling and after 1 year in low oxygen conditions. The uncertainty shown corresponds to the standard deviation (n=3).



The cationic fraction of Fe dominated the wines after 1 year as was the case at bottling (Figure 36b), and suggests that Fe is mainly bound by organic acids in wine conditions. A decrease was also evident in the proportion and concentration of the residual Fe present in the wines, significant for T1-T4, which accounts for the increase in the cationic fraction of Fe (Figure 36b). The presence of some Fe(III) at bottling, induced by the total packaged oxygen, and then the gradual conversion of Fe(III) to Fe(II) as the oxygen became depleted during bottle aging, may have contributed to the decrease in residual fraction of Fe.

The total, bound and free concentrations for hydrogen sulfide and methanethiol for samples T1-T6 after 1-year of bottle aging are shown in Table 8. The bound form of hydrogen sulfide is predominantly attributed to the formation of copper(I) sulfide (Kreitman et al. 2016), although other metal ions can contribute to the binding of hydrogen sulfide as well (Franco-Luesma and Ferreira 2014). The concentrations of the sulfur compounds were measured after the wine was aged 1-year in bottle (Table 8), but analysis was not performed at bottling (Rousseva et al. 2016). This means that it is not possible to directly distinguish between the amounts of sulfur compounds at bottling versus those generated during bottle aging. From the six protein-containing samples (T1-T6), there is a trend towards higher total and bound hydrogen sulfide and lower total methanethiol concentrations with increased total Cu concentration (Table 2). These relationships are evident in the correlations for total Cu and the sulfur compounds (r^2 = 0.75-0.99) shown in Table 8. The correlations are higher when the total Cu concentrations utilised were those measured at bottling rather than those measured a year later. Hence the total Cu that was initially present in the wine at bottling, rather the total Cu remaining in the wine after the gradual protein-induced loss, correlates best with total and bound hydrogen sulfide (positively) and total methanethiol (negatively) in the wine after 1 year.

The results (Table 8) also show that at bottling (year 0), it is the cationic and residual fractions of the total Cu that correlate best with total hydrogen sulfide concentration (positive correlation) and methanethiol concentration (negative correlation) after 1 year of aging. It was these same fractions of Cu that also correlated best with oxygen consumption in these wines (Rousseva et al. 2016), opening the possibility that they may be reactive in terms of both oxidative reactions (with ascorbic acid present) and reductive reactions (Bekker et al. 2016). The cationic fraction of Cu includes Cu(II) tartrate complexes, whilst the residual fraction of Cu has been observed to

Table 8. Concentration of total, free and bound forms of hydrogen sulfide (H_2S) and total and free methanethiol (H_3CSH) after 1 year of wine storage, and their correlation with total Cu and SPE ICPOES fractionated Cu (hydrophobic (Hyd), residual (Res), or cationic (Cat)) when measured at bottling (0 year) or 1 year (Y1) after bottling.

Sample	Total H_2S	Bound H_2S	Free H_2S	% Free H_2S	Total H_3CSH	% Free H_3CSH
T1	$3.4 \pm 0.6^{a,b,c}$	2.1	$1.3 \pm 0.3^{a,b}$	$38 \pm 10^{a,b,f}$	$4.3 \pm 0.3^{a,c}$	$71 \pm 12^{a,b}$
T2	3.8 ± 0.1^{a}	3.4	$0.5 \pm 0.2^{a,c}$	$12 \pm 4^{a,c,d}$	$2.2 \pm 0.3^{b,d}$	$59 \pm 14^{a,b,c}$
T3	$4.9 \pm 0.9^{a,b,c}$	4.2	$0.7 \pm 0.3^{a,b,c}$	$14 \pm 6^{a,c,d}$	2.0 ± 0.1^{b}	$55 \pm 15^{a,b,c}$
T4	$3.2 \pm 0.5^{a,b,c}$	2.0	1.1 ± 0.1^{b}	36 ± 5^{b}	$3.9 \pm 0.6^{a,c,d}$	66 ± 2^{a}
T5	2.7 ± 0.3^{b}	1.6	1.17 ± 0.05^{b}	43 ± 2^{b}	4.3 ± 0.1^{a}	$60 \pm 6^{a,b}$
T6	4.4 ± 0.2^{c}	4.1	0.32 ± 0.04^{c}	6 ± 1^{c}	1.9 ± 0.2^{b}	51 ± 3^{b}
WA	$3.4 \pm 0.6^{a,b,c}$	2.1	$1.3 \pm 0.3^{a,b}$	$38 \pm 10^{a,b,f}$	$4.3 \pm 0.3^{a,c}$	$71 \pm 12^{a,b}$
WB	$4.9 \pm 0.9^{a,b,c}$	4.2	$0.7 \pm 0.3^{a,b,c}$	$14 \pm 6^{a,c,d}$	2.0 ± 0.1^{b}	$55 \pm 15^{a,b,c}$
WC	4.4 ± 0.2^{c}	4.1	0.3 ± 0.1^{c}	6 ± 1^{c}	1.9 ± 0.2^{b}	51 ± 3^{b}
WA+bent	2.6 ± 0.4^{b}	2.2	$0.4 \pm 0.2^{a,c}$	$16 \pm 9^{a,c,d}$	$2.8 \pm 0.5^{c,b}$	$54 \pm 7^{a,b,c}$
WB+bent	2.6 ± 0.4^{b}	2.3	0.32 ± 0.04^{c}	12 ± 2^{d}	$1.4 \pm 0.2^{b,e}$	38 ± 3^{c}
WC+bent	$3.6 \pm 0.9^{a,b,c}$	3.3	0.3 ± 0.1^{c}	$7 \pm 3^{c,d}$	1.8 ± 0.3^{b}	$47 \pm 4^{b,c}$
WA ox	0.94 ± 0.04^{d}	0.3	0.66 ± 0.02^{a}	70 ± 2^{e}	1.06 ± 0.03^{e}	42 ± 1^{c}
WA+bent ox	1.13 ± 0.01^{e}	0.4	0.76 ± 0.05^{a}	68 ± 5^{e}	$0.94 \pm 0.05^{e,f}$	$42 \pm 10^{b,c,d}$
WB+bent ox	0.63 ± 0.02^{f}	0.6	0.32 ± 0.02^{c}	51 ± 2^{f}	0.71 ± 0.02^{g}	$34 \pm 16^{a,b,c,d}$
WC+bent ox	0.55 ± 0.04^{f}	0.3	0.31 ± 0.02^{c}	57 ± 4^{f}	$0.79 \pm 0.04^{f,g}$	20 ± 4^{d}
Correlation of sulfur compound concentration in T1-T6 after 1 year with total Cu and fractionated Cu after 0 or 1 years						
			Year 0			
Total Cu	0.9013	0.9863	(-) 0.8374	(-) 0.9446	(-) 0.9566	(-) 0.6440
Hyd Cu	0.5981	0.5204	(-) 0.6826	(-) 0.5997	(-) 0.4914	(-) 0.5136
Res Cu	0.9343	0.9512	(-) 0.6947	(-) 0.8851	(-) 0.8606	(-) 0.0287
Cat Cu	0.9108	0.9719	(-) 0.8041	(-) 0.9029	(-) 0.9135	(-) 0.6877
			Year 1			
Total Cu Y1	0.7506	0.8760	(-) 0.8393	(-) 0.8632	(-) 0.8088	(-) 0.6945
Hyd Cu Y1	0.3654	0.6311	(-) 0.6162	(-) 0.6474	(-) 0.6881	(-) 0.2106
Res Cu Y1	0.2899	0.3529	(-) 0.3639	(-) 0.4517	(-) 0.3223	(-) 0.3899
Cat Cu Y1	0.9500	0.9799	(-) 0.7175	(-) 0.8437	(-) 0.8783	(-) 0.6666

The uncertainty indicated is the standard deviation. Concentrations of a given metal with the same superscript letters in a given column are not significantly different at p=0.05.

increase with concentrations of organic acid Cu complexes that may have a negative charge at wine pH (Pohl and Sergiel 2009) (i.e. citrate complexes, Supplementary Figure 1). Correlation of the total hydrogen sulfide after a year with these two different fractions of Cu at bottling suggests they may be active in the production of hydrogen sulfide in the protein-containing wine during bottle aging. This is consistent with higher cationic Cu fractions in the red wines, of the previous Section 6.9.1, inducing more hydrogen sulfide during wine aging. A similar outcome is evident for methanethiol and the cationic and residual fractions of Cu, although in this case the correlations are negative instead of positive. Consequently, it is the cationic and residual fractions of Cu that have an inverse relationship with the concentration of methanethiol. The results for bound hydrogen sulfide concentration at 1 year versus Cu fractions are similar to that of total hydrogen sulfide, which is consistent with the fact that the majority of hydrogen sulfide in the wine is in the bound form (Table 8). The correlation between the total Cu concentration and the free hydrogen sulfide concentration (in both μg/L and percentage of total) was negative as expected by the ability of Cu to convert free hydrogen sulfide to bound hydrogen sulfide (Franco-Luesma and Ferreira 2014). The cationic fraction of total Cu had the highest magnitude of the negative correlation inferring the more of this fraction of Cu the less free hydrogen sulfide.

Fe at bottling or after 1 year, and regardless of the fractionated form, showed negligible correlation with total hydrogen sulfide, methanethiol or any of the free and bound forms (data not shown). For example, the correlation of total Fe at bottling with total hydrogen sulfide after a year in bottle was $r^2 = 0.0924$.

6.9.2.2 The 1-year bottle aging of Chardonnay: Impact of protein stabilisation and oxidation

The wines treated with bentonite exhibited significantly lower Cu concentrations at bottling compared to those wines not treated with bentonite as already reported (Section 6.5.2). For the wines with bentonite added there was minimal Cu lost during aging (Figure 37a), unlike the non-protein stabilised wines. The lower Cu concentration in the wines treated with bentonite (Figure 37a), in combination with the lack of protein present in the wine, was most likely responsible for the minimal Cu lost during bottle aging.

After 1 year of aging there were decreased concentrations and proportions of hydrophobic and cationic fractions of Cu in the wines with bentonite treatment compared to the wine at bottling, while the concentration of residual Cu remained constant (data not shown) with a consequent increase in its proportion (Figure 37b). This residual fraction of Cu was previously shown to have the strongest positive correlation with oxygen consumption in the wine when ascorbic acid was present, and the previous Section 6.9.2.1 showed that its concentration at bottling was correlated with hydrogen sulfide production in the protein-rich wine after 1-year of bottle aging. The lack of increase in hydrophobic Cu (i.e., copper(I) sulfide) in the bentonite treated wines with bottle age is consistent with the ability of bentonite to remove both Cu and proteins, the latter being a potential source of hydrogen sulfide. Therefore, bentonite can limit formation of copper(I) sulfide that contributes to the hydrophobic fraction of Cu, and furthermore, with a reduction in protein concentration and hence source of hydrogen sulfide, the initial copper(I)

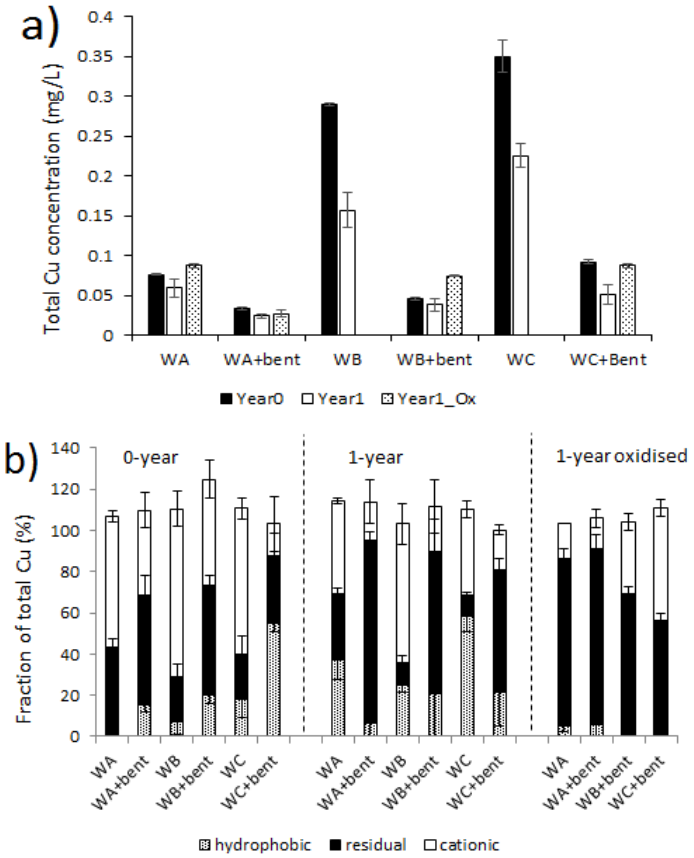


Figure 37. The total Cu concentration (a) and proportion of fractionated forms (b) in wines A-C, with and without bentonite treatment, at bottling and after 1 year in low and high oxygen conditions. The uncertainty shown corresponds to the standard deviation (n=3).

sulfide present at bottling may diminish if the wine is bottled with some oxygen (i.e., total packaged oxygen). Indeed, Table 8 shows a trend to lower concentrations of total hydrogen sulfide and methanethiol in the wines after 1-year that underwent bentonite treatment during production. If an alternative source of hydrogen sulfide, besides proteins, are present in the wine, such as polysulfides and/or polysulfanes, then the residual Cu favoured by bentonite treatment of the wine may still be able to undergo conversion to copper(I) sulfide during aging of the wine in low oxygen conditions.

The Fe speciation was similar to the wines without bentonite treatment, after 1 year of aging (Figure 38a and 38b). The bentonite treated wines showed slight decreases in the Fe concentration of the residual and hydrophobic fractions and increases in the cationic fraction, the latter being the dominant fraction in the wine (Figure 38b).

Wines A-C, with bentonite treatment, and wine A, without bentonite treatment, were aged in high oxygen conditions for 1 year. In high oxygen conditions (Figure 37a), no loss of Cu during aging was evident, supporting the requirement of low oxygen conditions, in combination with protein, to afford the loss of Cu in the form of a protein haze. Of particular note was the near absence of the hydrophobic fraction of Cu in the oxidised wines, and the dominance of the residual fraction of Cu both in terms of concentration and proportion (Figure 37b). Consistent with this was the significantly lower concentration of total hydrogen sulfide in the oxidised wines compared to those aged in low oxygen conditions (Table 8). In this present study, it is likely that the loss of the hydrophobic fraction of Cu during oxidation is a consequence of Cu(I)

sulfide being gradually converted to Cu(II) and oxidised products of sulfide (such as elemental sulfur, sulfate or polysulfides (Kreitman et al. 2016)), and the released Cu(II) then binding with other wine components to form the residual fraction of Cu. An oxidation product of organic acids in wine (i.e., oxalic acid) was shown to induce residual Cu in wine at pH 3.25 (the same pH as the wine under study) (see Supplementary Figure 1), but only when used at a concentration far exceeding that normally found in wine. It is more likely that a range of wine oxidation products are generated that are capable of converting Cu to the residual form as measured by SPE ICPOES.

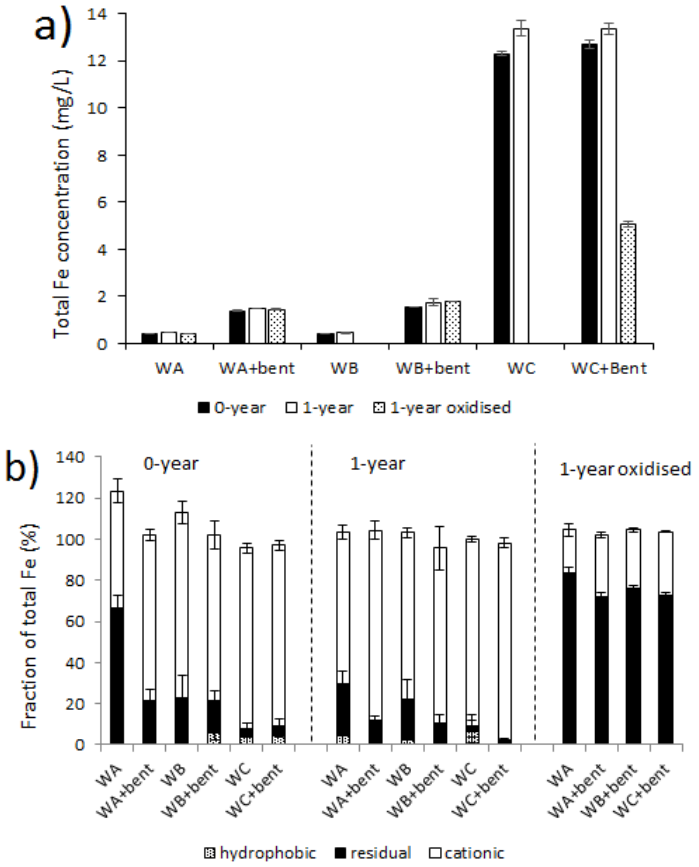


Figure 38. The total Fe concentration (a) and proportion of fractionated forms (b) in wines A-C, with and without bentonite treatment, at bottling and after 1 year in low and high oxygen conditions. The uncertainty shown corresponds to the standard deviation (n=3).

For the wine aged in high oxygen conditions, the total Fe concentration remained unchanged from their initial level at bottling, apart from the sample with highest Fe concentration, which decreased dramatically (by ~60 %) (Figure 38b). This is consistent with the ability of a high Fe concentration in wine to induce haze formation in oxidative conditions, which favours the conversion of Fe(II) to Fe(III) and its interaction with phosphate in white wines to afford a deposit (Joslyn & Lukton, 1953). In the oxidized wines, the concentration and proportion of the residual fraction of Fe dominated at the cost of the cationic fraction, and the hydrophobic fraction was largely absent (Figure 38b). This result suggests that oxidative conditions favours the conversion of Fe into its residual fraction, regardless of any precipitation of Fe in the form of a haze. As observed in Section 6.3.2, the residual fraction of Fe can be linked to an increase in oxidised organic acids.

6.9.2.3 The 1-year bottle aging of Chardonnay: Summary

The results allow insight into the impact of the initial concentration of metals in the juice prior to fermentation, and also wine protein, on metal fractionation during wine aging. The experimental strategy developed in this study has shown that the initial juice concentrations of metals, bentonite treatment and extent of oxygen supply to wines during bottle aging can influence both the total and the fractionated metal concentrations after 1 year. Low oxygen conditions in wines with protein present induced a significant loss of Cu particularly for those at elevated concentrations and a general increase of the hydrophobic fraction, consistent with the formation of copper(I) sulfide during aging. The concentration of residual and cationic Cu fractions at bottling correlated well with the total hydrogen sulfide in the wine after 1 year. Alternatively, no loss of total Cu or increase in the hydrophobic fraction of Cu was evident in bentonite fined wines but instead an increase in the residual fraction of Cu was favoured. That is, an increase in the fraction of Cu that correlated best with consumption of oxygen in wines with ascorbic acid added and the fraction most efficient at increasing hydrogen sulfide. Oxidative storage of the wines exhibited no loss of total Cu and caused a significant increase in the residual fraction and essentially eliminated the hydrophobic fraction.

In contrast to Cu, Fe was lost in oxidative conditions when at high concentrations rather than in low oxygen conditions. The cationic fraction of Fe dominated when aging occurring in low oxygen conditions, regardless of the presence of bentonite, and in high oxygen conditions the residual fraction of Fe was favoured, and linked to oxidised wine components complexing Fe(III).

6.10 The ratios of bound Cu to bound H_2S in red and white wines

There appeared to be an incongruity in the data presented within this report between the amounts of sulfide-bound copper measured versus the amount of total or bound hydrogen sulfide measured. Firstly, if it is assumed that only Cu is binding hydrogen sulfide in wine then a CuS or Cu_2S complex would be produced with Cu in either the +2 or +1 redox state, and consequently a Cu to sulfide ratio of either 1:1 or 2:1 would be expected. A ratio in between these two (~ 1-2:1) would be possible if Cu existed in both redox states during the binding with sulfide, although the Cu_2S form (i.e., Cu in a copper(I) state) has be proposed to be favoured in wine conditions.

However, if the wine composition is influencing the binding of Cu or sulfide, then further variations in the measured ratio would be observed. A lower apparent ratio could be possible if other metal ions were also binding sulfide, and thereby inducing a lower amount of Cu binding for a given amount of sulfide. However, of the metals in wine, Cu is seen as the strongest binder (Franco-Luesma and Ferreira, 2014). A higher apparent ratio could be also possible if other binding agents were present in the wine that contributed to Cu as being ‘bound’ by the speciation measures adopted. However, besides sulfide, no other binders were identified within this study. It must be mentioned that many of the binding components were only assessed in isolation and may provide more binding activity if combined, and this is an area of further work. Also, the ability of polysulfides and polysulfanes to bind Cu has not been assessed by the speciation techniques of this report. The results of this report do show that virtually all bound Cu in the wine can be converted to a non-bound form by storage of the wine in conditions to

induce oxidation and/or volatile losses of components. This again is consistent with bound copper being in the form of copper(I) sulfide and release of copper(II) through oxidative and/or volatile losses of hydrogen sulfide. Any additional unknown binder of copper in wine would also need to have a similar behaviour of binding that was dependent upon oxygen and/or aeration.

Finally, inaccuracies in the techniques used to measure the bound Cu and bound hydrogen sulfide concentrations could also contribute to higher or lower than expected ratios. This would include high ratios for measured bound Cu to measured bound hydrogen sulfide if the bound Cu was overestimated or the bound hydrogen sulfide underestimated.

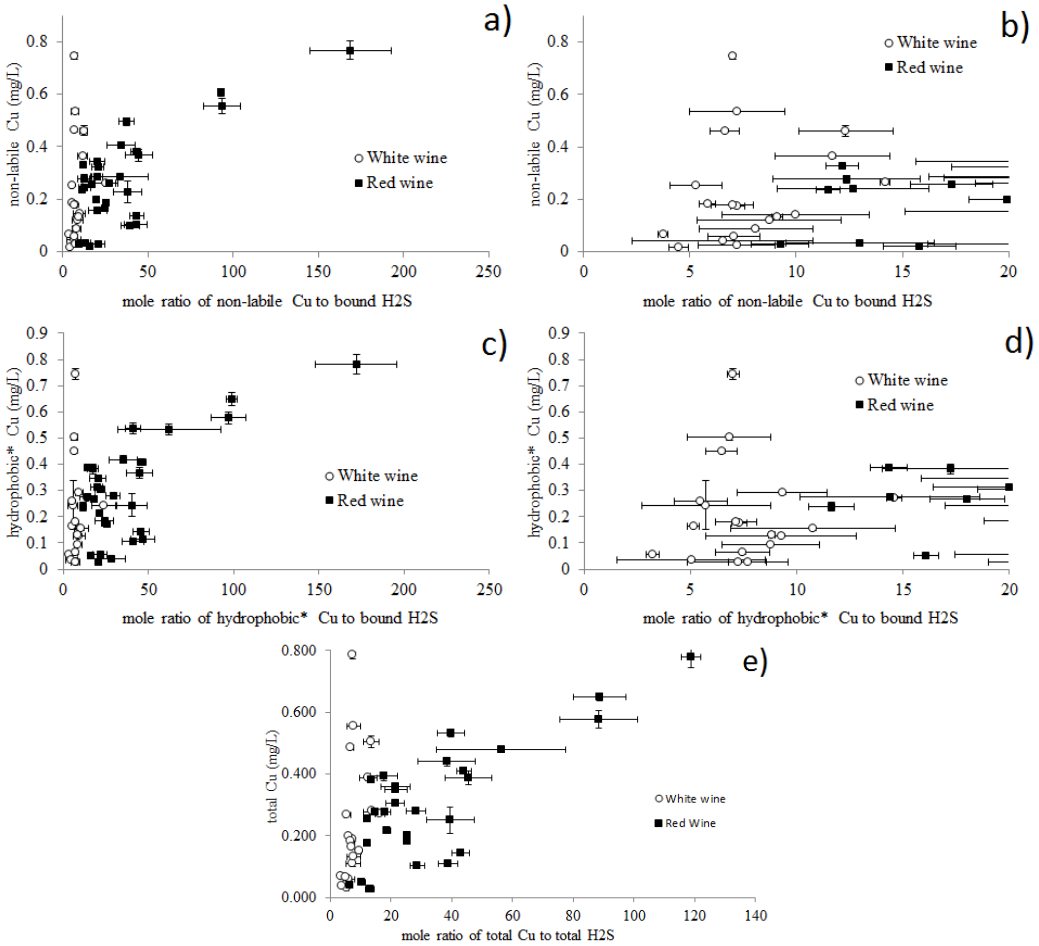
The ratios for the amount of measured bound Cu to bound hydrogen sulfide in a variety of studies is shown in Table 9. For the electrochemical technique, the non-labile Cu is taken as the bound Cu in both red and white wines, and non-labile Cu is determined by directly measuring labile Cu by electrochemistry and subtracting this from the total Cu in the wine (determined by ICPOES). For SPE ICPOES, the hydrophobic Cu (that trapped by a divinylbenzene SPE cartridge) is taken as the bound Cu in white wine, while for red wine it was the combined hydrophobic and residual Cu (the latter being Cu passing sequentially through divinylbenzene and cation exchange SPE cartridges, See Section 6.3). The SPE ICPOES also requires the measurement of total Cu in the wine by ICPOES in the calculation to determine the hydrophobic fraction of Cu. The bound hydrogen sulfide was determined by measuring the total hydrogen sulfide in wines with GC-SCD (wines+salt+heating, as reported in Kontoudakis, Smith et al. (2017) and adapted from Siebert et al. (2010)), and subtracting the free hydrogen sulfide (wine+heating, as reported in Kontoudakis et al. (2017) and adapted from Franco-Luesma and Ferreira (2014)). From Table 9, it is evident that the ratio of bound Cu to bound hydrogen sulfide is around 7-9 : 1 for white wine, 35-50 : 1 for red wines and around 25:1 when averaged over all the wines, and this was in agreement for the both Cu speciation measures utilised. This ratio is clearly much higher than the 1:1 or 2:1 expected of purely a Cu and S interaction, especially for red wine.

Table 9. Average ratios of measured bound Cu to measure bound hydrogen sulfide. Uncertainty is the standard deviation.

Section	Technique	Samples	White wine	Red Wine	All Wines
6.2	Electrochemistry	20 white, 29 red	(9 ± 2):1	(39 ± 7):1	(26 ± 5):1
6.2	SPE ICPOES	20 white, 29 red	(8 ± 2):1	(35 ± 5):1	(24 ± 4):1
6.9.1	SPE ICPOES	12 red	n/a	(50 ± 40):1	n/a
6.9.2	SPE ICPOES	9 white	(7 ± 4):1	n/a	n/a

Figure 39 provides a graphical representation of all the individual wines contributing to the ratios for the wines from Section 6.2 of the report. White wines (Figure 39b and 39d) show variations in the ratios but are consistently around 8:1 regardless of Cu concentrations. Alternatively, the red wines (Figure 39a and 39c) show that the ratio of Cu to hydrogen sulfide dramatically increases as the bound Cu concentration increases. Given that the two different measures of bound Cu behave similarly in their relationship to hydrogen sulfide, it decreases the likelihood that the methods are overestimating the measurement of bound Cu. As the concentration of free hydrogen sulfide and non-sulfide bound Cu are relatively low in the wines compared to the bound forms (See Section 6.3, Figure 1), plotting total Cu versus total hydrogen sulfide (Figure 39d) provides a similar ratio plot to that of the bound measures. In summary of the data in Figure 39, it appears that the higher the total or bound concentration of Cu in the

Figure 39. The ratio of measured Cu to measured hydrogen sulfide in red and white wines from Section 6.2. The (a) and (b, zoom) non-labile Cu (electrochemistry) versus bound hydrogen sulfide, (c) and (d, zoom) hydrophobic* Cu (SPE ICPOES) versus bound hydrogen sulfide, and (d) total Cu versus total hydrogen sulfide.



* For red wine, the 'hydrophobic Cu' was taken by SPE ICPOES as hydrophobic + residual fractions, while for white wine it was just the hydrophobic fraction.

red wine the higher the ratio of bound Cu to bound hydrogen sulfide, while in white wine the ratio of bound Cu to bound hydrogen sulfide is higher than expected but largely independent of Cu concentration.

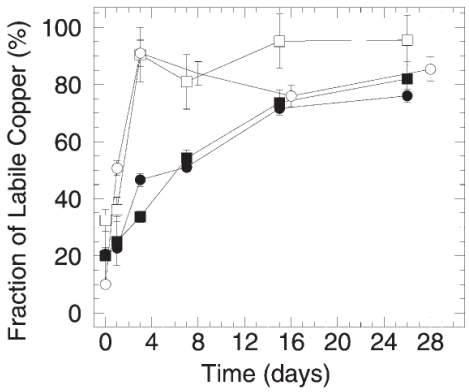
To provide an additional perspective, speciation data was assessed for the change in bound Cu relative to the amount of hydrogen sulfide added to wines. In essence, to provide ratios of measured bound Cu to added hydrogen sulfide (rather than measured hydrogen sulfide) and avoiding the complication of needing to release Cu from sulfide. From Table 10 it is evident that the ratios obtained are much lower than those in Table 9 and lower than the expected 1:1 or 2:1. In this case the lower ratios may have been due to other metals in the wine competing for binding to the sulfide. Alternatively, losses of hydrogen sulfide through volatility or oxidation may also have been possible. Kreitman et al. (2016) propose oxidation of some sulfide during the reduction of copper(II) by sulfide to form copper(I) sulfide, and in a model wine they obtained a ratio of H_2S to Cu of ~1.4:1 which corresponds to a Cu to H_2S ratio of ~0.7. This is similar to the value obtained in Table 10 for the electrochemically measured bound Cu to added H_2S in the model wine from Section 6.6.

Table 10. The ratio of increase in measured bound Cu to added hydrogen sulfide.

Section	Technique	Samples	White wine	Red Wine	Model Wines
6.3.1	Electrochemistry	1 white	0.4:1		
6.6	Electrochemistry	2 model wine			(0.75 ± 0.04):1
6.7.2	Electrochemistry	6 red		(0.2 ± 0.2) : 1	
6.9.3	Electrochemistry	6 white	(0.17 ± 0.04):1		

As the high ratios observed in Table 9 and Figure 39 could be due to an underestimation in the amount of measured total hydrogen sulfide in the wines, the three wines with the highest bound Cu to bound hydrogen sulfide ratios in Figure 39 were measured by two different pre-treatment procedures to measure total hydrogen sulfide in wine by GC-SCD. During the extraction step, wines were treated with salt addition (as a solid) or a 10-fold dilution of the wine with a brine solution. The 10-fold dilution was chosen as it had been reported to allow complete dissociation of copper(I) sulfide at room temperature (Chen et al. 2017) when using commercially available colorimetric gas detection tubes. The results showed no trends towards lower concentrations of total hydrogen sulfide detected upon the salting of the wine compared to treatment with brine (Appendix 6, Supplementary Figure 5). Consequently, it would appear that the pre-treatment (salting vs brine dilution) of the wines prior to GC-SCD analysis could not account for the relatively low concentrations of bound hydrogen sulfide relative to non-labile Cu in red wines of high Cu concentration (Figure 39). However, it was still possible that both methods were still underestimating the metal-bound sulfide in some wines. It has been noticed that recovery of total hydrogen sulfide in model wines of copper(I) sulfide, prepared *in situ*, are often very low (<< 50 %) when analysed by GC-SCD and sample pretreatment involved heating with salt addition.

Figure 40. The measurement of labile copper fraction (of total copper concentration) during the accelerated oxidation of four white wines. Error bars represent the 95 % confidence limit.



The efficiency of release of Cu from copper(I) sulfide would appear to be relatively wine dependent. Previous research showed that the release of bound Cu, as determined electrochemically, by heating the wine at 45 °C could occur rapidly for some wines whilst others required days (Figure 40). Furthermore, the GFAAS speciation measure described in Section 6.2 (Figure 1c), showed that a 2-fold dilution of wine with 8 M ammonium thiocyanate with 5 minutes of mixing at room temperature, followed by a further 2-fold dilution with methyl

isobutyl ketone, only led to a measurement of a fraction of the total copper in some wines. Although this salting and diluting of the wine led to lower bound Cu concentrations than the more mild-pretreatment techniques of SPE ICPOES and electrochemistry, there were still some wines that had a large component of bound Cu by the harsh sample pretreatment regime of the GFAAS method. In particular, the three red wines with the highest bound Cu to bound hydrogen sulfide ratios in Figure 39, also had the highest bound Cu concentrations of all the wines by the GFAAS method. Other work has shown that the Cu within copper(I) sulfide in model wine is more difficult to detect by FAAS than in white wines and requires the addition of Ag(I) to aid its dissociation in model wine. Finally, at 45 °C, copper(I) sulfide was proposed to dissociate more easily in white wine than model wine to allow copper(II) to catalyse reactions between (+)-catechin and glyoxylic acid (Clark et al. 2015).

Despite the evidence presented, at this stage it is not possible to ascertain the exact reason for the high bound Cu to bound hydrogen sulfide concentrations observed in Table 9 and Figure 39. The possibilities remain that i) some other binder of Cu exists in wines that has not yet been determined with respect to the Cu speciation measures used, ii) the measured total hydrogen sulfide is being underestimated, and/or iii) the measured bound Cu is being overestimated. This area of research is worth further investigation.

6.11 Colorimetric assay for measurement of Cu in wine

Based on the work conducted within the project, it was deemed necessary to develop a method for total Cu determination in wine that would be easily adopted by the majority of Australian winemakers. Consequently, it was decided to optimise a colorimetric method that would require equipment no more specialised than a spectrophotometer. Many wineries currently have access to spectrophotometers for wine colour measurements and enzymatic test-kit analyses. The major hurdle in such a colorimetric measure was the ability to detect Cu at sub-mg/L concentrations despite the background colour of wine. In addition to a manual colorimetric measure, an alternative automated method that is already available, but requires access to a more expensive chemical analyser (i.e., Konelab system), was also assessed for accuracy.

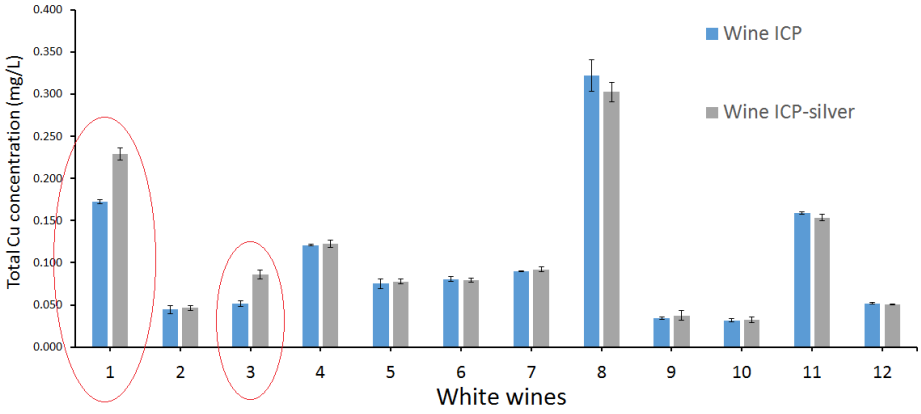
6.11.1 Measurement of total Cu in white wines: Silver improves measurement

During the project, it was found that addition of silver(I) nitrate ($AgNO_3$) to model wine samples containing copper(I) sulfide aided the accuracy of FAAS determination of total copper in these samples. The rationale was that the added silver (I) can bind sulfide in preference to copper(I) ions, as it has a higher affinity for binding sulfide than copper(I), and thereby release copper(II) into the non-sulfide bound form for easier detection by FAAS.

Consequently, given that the majority of copper in wine is in the form of copper(I) sulfide (Figure 1), it was decided to investigate whether silver(I) may aid the determination of total Cu by ICPOES. Previously, wines were diluted 4-fold by 5 %(v/v) aqueous nitric acid prior to determination. As the copper(I)/copper(II) in wine is atomised within a higher temperature plasma by ICPOES versus a flame in FAAS, ICPOES was expected to be less impacted by sulfide

binding copper(II) than FAAS. The results in Figure 41 show that the majority of white wines analysed showed no impact of Ag(I) on the ICPOES determination, however, there were several wines in which the impact of Ag(I) was significant (wines 1 and 3, Figure 41). This supported previous observations that in some wines, copper can be more difficult to remove from sulfide binding than in other wines. It also meant that for any colorimetric reagent to efficiently access Cu, it was most likely that the Cu first had to be released from any sulfide-binding it, and that Ag(I) was an efficient means of releasing the Cu.

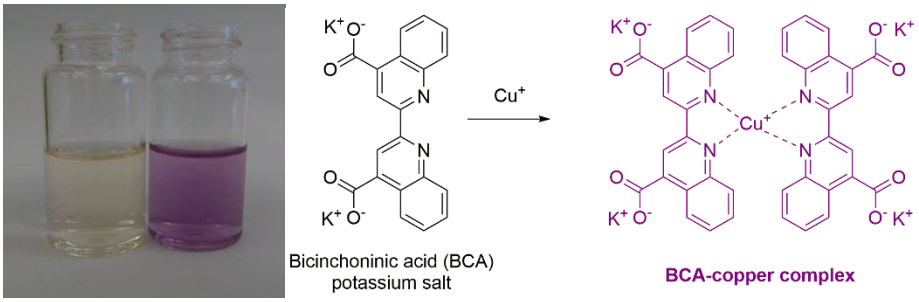
Figure 41. Impact of Ag(I) on the determination of total Cu in white wines by ICPOES. The error bars indicate the standard deviation (n=3).



6.11.2 Measurement of total Cu in white wines by BCA: Manual spectroscopic method

After assessment of various colorimetric reagents for Cu, the reagent bicinchoninic acid (BCA) (Figure 42) was found to have the required selectivity for Cu whilst also providing sufficient colour intensity at 563 nm for a given amount of Cu. Two ions of BCA interact with copper(I) providing the complex shown in Figure 42. The formed complex appears purple in colour with a maximum of absorbance at 563 nm. This means that the reagent is well suited to detect Cu in white wines that have little background absorbance at the wavelength of 563 nm, but less suited to detection in red wines (See section 6.11.1.3). During the traditional use of this colorimetric analysis for Cu, reducing agents such as ascorbic acid or hydroxylamine are required to induce formation of copper(I) and subsequent complexation by BCA.

Figure 42. Wine samples with and without BCA (left hand side), and the reaction for the formation of the purple BCA-copper(I) complex (right hand side).

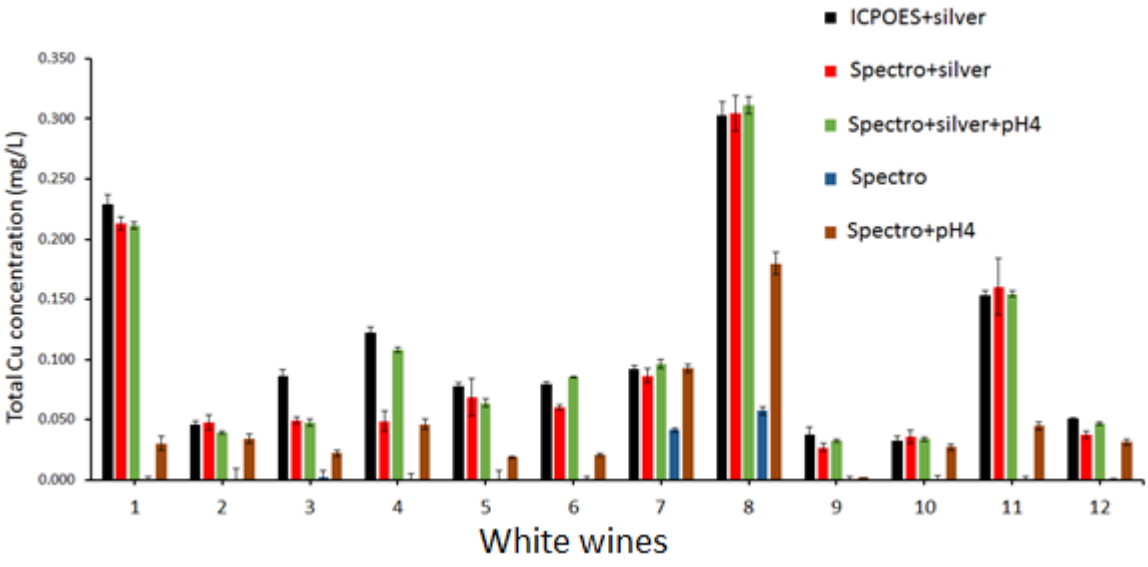


For analysis of total copper concentration in wine, a number of parameters of the colorimetric measure were optimised including reaction time, reagent concentrations, reducing agents and

associated concentrations, temperature, and sample pH were investigated. During optimisation, it was noticed that a precipitate often formed after addition of the reagents to wine, and that it was critical to measure the absorbance of samples soon after their filtration. Turbidity measurements showed that precipitate gradually formed after filtration and that eventually this turbidity could impact absorbance measurements. Also, despite the inherent sensitivity of the colorimetric reagent, the determination was best performed with a 40 mm glass cuvette. Such cuvettes are not expensive (~ $30-40) but do require a spectrophotometer that can hold them. Many spectrophotometers already have the capability to use cuvettes larger than the standard 1 and 10 mm path length cells, but some varieties of spectrophotometer may require a specialised cuvette holder or may simply not be amenable to such a cuvette. As will be shown, the concentrations determined by the BCA method were accurate down to 0.040 mg/L, and therefore if a 10 mm instead of 40 mm cuvette was utilised, this equivalent lower limit for Cu determination would be expected to be around 0.12 mg/L. Quantification was achieved in the following results with the use of three standard additions of Cu to the wine, but analysis of the data showed that the use of two or one standard addition/s would have provided equivalent results (data not shown). However, two standard additions would be recommended to limit the onset of experimental errors during the determination of copper concentration.

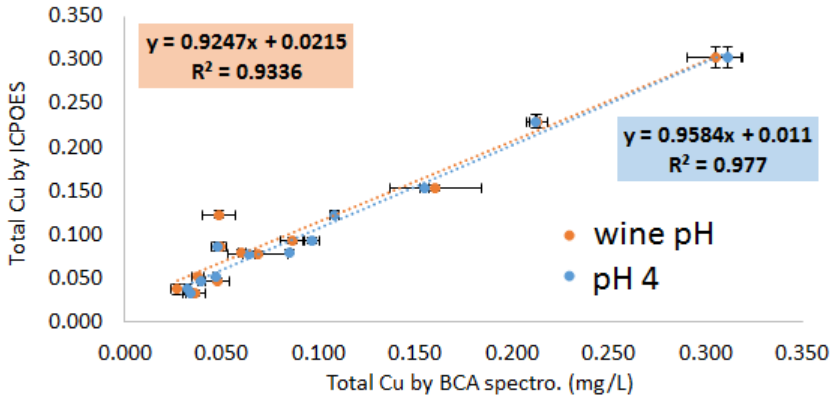
Figure 43 shows the application of the BCA colorimetric method to 12 white wines. The results show the best agreement between the ICPOES data (black bars, Figure 43) with the wines that were adjusted to pH 4 and had silver addition prior to analysis by the BCA colorimetric method (green bars, Figure 43). The impact of wine pH was not large, with only one wine (wine 4, Figure 43) showing improved determination at pH 4 compared to wine pH. The addition of silver(I) to samples prior to analysis had a huge effect and appeared to be a critical component in the analysis to ensure that copper was in a non-sulfide bound form able to be complexed by the BCA reagent. Interestingly, in the absence of silver addition, more copper could be detected by the BCA reagent at pH 4 compared to wine pH.

Figure 43. The determination of total Cu in white wines by ICPOES versus the BCA colorimetric method ('Spectro'). The colorimetric method was performed at wine pH and pH 4, and also with and without silver addition. The error bars indicate the standard deviation (n=3).



The correlation plots for the ICPOES and BCA colorimetric analysis data are shown in Figure 44, with the latter analysis performed with and without pH modification of wines to pH 4.0. For the wines adjusted to pH 4 (i.e., best results), the majority of the BCA colorimetric results were within 0.02 mg/L of the ICPOES results with only one wine outside this range and at a discrepancy of around 0.04 mg/L Cu (Wine 3, Figure 43). There appeared to be a slight trend towards underestimation by the BCA colorimetric technique but based on the results in Figure 43, this underestimation was only significant for wine 3.

Figure 44. The correlation between measures of total Cu by ICPOES compared to the BCA colorimetric method. The BCA method was conducted at wine pH and also with adjustment of the wines to pH 4 prior to analysis. All samples had Ag(I) addition prior to analysis.



The validation parameters of the technique are as follows:

- ***Recovery.*** The average recovery was 104 ± 9 % for 12 white wines with addition of 0.1 mg/L copper(II).
- ***Accuracy.*** The accuracy of the technique for determination of total copper in white wines was equivalent to that of ICPOES as shown in Figures 43 and 44.
- ***Repeatability.*** The repeatability was assessed in terms of the average relative standard deviation of total copper concentrations, and was 3 ± 2 % for 12 white wines.
- ***Specificity.*** The BCA reagent is specific for Cu in wine, with no influence from Mg, Mn, Fe, Zn and Al.
- ***Linearity.*** The technique is linear within the range tested from 0.04 to 1.0 mg/L copper.
- ***Stability.*** Samples should be filtered 30 minutes after addition BCA to wines, and then measured immediately afterwards.
- ***Matrix effects.*** Eliminated with silver(I) additions to wine and with the use of quantification via standard additions.

6.11.3 Digestion of red wine prior to analysis by the BCA method

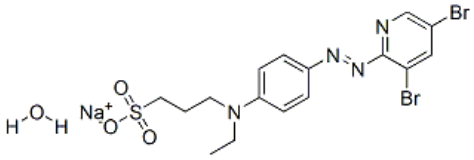
In order to allow the measurement of total Cu in red wines by the BCA method, a significant portion of the red phenolic pigmentation had to be removed in order to provide a sufficiently low absorbance background at 563 nm. This meant that the native anthocyanins and polymeric pigment required bleaching prior to analysis. The best approach appeared to be the incubation of 200 mL of red wine with 20 mL of 1 mol/L sodium hydroxide and 20 mL of 30 %(v/v) hydrogen peroxide for 3-5 hours at 60 °C.

Further work is required to validate whether the BCA method can accurately determine the total Cu concentration in red wine after its digestion.

6.11.4 Measurement of total Cu in white wines by DiBr-PAESA: Automated spectroscopic method

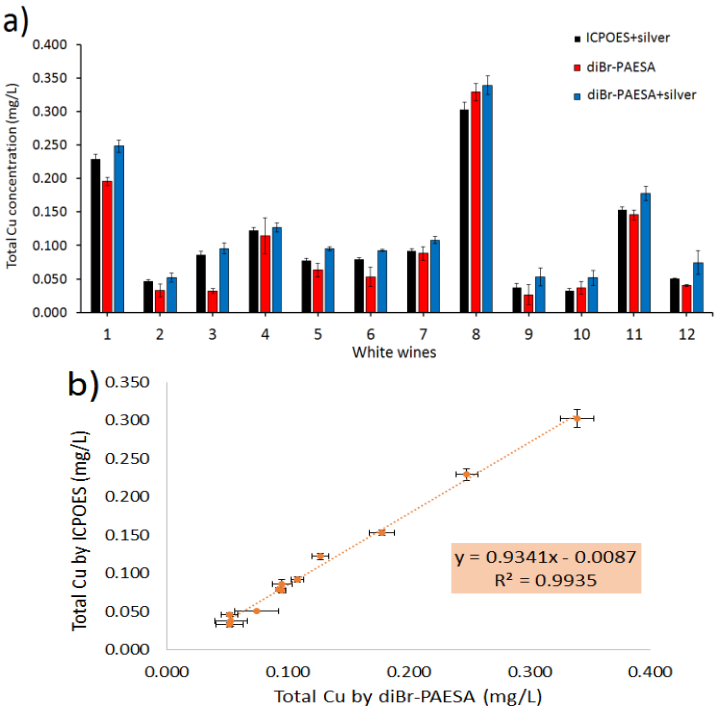
An automated method was also investigated which used the colorimetric reagent 4-(3,5-dibromo-2-pyridylazo)-N-ethyl-N-sulfopropylanaline-monosodium salt (diBr-PAESA, Figure 45). This method required an automated chemical analyser, and the results below were obtained utilising a Thermo Scientific™ Konelab™ 20 Clinical Chemistry Analyser system with the Thermo Scientific copper test kit (984628).

Figure 45. The diBr-PAESA reagent.



A comparison of results was made with and without the addition of silver(I) nitrate to wines, to provide silver(I) concentrations of 10 mg/L, in order to establish whether better results would be achieve in conditions more conducive to non-sulfide bound copper. The results in Figure 46a show that the samples analysed with the diBr-PAESA reagent and with silver addition compared better to the ICPOES results than the equivalent samples without silver. Without silver(I) there was a general underestimation of total Cu, although it wasn't to the same extent as observed for the BCA reagent. The correlation plot (Figure 46b) shows good agreement between the two techniques.

Figure 46. The a) determination of total Cu in white wines by ICPOES versus the automated diBr-PAESA colorimetric method, and b) the associated correlation plot. The error bars indicate the standard deviation (n=3).



This method may also be well suited to the analysis of total Cu in red wine, particularly after the addition of silver(I) to the wine. However, further work is required to validate such an approach. Also, further work is required to assess if digestion of the red wine will enable more accurate measures by the automated colorimetric method rather than just direct addition of the red wine with silver(I) addition.

6.12 'How to' guide for winemakers

A 'How to' guide for the determination of total Cu in white wine by the BCA method was prepared. The 'How to' guide provides a list of the equipment and reagents required, including associated photos. It also provides the detailed instructions and calculations to allow accurate determination of Cu in white wine. The 'How to' guide is attached within Appendix 6 of this report. The method will be published in a Scientific Journal and also made available on the NWGIC website (https://www.csu.edu.au/nwgic/engagement/oenology-resources). An industry article will be written to describe the method and to indicate its accessibility on the NWGIC website and within the Journal Article. The method will also be taught to students within the CSU Wine Science course.

6.13 Teaching materials

A range of material presented within this report have been inserted into the teaching materials for the Wine Science course at Charles Sturt University. This is one of the major educators of winemakers in Australia. Specifically the material within this report have been inserted into the subject WSC304 Wine Chemistry, including both general course work and practical work material. Table 6 and Figures 1, 5, 9, 11 and 17 of this report have been included in the teaching materials. Some key aspects covered within the teaching materials are:

- The ability of yeast to remove the majority of grape-derived Cu.
- That copper(I) sulfide formed in wine is largely not visible, does not settle and is mostly smaller than the pores of membrane filtration material used in wineries.
- Grape-derived copper that is present in protein-unstable white wine can be significantly lowered in concentration by bentonite treatment.
- PES and nylon membrane filters can remove some copper(I) sulfide from wine by adsorption rather than size discrimination.
- Non-sulfide bound copper(II) within wine readily evolves to copper(I) sulfide during the bottle aging of most wines.
- The majority of copper in bottled wine is in the form of copper(I) sulfide.
- A non-sulfide bound copper concentration of 0.02 mg/L can ensure that the free hydrogen sulfide concentrations are below the aroma threshold. However, as the non-sulfide bound copper concentration decreases during bottle aging, the low free hydrogen sulfide concentration can only be guaranteed in the short-term.
- For white wines with ascorbic acid, non-sulfide bound copper is a much more efficient mediator for the reaction with oxygen than copper(I) sulfide.

The colorimetric measurement of total Cu in wine will also be incorporated into the practical class for WSC312 Wine Science2.

7 OUTCOMES AND CONCLUSIONS

The overall objectives of this project were to:

1. Survey a range of wines for copper and iron speciation.
2. Identify the components of wine that influence the speciation of copper and iron.
3. Relate the metal speciation measures of copper and iron to the rate of wine oxidation, and the formation of volatile sulfur compounds in reductive conditions.
4. Relate the metal speciation measures to existing colorimetric assays of metal reactivity and modify the assays as required to reflect the ability of metals to affect spoilage processes.

The specified outputs and activities of the project are addressed in the various sections of this report as indicated in Table 1.

7.1 Assessment of speciation techniques

A larger number of published research-orientated Cu and Fe speciation measures were assessed for their suitability and applicability to both red and white wines. However, based on their level of sensitivity and repeatability many were found to be not suitable. Others were modified to improve their application to wine and included the development of a novel electrochemical approach for the measurement of Cu speciation in wine using screen printed carbon electrodes.

7.2 Survey of Australian wines for metal speciation

A survey was performed on 49 wines donated by Australian wineries, from the 2007 – 2014 vintages (i.e., a bottle age ranging from 1 to 8 years old when measured), and showed average concentrations of 0.28 mg/L Cu and 1.8 mg/L Fe. Electrochemical and SPE ICPOES measure of the Cu speciation in the wines showed that a single form of Cu dominated the vast majority of wines. In contrast, the SPE ICPOES measure of Fe speciation showed that Fe was distributed in all different fractions assessed.

7.3 Identification of binding components of metals

The assessment of individual wine components for the potential to bind Cu and Fe in model wine systems showed that the main binder of Cu in wine was hydrogen sulfide generating copper(I) sulfide as the product. Several red wines provided evidence suggesting either an alternative binder for Cu in these wines or that the concentration of the metal-bound hydrogen sulfide was being underestimated in these wines, but further work is required to investigate these possibilities. Overall, this study showed that the main form of Cu in the vast majority of Australian wines is copper(I) sulfide and this observation was largely independent of closure type. While only six wines in the survey were closed under cork, all still had the vast majority of Cu in the form of copper(I) sulfide. The implication of these observations is that if any wine had late additions of Cu prior to bottling, the Cu must have evolved to a sulfide-bound state. It also suggests that the majority of wines have more than enough sulfide-precursors to enable the gradual binding of Cu upon aging.

For Fe, the classification of its form depended on pH and phenolic concentration, and with clear differences between red and white wines. In white wines, of low pH and phenolic concentration, the major fraction of Fe was characteristic of Fe-organic acid complexes, whilst in red wines of high pH and high phenolic concentration, the major fraction was characteristic of Fe-phenolic interactions. Citric acid was observed to be an efficient binder of Fe and induced different fractionation of Fe compared to other organic acids in model wine. There was no influence of sulfur compounds, including hydrogen sulfide, on the speciation classification of Fe by the SPE ICPOES technique.

7.4 Removal of Cu from protein-rich wine by bentonite treatment

In a survey of 100 Chardonnay juices during the 2009 Australian vintage, the median Cu concentration was around 1.0 mg/L, with 50% of juices between approximately 0.5 and 1.5 mg/L, and the highest juice at ~7.0 mg/L (Schmidt et al. 2011). Based on this range of Cu in juice, Chardonnay juice was fermented with concentrations of 1, 3 and 6 mg/L Cu to provide corresponding wines with 0.083, 0.233 and 0.293 mg/L Cu, respectively. As already known, yeast is efficient at removing most Cu during fermentation, but a general trend was evident whereby wine made from juice with high Cu concentrations had higher Cu in the wine than if made from juice with low Cu. This demonstrates that a Cu concentration higher than the average for the surveyed 49 Australian wines (0.28 mg/L) can be achieved simply through the level of Cu in the juice and without Cu addition to the wine. In contrast, little Fe is removed by yeast fermentation and the wine Fe concentration was quite similar to that of the juice. During production of the Chardonnay wines, bentonite led to an increase in Fe concentration, by around 0.4-1.0 mg/L, but was quite efficient in removing the Cu from the wines with reductions of 50-85 % (to less than 0.1 mg/L); it was however proposed that grape-derived proteins in the wine were important for the efficiency of copper-removal by bentonite. Further work in establishing this link between bentonite-treatment, and the need for proteins and type of proteins, for copper removal in wine is required. The removal of Cu, especially that in the form of copper(I) sulfide, during processing may be an effective way of removing a potential reservoir of hydrogen sulfide in the wine.

7.5 Removal of copper(I) sulfide from wine by filtration

Particle size measurement on model wines and white wines showed that removal of copper(I) sulfide from wine by membrane filtration was due to adsorption rather than size discrimination. Indeed, the copper(I) sulfide particles in model and white wines were smaller than the 0.45 µm pores of membrane filters used in wine production, and even 0.20 µm membrane filters would be largely ineffective based on size exclusion. The small copper(I) sulfide particles were able to remain dispersed in the wine and did not settle. White wine proteins and polysaccharides were components of white wine that could hinder the adsorption of copper(I) sulfide onto the filtration media. The composition of the filtration media was important with PES and nylon being more efficient in adsorbing copper(I) sulfide from white wine than cellulose and Teflon based membrane media. PES and nylon are currently used as the favoured membrane filters in wineries. Further research is required, preferably on a larger applied scale, to investigate depth filtration media, as this type of filter should be more efficient in adsorbing copper(I) sulfide than membrane filters, and also cross flow filtration. The composition of depth filtration media,

which are usually cellulose based and with or without bentonite present, would be worthy of investigation, especially the viability of depth filters composed of PES or nylon. Equivalent studies on copper(I) sulfide removal in red wine are also necessary.

7.6 Minimising Cu additions for the removal of free hydrogen sulfide in wine

Importantly, the Cu speciation measurements showed that for the 49 wines assayed, when the concentrations of non-sulfide bound Cu were above 0.02 mg/L, the free hydrogen sulfide was below 1 µg/L, which is a typical aroma threshold for hydrogen sulfide. This could provide a means of limiting the amount of Cu added to wine during fining in order to achieve removal of free hydrogen sulfide. Wine with non-sulfide bound Cu concentrations already above 0.02 mg/L could be identified as not requiring further Cu addition. Alternatively, wines requiring Cu addition based on sensory assessment could undergo a Cu fining trial (as per the typical procedure) and the wine with the desired Cu rate tested for non-sulfide bound Cu to ensure that it is not excessively over 0.02 mg/L.

7.7 The transformation of Cu species between different forms

The results in this work showed that Cu can readily convert between sulfide and non-sulfide bound forms, with aeration and oxidation favouring the non-sulfide bound form while anaerobic conditions favoured the sulfide-bound form. A significant amount of conversion (> 50%) can occur within hours, for transition of sulfide-bound Cu to the non-sulfide bound form, or within days, for the reverse transition. Ascorbic acid appeared to enhance the stability of copper(I) sulfide in the presence of oxygen, allowing it to survive longer for a given amount of oxygen compared to the sole presence of phenolic compounds in model wines. The different behaviour of copper(I) sulfide in wine with ascorbic acid present may be important for the Australian wine industry given that a snap shot of 16 white wines (price point <$15) showed most contained ascorbic acid. Further research would investigate how specific winery operations could impact the Cu forms.

7.8 Cu speciation and the reductive aging of wine

A bottle trial of Shiraz and Cabernet Sauvignon showed that late additions of Cu to the wine provided wines with a higher proportion of non-sulfide bound Cu prior to bottling. Within two months, the non-sulfide bound Cu in the wines had converted to a sulfide-bound form, and concurrently, the measured total hydrogen sulfide (i.e., free + metal-bound forms) in the wines increased. The increase in total hydrogen sulfide was larger for those wines with Cu addition at bottling than those without. As this bottle trial was commenced at the start of the project, when the speciation measures were less refined, it would be worth repeating and over a longer time scale with more frequent measurements. A bottle trial for Chardonnay also showed that the non-sulfide forms of Cu at bottling correlated most with the total hydrogen sulfide concentrations in the wines after 1-year. These results suggest that the non-sulfide bound form of Cu is the form of Cu most efficient in promoting increases in total hydrogen sulfide observed during bottle aging. It is however unclear if the sulfide-bound form of Cu has any activity in inducing the formation of sulfide that can accumulate as free hydrogen sulfide in the wine. It would be of interest to assess if high concentrations of other metals, such as Fe, Mn, Zn and Al, could induce accumulation of higher concentrations of free hydrogen sulfide if all the Cu in the

wine is in a sulfide-bound form. Related studies on total concentrations of these metals already shows a synergistic effect on total hydrogen sulfide production (Viviers et al. 2013).

The Chardonnay wines prepared with bentonite fining, and subsequent reduced Cu and protein concentrations, tended to lower sulfide-bound forms after 1-year of bottle aging, contrary to what was observed for the non-bentonite fined wine. In these wines at least, the ability of Cu to sequester sulfide appeared to be limited by the bentonite treatment and presumably the removal of protein from the wine. Further research on the impact of residual concentrations of protein in wine as a source of sulfide should be conducted along with the concentrations at which they can still act as a significant source of sulfide. As the Chardonnay wines were not aged with ascorbic acid, it would be of interest to assess the impact of ascorbic acid on the behaviour of copper(I) sulfide during bottle aging.

7.9 Metal speciation and the oxidation of wine

In terms of oxidation, a specific form of Cu was quite important for mediating oxidation in white wines containing ascorbic acid. The non-sulfide bound form of Cu was a much more effective oxidation catalyst than the sulfide-bound form. This means that white wines containing ascorbic acid will consume oxygen much more rapidly if copper additions are made to the wine whereby large concentrations of non-sulfide bound Cu remain. Under normal bottle aging conditions, Cu will exist in sulfide-bound form which is a non-efficient catalyst of oxidation, but upon oxygen exposure, the sulfide bound form will gradually transform to the more catalytic non-sulfide bound form. The ramifications are probably more important in a winery in terms of minimising access of the wine to oxygen prior to additions of ascorbic acid and Cu, by limiting aeration, splashing and/or oxygen exposure thereafter. The concentrations of Fe in white wines did not appear to have a substantial impact on the rates of oxygen consumption when ascorbic acid was present. Interestingly, the Chardonnay prepared from juice of different Cu concentrations showed faster rates of oxidation in the wines prepared from juices of higher Cu concentrations. Therefore, even without addition of Cu to the wines, the juice Cu concentrations could still impact oxygen consumption rates in the wines.

In the absence of ascorbic acid, both white and red wines showed little dependence on non-sulfide bound Cu for the oxidation rates. In fact, if anything, there was a greater influence of the sulfide-bound Cu on oxygen consumption rates in these wines, but further research is required on this point. It however demonstrates the significant influence ascorbic acid can exert on oxidation mechanisms in white wines and the relative importance of the different forms of metals. In the wines without ascorbic acid, the amount of Fe added as Fe(II) to red wine appeared to be more critical on the rates of oxidation than Cu. Further research is required on the impact of Fe speciation in these types of wines. A novel measure is also required for the quantification and discrimination of Fe(II) and Fe(III) in red wines. Using the catalytic ability of Cu and Fe to accelerate the reaction between (+)-catechin and glyoxylic acid as a tool for metal activity in wine was not found feasible at this stage due to conversion of the metal forms during the assay.

7.10 A colorimetric measure of Cu in wine

A colorimetric assay to detect the total concentration of Cu in wine was developed. Previously, no simple measure of total Cu in wine was available to winemakers. The method utilises an addition of silver(I) to wine in order to release the Cu from sulfide and standard additions to the wine to allow quantification. The test will allow winemakers to assess the amount of Cu present in wine after fermentation, after bentonite fining and also how much Cu remains in wine after Cu fining trials. Currently, the technique is also being modified to allow a measure of the non-sulfide bound fraction of Cu. Measurement of this form of Cu may allow winemakers to determine if Cu addition to the wine is necessary (i.e., no addition is necessary if it already contains sufficient non-sulfide bound Cu), and if the wine is prone to high rates of oxygen consumption (i.e., high non-sulfide bound Cu, ascorbic acid and high dissolved oxygen) at any given point in the production process.

8 RECOMMENDATIONS

The following are the recommendations from this project:

Broad Industry Practises

- Winemakers need to recognise that copper fining of wine results in dispersed copper(I) sulfide that does not settle and cannot be removed from white wine via filtration on the basis of particle size exclusion. Instead, filtration may remove some copper(I) sulfide via adsorption rather than size exclusion. PES and nylon membrane filters showed a higher efficiency of copper(I) sulfide adsorption than cellulose and Teflon while pore size of the membrane (0.20 µm vs 0.45 µm) did not have a significant influence.
- Bentonite appears particularly efficient in removing Cu that has survived fermentation in a protein-rich wine. This was particularly the case for the Chardonnay wines studied whose Cu concentration was above 0.25 mg/L.
- This research shows that late copper additions to wine, such as those made at bottling, will invariably result in an increase in the non-sulfide bound Cu. During bottle aging, this Cu will evolve into the sulfide-bound form. It would appear that most wines have sufficient sulfide precursors present that are far in excess of any allowable Cu addition rates at bottling. Further research is required to assess the ability of sulfide-bound Cu, as opposed to non-sulfide bound Cu, to continue to increase hydrogen sulfide production during wine aging.
- A concentration of 0.02 mg/L for non-sulfide bound Cu appears to ensure the concentration of free hydrogen sulfide is reduced below sensory detection thresholds. This may provide winemakers a target level of non-sulfide bound Cu to aim for during Cu fining in order to minimise Cu additions to wine. Wines found to be above 0.02 mg/L non-sulfide bound Cu could be identified as not requiring any further Cu fining, whilst wines requiring Cu could undergo addition to achieve concentrations just above the 0.02 mg/L non-sulfide bound Cu concentration.

- The non-sulfide form of Cu is much more efficient in catalysing the reaction between oxygen and ascorbic acid than the sulfide-bound form of Cu. This is not the case for ascorbic acid-free wine, where the non-sulfide form of Cu did not show any correlation with oxygen consumption rates. The ramifications of the oxygen consuming efficiency of the 'ascorbic acid/non-sulfide bound Cu' duo are more important where white wine may have access to oxygen during wine production. This situation is also crucial in order to avoid additions of ascorbic acid and copper until oxygen supply to the wine is prevented (i.e., ullage removed and/or dissolved oxygen lowered). Otherwise, close attention should be made to replenish sulfur dioxide in the wine as the dissolved oxygen concentration in the wine is lowered through rapid reaction with ascorbic acid. During bottle aging under non-compromised screw cap closures, oxygen should not be problematic unless large amounts of total packaged oxygen is present at bottling.
- The colorimetric assay can be utilised by winemakers to assess the levels of Cu in their wines post-fermentation even if Cu additions to the wine have not been made. The colorimetric assay may also be used to assess the amount of Cu post-bentonite treatment and/or after copper fining.

Future research

- The impact of membrane filtration on copper(I) sulfide removal should be investigated on a larger scale than the laboratory scale used within this report. Assessment of cross flow filtration, cellulose depth filters impregnated with bentonite for copper(I) sulfide removal is required, and if possible, PES and nylon depth filter media.
- Further research is required to establish whether some non-sulfide bound Cu may be beneficial in certain wines with high concentrations of other metals that may still induce hydrogen sulfide formation but not bind it. Furthermore, the impact of Cu speciation on methanethiol production should be established and assessment made if production of this thiol is more detrimental to wine as Cu cannot bind it as efficiently as hydrogen sulfide.
- Further research is required on the high measured bound Cu to measured bound hydrogen sulfide ratios found in this study. The high ratios suggest either some unknown binder of Cu is present in wine, the measure of total hydrogen sulfide in wine is being underestimated by currently established techniques, or that the measures of bound Cu in this report are over-estimated.
- Further work is required on the efficacy of bentonite to remove Cu in wine during production, including the influence of grape-derived proteins, other types of proteins (isinglass, gelatine), concentration of proteins, and Cu form. Furthermore, the use of small amounts of bentonite in red wine could be investigated for Cu removal.
- More research should be conducted on the impact of ascorbic acid on the sulfide-sequestering ability of non-sulfide bound Cu and general comparison to white wines bottled without ascorbic acid.
- Given the reversibility of the Cu forms in wine, investigations should be conducted into the rate of release of sulfide and hydrogen sulfide from copper(I) sulfide and whether the amounts released can impact wine sensory assessment. For example, 0.25 mg/L of

copper in wine as copper(I) sulfide could have the potential to release 65 μg/L hydrogen sulfide. Is there a rate of release of sulfide from the copper(I) sulfide that could impact sensory features of the wine such as fruit repression or induce off-aromas? Is the decanting of wine prior to tasting, and subsequent improved flavour (i.e., opening up of the wine), related to the release of excess bound sulfide from copper(I) sulfide?

- A longer term bottle trial on red and white wines, with copper additions prior to bottling, should be conducted with the established speciation measures within this report. This could combine the impact of ascorbic acid in white wines. The influence of Cu speciation on methanethiol production should also be explored further.
- Further research is required on the role of Fe speciation on the oxidation of white wines and red wines. Coupled to this, is the need for a technique to measure Fe(II) and Fe(III) in red wines.
- The colorimetric assay needs to be refined in further work to allow the measurement of low levels (e.g. 0.02 mg/L) of non-sulfide bound Cu.
- Sensory studies should be performed to establish when reductive aromas in wine become evident with respect to the evolution of Cu species in wine. For example, does reductive aroma dominate before or after Cu becomes predominantly bound to sulfide in wine? Is reductive aroma mainly due to methanethiol which may accumulate before hydrogen sulfide in wine or does reductive aroma require hydrogen sulfide to accumulate? Is there a repression in fruit flavour prior to the overt detection of reduced aromas in wine, and does this relate to the conversion of Cu from non-sulfide bound to completely bound by sulfide?

9 Appendix 1: Communications

Publications in international peer review journals:

Guo, A., Kontoudakis, N., Scollary, G. R., and Clark, A. C. (2017). Production and isomeric distribution of xanthylium cation pigments and their precursors in wine-like conditions: Impact of Cu(II), Fe(II), Fe(III), Zn(II), and Al(III). Journal of Agricultural and Food Chemistry **65**, 2414-2425.

Kontoudakis, N., Guo, A., Scollary, G. R., and Clark, A. C. (2017). The impact of aging in high and low oxygen conditions on the fractionation of Cu and Fe in Chardonnay wine. Food Chemistry **229**, 319-328.

Kontoudakis, N., Smith, M., Guo, A., Smith, P.A., Scollary, G.R., Wilkes, E. and Clark, A.C. (2017). The impact of wine components on fractionation of Cu and Fe in model wine systems: Macromolecules, phenolic and sulfur compounds. Food Research International **98**, 95-102.

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

Rousseva, M., Kontoudakis, N., Schmidtke, L., Scollary, G.R. and Clark, A.C. (2016). Impact of wine production on the fractionation of copper and iron in Chardonnay wine: Implications for oxygen consumption. Food Chemistry **203**, 440-447.

Submitted.

Kontoudakis, N., Schmidtke, L. M., Bekker, M. Z., Smith, P.A., Scollary, G.R., Wilkes, E. and Clark, A.C. (2018). Analytical strategies for the measurement of different chemical forms of Cu and Fe in wine: Comparison between approaches in relation to wine composition. Food Chemistry, *submitted 11th January 2018.*

Kontoudakis, N., Mierczynska-Vasilev, A., Guo, A., Smith, P.A., Scollary, G.R., Wilkes, E. and Clark, A.C. (2018). The removal of copper(I) sulfide from white wine by membrane filtration. The Australian Journal of Grape and Wine Research, *submitted 30th January 2018.*

Publications in journals dedicated to the wine industry:

Clark, A.C., Cleghorn, N., Grant-Preece, P., and Scollary, G. R. (2015). Copper fining of sulfidic off-odours – some complex white wine chemistry with practical outcomes. Wine and Viticulture Journal, January/February, 14-16.

Oral communications at conferences:

Clark, A. (2017). Keynote presentation: Analytical strategies for the measurement of different chemical forms of Cu and Fe in wine: Relevance to wine oxidation and copper(I) sulfide stability. Xth In Vino Analytica Scientia, Salamanca, Spain, 17-20th July.

Kontoudakis, N. (2017) Filterability of copper sulfide in white, red and model wines. Crush, Adelaide, 13-14th November.

Kontoudakis, N., Smith, M., Smith, P., Scollary, G. R., Wilkes, E., and Clark, A. C. (2016). Fractionation of copper and iron in wine: Assessment of potential macromolecule and sulfur binding agents. Macrowine, Changins, Switzerland, 27-30 June.

Kontoudakis, N. (2015) The SPE fractionation of Cu and Fe in Chardonnay wine: Impact of wine production and the influence on oxygen consumption. Crush, Adelaide, 20th November.

Clark, A., Rousseva, M., and Schmidtke, L. M. (2015). Copper and iron speciation in wine by SPE-ICPOES: The influence of juice metal concentrations and bentonite-treatment on speciation, and the subsequent impacts on oxygen consumption. IXth In Vino Analytica Scientia, Trento, Italy, 14-17 July.

Oral communications for the industry:

Clark, A. C. (2017). Copper fining. WSC304 Wine Chemistry residential school, Charles Sturt University, Wagga Wagga, 1-3rd July.

Kontoudakis, N. (2017). Metal ion speciation: Understanding its role in wine development and generating a tool to minimise wine spoilage. NWGIC Seminar Series, Wagga Wagga, 6th June.

Clark, A. C. (2016). Radicals and singlet oxygen: Fundamental research, questions in wine. The University of Melbourne, November.

Clark, A. C. (2016). Copper fining. WSC304 Wine Chemistry residential school, Charles Sturt University, Wagga Wagga, 2-4th July.

Kontoudakis, N. (2015). Metal ion speciation: Understanding its role in wine development and generating a tool to minimise wine spoilage. NWGIC Seminar Series, Wagga Wagga, 11th November.

Clark, A. C. (2015). Wine, models, mood-lighting and heavy metal: A wine chemists dream. Australian Wine and Research Institute, March.

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Kontoudakis, N., Guo, A., Scollary, G. R., and Clark, A. C. (2017). The impact of emtals on the production and isomeric profile of wine phenolic oxidation products: LC-HRMS, electrochemistry and SPE ICPOES. Xth In Vino Analytica Scientia, Salamanca, Spain, 17-20th July.

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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

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13 Appendix 5: Additional Tabulated Data

13.1 Supplementary Table 1. Copper concentration (mg/L) in 10 commercial wines after copper(II) addition and after hydrogen sulfide addition at a ratio of 1:1 (hydrogen sulfide to copper(II)).

Wine	Adjusted copper conc.	Setting 1 day			Settling 5 days
		Racking†	F (0.45) †	F (0.20) †	Racking†
W5	0.953±0.006	0.96±0.01	0.87±0.03	0.84±0.02	0.94±0.01
W6	0.97±0.01	0.953±0.006	0.86±0.02	0.77±0.01	0.98±0.01
W7	1.04±0.06	0.94±0.02	0.87±0.06	0.8±0.1	0.97±0.01
W8	1.00±0.01	0.966±0.006	0.95±0.02	0.89±0.02	0.974±0.005
W9	0.97±0.02	0.882±0.006	0.959±0.006	0.923±0.006	0.974±0.001
W10	0.926±0.006	0.85±0.03	0.92±0.02	0.897±0.006	0.928±0.001
W11	0.97±0.02	0.943±0.006	0.90±0.02	0.85±0.01	0.942±0.005
W12	0.94±0.01	0.93±0.02	0.90±0.02	0.88±0.03	0.928±0.001
W13	0.94±0.02	0.95±0.01	0.903±0.006	0.913±0.001	0.95±0.01
W14	0.95±0.02	0.927±0.006	0.92±0.01	0.93±0.01	0.960±0.005

All uncertainty values are given as the standard deviation (n=3).
† Clarification type: racking, F(0.45) = filtration with pore size 0.45 µm, and F(0.20) = filtration with pore size 0.20 µm

13.2 Supplementary Table 2A. All wines (red and white wines combined): Correlation table for compositional parameters versus metal analyses. Positive correlation is shaded in red and negative correlation in blue.

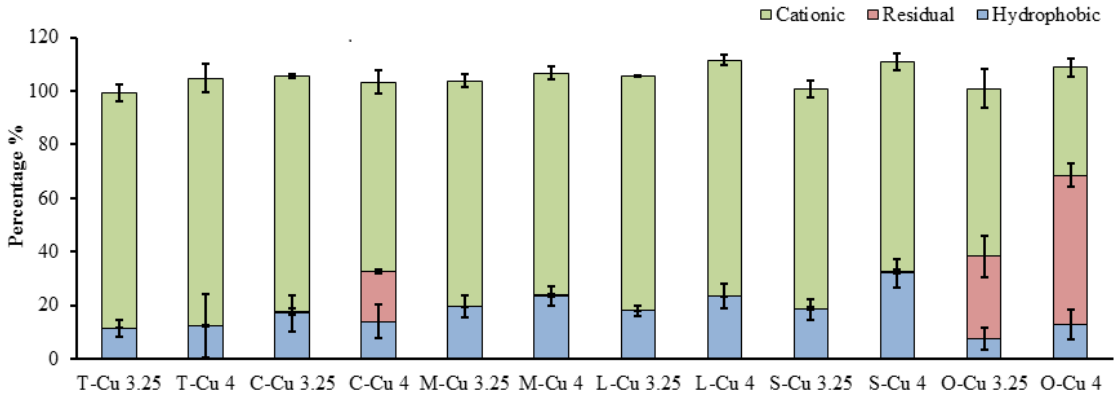
	Cu	Cu Hyd	Cu Res	Cu Cat	LabCu GF	NonLabCu GF	LabCu Elec	NonLabCu Elec	Fe	Fe Hyd	Fe Res	Fe Cat	FePadapHPLC	Mn	Al	Zn	Mg
Free SO_2	0.0688	0.0014	0.2061	0.1389	0.3174	-0.2119	0.1007	0.0558	0.2834	0.0156	0.1310	0.3975	0.3065	0.2702	0.1408	0.0294	0.0493
Total SO_2	-0.0166	-0.0088	-0.0381	0.0694	0.2023	-0.2302	-0.0015	-0.0173	-0.1403	-0.1102	-0.4001	0.3290	-0.1555	-0.0234	0.1040	0.1882	-0.4714
pH	0.1633	0.0425	0.4492	0.0954	0.1059	0.1505	0.1867	0.1414	0.3396	0.0926	0.5759	-0.1109	0.4193	0.1062	-0.0992	-0.3119	0.5872
TA	0.2860	0.2274	0.2739	0.1810	0.2589	0.1899	0.3561	0.2429	0.0338	0.1924	0.1239	-0.2597	-0.1009	0.1674	-0.0598	0.4436	0.2218
Ethanol	0.1070	-0.0019	0.3920	0.1018	0.0898	0.0777	0.2198	0.0766	0.2073	0.0398	0.3960	-0.1447	0.2603	-0.1275	-0.0517	-0.4853	0.5071
Total H_2S	0.4657	0.5029	-0.0383	0.2997	0.5612	0.1680	0.0692	0.4789	0.0478	0.4532	-0.2924	0.2128	-0.1521	0.1714	0.1356	0.4910	-0.4065
Free H_2S	-0.3559	-0.2886	-0.2496	-0.2550	-0.4266	-0.1311	-0.1693	-0.3469	-0.1287	-0.0953	-0.2640	0.1778	-0.1131	-0.1999	0.0065	-0.2379	-0.1642
Bound H_2S	0.4929	0.5223	-0.0117	0.3199	0.5937	0.1781	0.0852	0.5049	0.0601	0.4538	-0.2591	0.1901	-0.1373	0.1884	0.1321	0.5055	-0.3812
Total MeSH	-0.2307	-0.1806	-0.2116	-0.1818	-0.2616	-0.1001	-0.1428	-0.2195	-0.0587	-0.1465	-0.2556	0.2998	-0.0047	-0.1643	0.0753	-0.1275	-0.2315
Free MeSH	-0.2181	-0.1464	-0.2774	-0.2061	-0.2669	-0.0748	-0.1947	-0.1977	-0.0521	-0.0994	-0.2520	0.2910	0.0249	-0.1655	0.0246	-0.1473	-0.1901
Total DMS	-0.0788	-0.0345	-0.1514	-0.1493	-0.2700	0.1483	-0.0743	-0.0705	0.0285	0.0158	0.1388	-0.2100	0.0988	-0.1087	0.0791	-0.2003	0.1386
Total CS_2	0.0022	0.0258	-0.0867	-0.0615	0.0379	-0.0350	-0.0667	0.0131	0.1859	0.0530	-0.0621	0.4171	0.2361	0.1820	0.1535	-0.0558	-0.0811
Citric acid	-0.0106	0.0784	-0.3073	0.0932	-0.0211	0.0047	-0.1126	0.0073	-0.0038	0.3017	-0.5108	0.4161	-0.1679	0.0518	0.2608	0.2655	-0.5057
Glucose	-0.1578	-0.1009	-0.2071	-0.0951	-0.1398	-0.1073	-0.1323	-0.1442	-0.2364	-0.1453	-0.1939	-0.0958	-0.2624	0.1746	-0.0377	0.4759	-0.3143
Fructose	-0.1853	-0.1282	-0.2179	-0.1093	-0.1775	-0.1126	-0.1292	-0.1738	-0.3009	-0.1790	-0.2478	-0.1379	-0.3154	0.0755	-0.0791	0.4152	-0.3373
Succinic acid	0.1164	0.0086	0.4156	0.0245	0.0388	0.1442	0.3067	0.0723	0.2495	0.0697	0.5679	-0.3282	0.2939	0.0589	-0.1816	-0.2796	0.6794
Lactic acid	0.1334	0.0913	0.2057	-0.1243	-0.0470	0.2576	0.1254	0.1200	0.2895	0.0909	0.5292	-0.1681	0.3842	0.1553	-0.1274	-0.2047	0.6277
Glycerol	0.1671	0.0618	0.4149	0.0456	0.0520	0.2107	0.3053	0.1259	0.2588	0.0829	0.6250	-0.3578	0.2977	0.0329	-0.1809	-0.3106	0.7704
Acetic acid	0.1010	0.0380	0.2917	-0.0744	-0.0198	0.1791	0.2526	0.0650	0.0414	0.0659	0.3886	-0.4220	0.0652	-0.0831	-0.3637	-0.1614	0.5761
Tartaric acid	0.0412	-0.0070	0.1241	0.1106	-0.0138	0.0787	0.0333	0.0378	-0.0799	-0.0090	0.1818	-0.4031	-0.1165	-0.0475	-0.2992	0.2347	0.2426
Malic acid	-0.0777	-0.0002	-0.2910	0.0446	0.0062	-0.1285	-0.1713	-0.0537	-0.2792	-0.0436	-0.6624	0.3444	-0.3422	-0.0819	0.1742	0.3386	-0.7212

13.2 Supplementary Table 2B. Red wines: Correlation table for compositional parameters versus metal analyses. Positive correlation is shaded in red and negative correlation in blue.

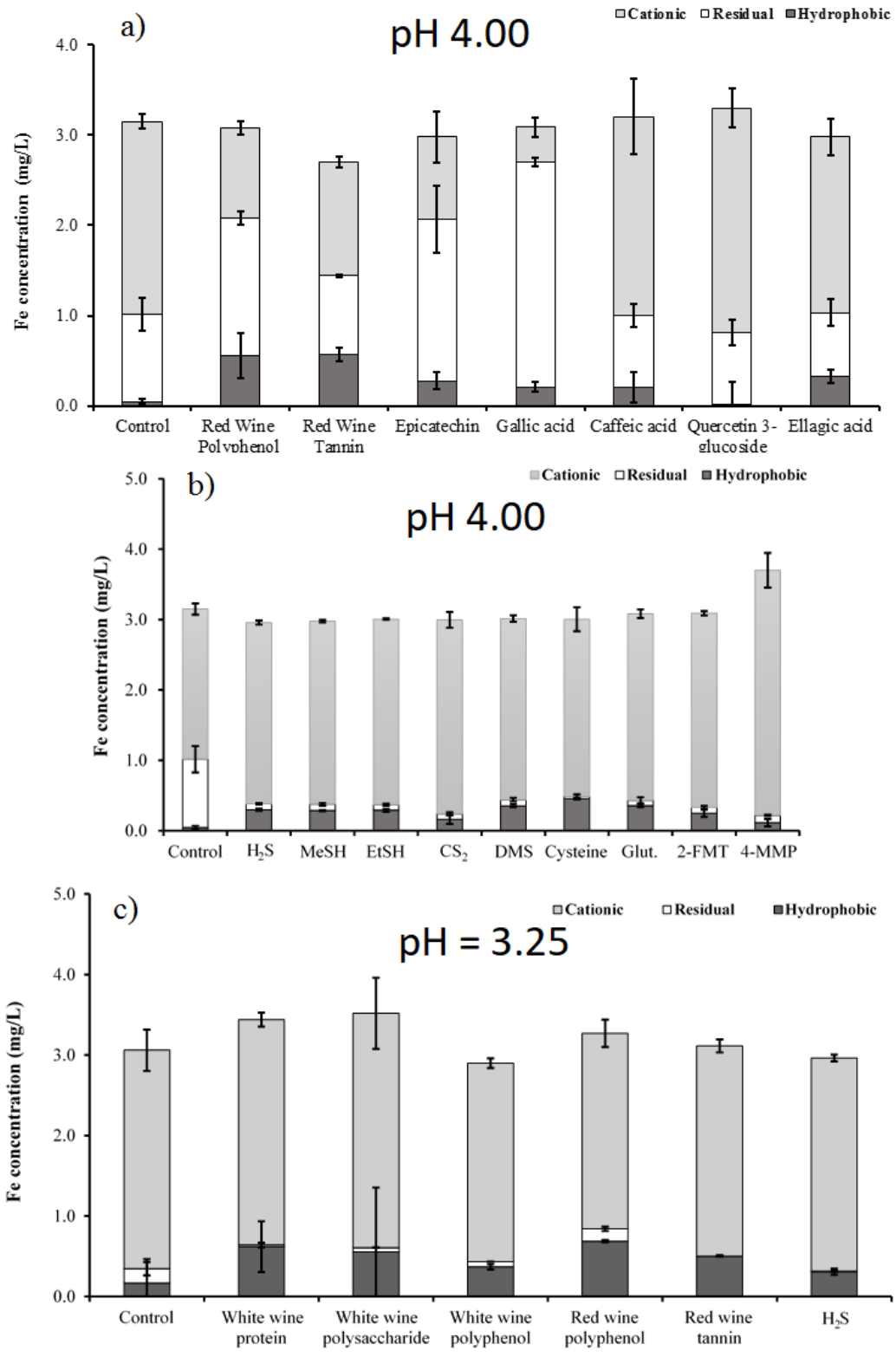
	Cu	Cu Hyd	Cu Res	Cu Cat	LabCu GF	NonLabCu GF	LabCu Elec	NonLabCu Elec	Fe	Fe Hyd	Fe Res	Fe Cat	FePadapHPLC	Mn	Al	Zn	Mg
Free SO_2	0.2122	0.0747	0.4187	0.2710	0.5996	-0.1823	0.1939	0.1837	0.3432	0.1576	0.4138	0.2787	0.3067	0.4032	0.0056	0.3484	0.2610
Total SO_2	0.1903	0.0754	0.3964	0.1347	0.5548	-0.1765	0.3054	0.1371	0.3288	0.1652	0.3876	0.2675	0.2993	0.3182	-0.0202	0.2971	0.2382
pH	0.1030	0.0066	0.3121	0.1102	0.0871	0.0666	-0.0499	0.1194	0.1289	0.2555	0.0746	0.1349	0.2138	0.0507	-0.0996	0.3383	0.1252
TA	0.4745	0.3399	0.5669	0.1521	0.5360	0.2045	0.5356	0.3894	-0.0053	0.1563	-0.0382	-0.0906	-0.1302	0.1391	-0.0237	0.4358	0.2168
Ethanol	-0.0259	-0.1056	0.2857	0.1096	0.0845	-0.0983	0.1949	-0.0684	-0.4319	-0.3043	-0.3982	-0.3921	-0.3821	-0.3136	-0.0240	-0.1226	-0.2500
Total H_2S	0.2534	0.1802	0.2492	0.2466	0.4978	-0.0523	0.2948	0.2057	0.3052	0.2415	0.4076	0.1942	0.2622	0.1156	0.2843	0.1702	0.1341
Free H_2S	-0.2966	-0.2331	-0.1995	-0.2033	-0.4587	-0.0339	-0.1407	-0.2841	-0.1692	-0.0216	-0.1619	-0.1284	-0.2148	-0.2154	-0.0493	-0.3058	0.0859
Bound H_2S	0.3061	0.2224	0.2810	0.2795	0.5744	-0.0425	0.3115	0.2580	0.3277	0.2346	0.4237	0.2132	0.2967	0.1571	0.2816	0.2288	0.1089
Total MeSH	0.2636	0.2899	-0.0749	-0.0897	0.0643	0.2905	0.0090	0.2766	0.4720	0.4665	0.4939	0.3514	0.4140	0.3740	0.4501	0.5034	0.4702
Free MeSH	0.1171	0.1917	-0.2380	-0.1420	-0.1663	0.2770	-0.1410	0.1532	0.2580	0.3328	0.2836	0.2066	0.3636	0.1654	0.1329	0.2117	0.4659
Total DMS	-0.1930	-0.0687	-0.4091	-0.2264	-0.4658	0.1054	-0.1857	-0.1646	-0.1309	0.0191	-0.1968	-0.2548	-0.0518	-0.2393	0.1720	-0.2587	-0.2288
Total CS_2	0.0512	0.1081	-0.2006	-0.1633	0.1346	-0.0363	0.0574	0.0421	0.5920	0.3197	0.6056	0.4232	0.5272	0.4989	0.4281	0.1622	0.3310
Citric acid	-0.0021	0.0758	-0.2133	-0.2076	-0.1838	0.1373	-0.0168	0.0017	0.0061	0.0268	-0.0524	-0.1009	-0.0715	0.1115	0.2553	-0.1304	0.0883
Glucose	-0.0268	-0.0156	-0.0776	0.0975	0.1744	-0.1673	-0.1209	-0.0029	0.5005	0.3464	0.5222	0.2965	0.2935	0.5895	0.3620	0.0437	0.1898
Fructose	-0.0629	-0.0903	0.0194	0.1581	0.1652	-0.2069	-0.0521	-0.0555	0.5365	0.2620	0.5636	0.3624	0.3243	0.6568	0.3799	0.1226	0.1918
Succinic acid	0.0070	-0.0688	0.2994	0.0190	0.0003	0.0091	0.2647	-0.0477	-0.1655	-0.1229	-0.2282	-0.1500	-0.1437	-0.0858	-0.1688	-0.0055	0.0008
Lactic acid	0.1897	0.2371	-0.0684	-0.2451	-0.1023	0.3227	-0.0232	0.2056	0.0784	0.2019	0.0395	0.0665	0.1760	0.1476	-0.0093	0.3348	0.2536
Glycerol	0.2236	0.1464	0.3744	0.0805	0.0290	0.2662	0.3971	0.1531	-0.4171	-0.1368	-0.4266	-0.3857	-0.4257	-0.2631	-0.2420	-0.0070	0.1869
Acetic acid	-0.0279	-0.0526	0.1682	-0.1312	-0.1319	0.0645	0.1588	-0.0628	-0.4235	-0.1653	-0.4299	-0.2734	-0.3760	-0.4355	-0.3330	0.0808	0.0057
Tartaric acid	0.3755	0.2098	0.5011	0.4618	0.4512	0.1407	0.0590	0.3838	-0.0005	0.0103	0.0229	0.1217	-0.0370	-0.0676	-0.3281	0.2508	0.0701
Malic acid	0.1506	0.1587	-0.0220	0.1075	0.1876	0.0513	0.0641	0.1459	0.4536	0.4044	0.4720	0.1653	0.3261	0.5794	0.3709	0.2772	0.3733

14. Appendix 6: Additional Figures

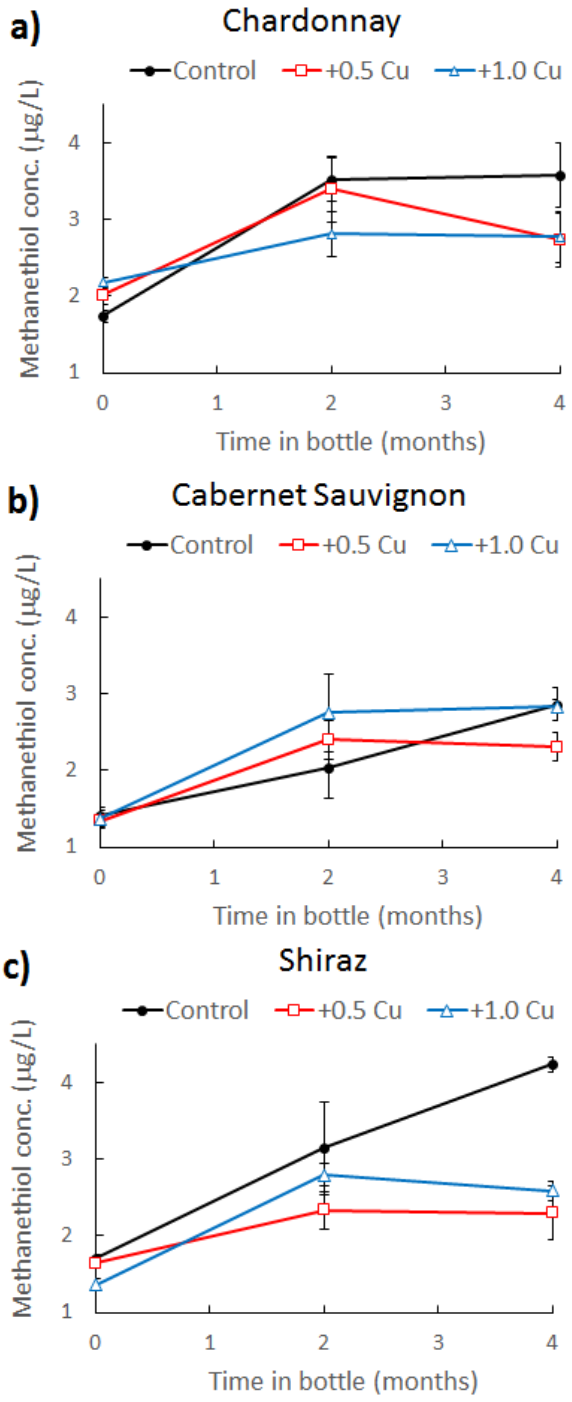
14.1 Supplementary Figure 1. The impact of pH (3.25 and 4.00) and organic acids (0.018 M) in model wine solutions on the speciation of 0.40 mg/L Cu as determined by SPE ICPOES. The organic acids utilised were tartaric acid (T), citric acid (C), malic acid (M), lactic acid (L) and oxalic acid (O). The concentration of 0.40 mg/L is represented as 100 % on the y-axis.



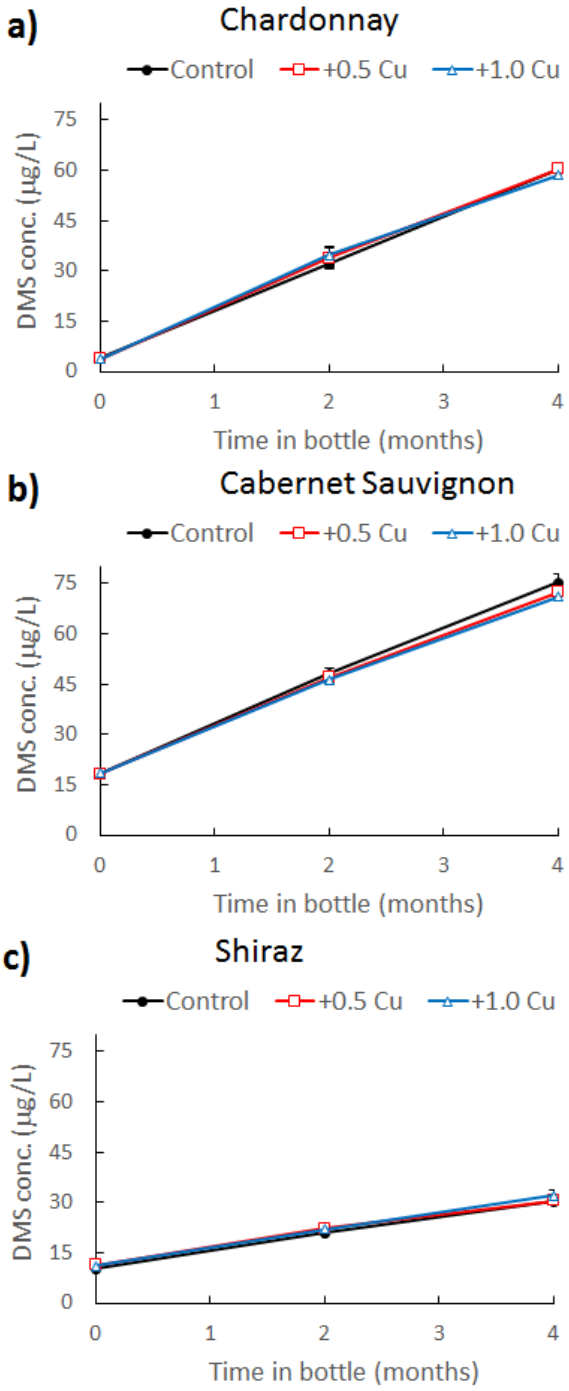
14.2 Supplementary Figure 2. The concentrations of hydrophobic, residual and cationic forms of Fe in model wine systems at pH 4.00 with a) extracted red wine macromolecules and monomeric phenolic compounds, and b) sulfur-containing compounds, and c) the model wine at pH 3.25 with extracted white and red wine macromolecules and hydrogen sulfide. The error bars are the st.dev. (n=3).



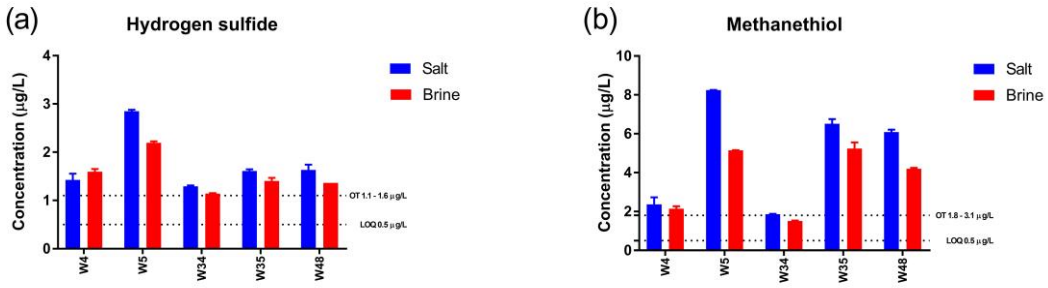
14.3 Supplementary Figure 3. The concentrations of methanethiol in two red wines and one white wine with modified Cu concentrations.



14.4 Supplementary Figure 4. The concentrations of methanethiol in two red wines and one white wine with modified Cu concentrations.



14.5 Supplementary Figure 5. Comparison of addition of salt or 10-fold addition with brine for the determination of (a) total hydrogen sulfide and (b) total methanethiol in wine.



15. Appendix 7. The Determination of Total Cu in White Wine by BCA Colorimetric Analysis

Nikolaos Kontoudakis[a,b], Mark Smith[c], Paul A. Smith[c], Eric N. Wilkes[c], Andrew C Clark[a,b]

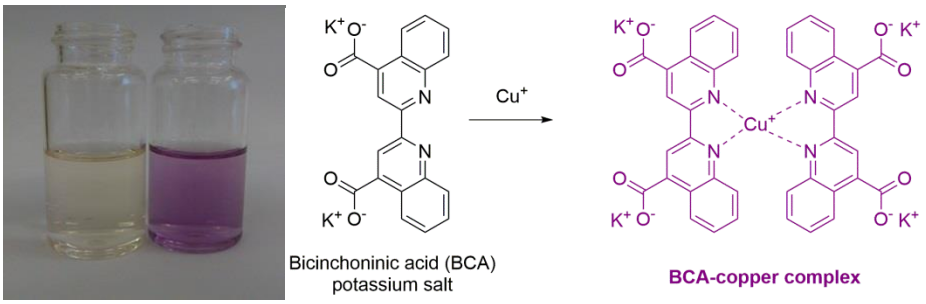
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[b] School of Agricultural and Wine Sciences, Charles Sturt University, Locked Bag 588, Wagga Wagga, NSW 2678, Australia

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Method Overview

This method utilises the colorimetric reagent 2,2′-bicinchoninic acid dipotassium salt (BCA) to react with copper(I) and form a purpled coloured complex, which absorbs at 563 nm. During the analysis, ascorbic acid is added to aid the conversion of copper(II) to copper(I). Also, silver(I) nitrate is added in order to induce dissociation of suspended copper(I) sulfide in wine, and thereby to provide non-sulfide bound copper ions and silver(I) sulfide. The background absorbance at 563 nm is sufficiently low in white wine to allow successful determination of total copper concentrations, whilst the same is not the case in red wine.



Wine samples with and without BCA (LHS), and the reaction for the formation of the purple BCA-copper complex (RHS).

Equipment and Reagents Required

- Preferably de-ionised water, and if possible ultra-pure water (18.2 μΩ), for the preparation of Cu standards and reagents. This will ensure as little Cu contamination within the water as possible.
- Analysis of metals at concentrations below 1 mg/L often involves soaking glassware with 10 %(v/v) nitric acid, and rinsing with copious amounts of ultra-pure water prior to use. However, in a winery setting, washing glassware with 1 %(w/v) citric acid dissolved in de-ionised water, followed by 5-6 rinses with deionised water should suffice.
- General glassware: 50 mL volumetric flask, 10 mL volumetric flask, 6 x 25 mL beakers, 1 L volumetric flask, 100 mL volumetric flask.
- Spectrophotometer: containing a standard visible lamp and the capability to insert a 40 mm cuvette. The spectrophotometer should be turned on 20 minutes before the analysis. The spectrophotometer should be zeroed on water in the 40 mm cuvette prior to analysis.
- Analytical balance.
- Micropipettes: 1 x 1-5 mL range, 1 x 0.1-1.0 mL range, and 1 x 0.02-0.20 mL range (or 0.01-0.10 mL range). The accuracy and precision of this technique will depend on the accuracy and precision of the micropipettes utilised. Pipettes must be calibrated for accuracy every 6 months.
- 40 mm glass cuvette: can be purchased for around $ 30-40. Quartz cuvettes (40 mm) can also be used but will be 10-times more expensive.
- 0.20 or 0.45 μm syringe filter. Regenerated cellulose is preferred but any should be fine.
- 10 mL syringes.

General Reagents

- 2,2′-Bicinchoninic acid dipotassium salt (BCA) regent: 0.05 %(w/v) in water. Weigh 25 mg in a 50 mL volumetric flask, dissolve in ~45 mL water and make up to 50 mL with water. Prepare reagent fresh.
- Ascorbic acid: 80 g/L in water. Weigh 0.8 g of ascorbic acid into a small beaker and add 10 mL of water. Prepare this reagent fresh. Accuracy of preparation is not critical for this reagent.
- Silver(I) nitrate: 1 g/L silver(I) in water (which is 1.57 g/L silver(I) nitrate). Weigh 32 mg of silver(I) nitrate into a small beaker and add 20 mL of water. Prepare this reagent fresh.

Reagents that must be prepared accurately.

- 500 mg/L copper(II): weigh 1.965 g copper(II) sulfate pentahydrate into a 1 L volumetric flask and dissolve in 800 mL of water and then make the volume accurate to 1L with water.
- 20.0 mg/L copper(II): accurately take 4.00 mL of 500 mg/L copper(II) and dilute it to 100 mL with water in a 100 mL volumetric flask.

Methodology

For each wine sample the four solutions indicated below require preparation. This includes: i) a wine blank, ii) wine non-blank, iii) wine + 0.1 mg/L Cu, and iv) wine + 0.3 mg/L Cu sample. A shortened version of the method may involve skipping the 'wine + 0.3 mg/L Cu' sample but this will increase the likelihood of experimental errors in the method. Replicate measures will provide an indication of precision of the measurement, and triplicate preparation of each solution is recommended. The spectrophotometer can be zeroed on water at 563 nm prior to the measurements below.

Solution to add	Wine blank	Wine non-blank	Wine + 0.1 mg/L Cu	Wine + 0.3 mg/L Cu
Sample	10	10	10	10
Ascorbic acid	0.05	0.05	0.05	0.05
Silver(I)	0.1	0.1	0.1	0.1
BCA	---	0.5	0.5	0.5
Copper	---	---	0.05	0.15
Water	0.65	0.15	0.10	---

All addition volumes listed are in mL.

After the additions in the table above, the samples are mixed and after 30 minutes at room temperature filtered with a syringe filter. A small portion of the sample (~0.5-1.0 mL) is used to rinse the 40 mm glass cuvette, and then the remaining sample loaded into the 40 mm glass cuvette and the absorbance measured at 563 nm.

Calculation

i) If any replicates for the samples above were recorded, the average values should be calculated for the 'Wine blank', 'Wine+0.1 mg/L Cu' and 'Wine+0.3 mg/L Cu' samples. The 'Wine non-blank' replicates can be left as individual absorbances at this stage.

ii) The average blank absorbance value should be subtracted from each of the 'Wine non-blank', 'Wine+0.1 mg/L Cu' and 'Wine+0.3 mg/L Cu' absorbances to provide A_{wine_rep}, $A_{0.1}$, $A_{0.3}$.

iii) Within excel, prepare a chart with x values of 0, 0.1 and 0.3, and then with y values with the absorbances corresponding to A_{wine_rep}, $A_{0.1}$, $A_{0.3}$.

iv) Fit a trendline to the data and show the equation for the trendline. The equation for the trendline will be in the format of y = **a** x + **b**, were **a** and **b** are numbers.

v) The copper concentration within the wine can be provided by calculating: (**b** / **a**), and the answer will be in mg/L.

vi) Perform steps iii)-v) for additional wine replicates and then average them for the final result.

Example Calculation for Sample Preparation in Triplicate

As an example the following data was obtained for samples prepared in triplicate:

Wine blank: 0.017, 0.023, 0.020 Wine non-blank: 0.121, 0.120, 0.120

Wine + 0.1 mg/L Cu: 0.165, 0.167, 0.167 Wine + 0.3 mg/L Cu: 0.260, 0.257, 0.257

Step i) The average is calculated for the Wine blank, and the samples with added Cu:

Average(Wine blank) = (0.017 + 0.023 + 0.020) / 3 = 0.020

Average(Wine + 0.1 mg/L Cu) = (0.165 + 0.167 + 0.167) / 3 = 0.166

Average(Wine + 0.3 mg/L Cu) = (0.260 + 0.257 + 0.257) / 3 = 0.258

Step ii) Subtract the average blank from each of the replicates of the 'Wine non-blank' and from the averages of the samples with added Cu.

A_{wine_rep1} = 'Wine non-blank'(replicate 1) – 'Wine blank' = 0.121 – 0.020 = 0.101

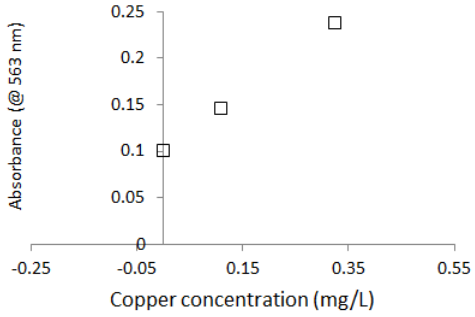
A_{wine_rep2} = 'Wine non-blank'(replicate 2) – 'Wine blank' = 0.120 – 0.020 = 0.100

A_{wine_rep3} = 'Wine non-blank'(replicate 3) – 'Wine blank' = 0.121 – 0.020 = 0.100

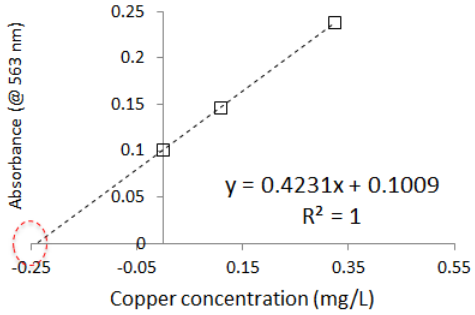
$A_{0.1}$ = 'Wine 0.1 mg/L Cu'(average) – 'Wine blank' = 0.166 – 0.020 = 0.146

$A_{0.3}$ = 'Wine 0.3 mg/L Cu'(average) – 'Wine blank' = 0.258 – 0.020 = 0.248

Step iii) In excel, prepare a graph for the quantification of each wine replicate. For Wine replicate1, the x values are the Cu concentrations of 0, 0.1 and 0.3, while the y values would be the absorbances of A_{wine_rep1}, $A_{0.1}$, $A_{0.3}$. For replicate1, the y values are 0.101, 0.146 and 0.248, providing the following graph:



Step iv) Fit a trendline to the graph and add the equation (as shown below). In this case, the equation for wine replicate1 is y = 0.4569 x + 0.1009 (make sure to show four decimal places in the equation). If the general equation format is written as y = **a** x + **b**, in this case **a** = 0.4231 and **b** = 0.1009.

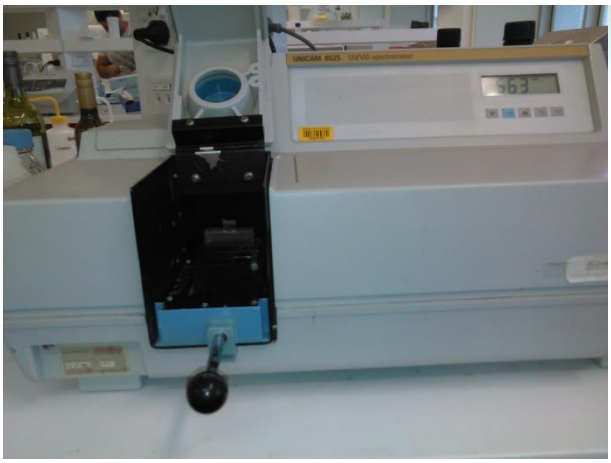


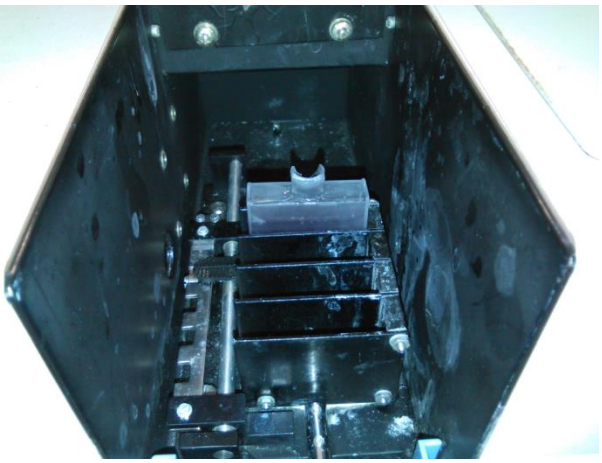
Step v) The intercept of the trendline on the x-axis will indicate the copper concentration in wine replicate1. In this case it can be given by **b** / **a** = 0.1009 / 0.4231 = 0.238 mg/L copper.

Step vi) The identical approach is taken for wine replicate2 and wine replicate3 is performed, utilising their A_{wine_rep1} and A_{wine_rep2} values (see step ii)) of 0.100 and 0.100, respectively. This should provide copper concentrations in wine replicates 2 and 3 of 0.235 and 0.235 mg/L copper. Therefore, the average of the three replicates would provide a concentration of (0.238 + 0.235 + 0.235) / 3 = 0.236 mg/L. The standard deviation of the replicates can also be calculated within excel (=STDEV(0.238, 0.235, 0.235) = 0.002 mg/L. Therefore, the answer can be quoted as 0.236 ± 0.002 mg/L (n=3).

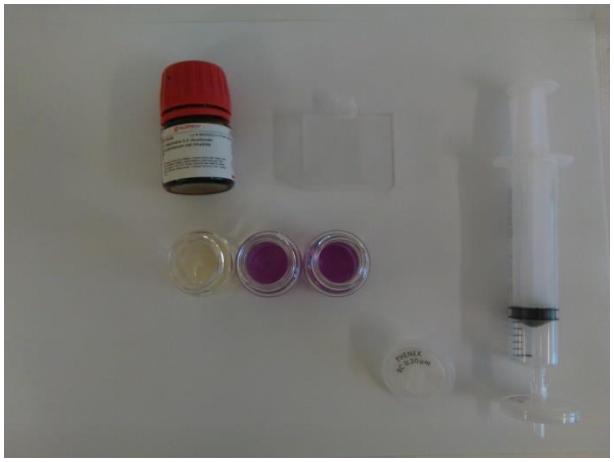
Photos of Equipment

Typical spectrophotometer used for the analysis (left hand side) and the 40 mm cuvette within the spectrophotometer (right hand side)





The BCA reagent, sample solutions, including the clear/yellow coloured blank sample, and syringe filter.



Filtration of the samples after the 30 minute reaction time.



Validation Parameters

The validation parameters of the technique are as follows:

- ***Recovery.*** The average recovery was 104 ± 9 % for 12 white wines with addition of 0.1 mg/L copper(II).
- ***Accuracy.*** The accuracy of the technique for determination of total copper in white wines was equivalent to that of ICPOES.
- ***Repeatability.*** The repeatability was assessed in terms of the average relative standard deviation of total copper concentrations, and was 3 ± 2 % for 12 white wines.
- ***Specificity.*** The BCA reagent is specific for Cu in wine, with no influence from Mg, Mn, Fe, Zn and Al.
- ***Linearity.*** The technique is linear within the range tested from 0.04 to 1.0 mg/L copper.
- ***Stability.*** Samples should be filtered 30 minutes after addition BCA to wines, and then measured immediately afterwards.
- ***Matrix effects.*** Eliminated with silver(I) additions to wine and with the use of quantification via standard additions.