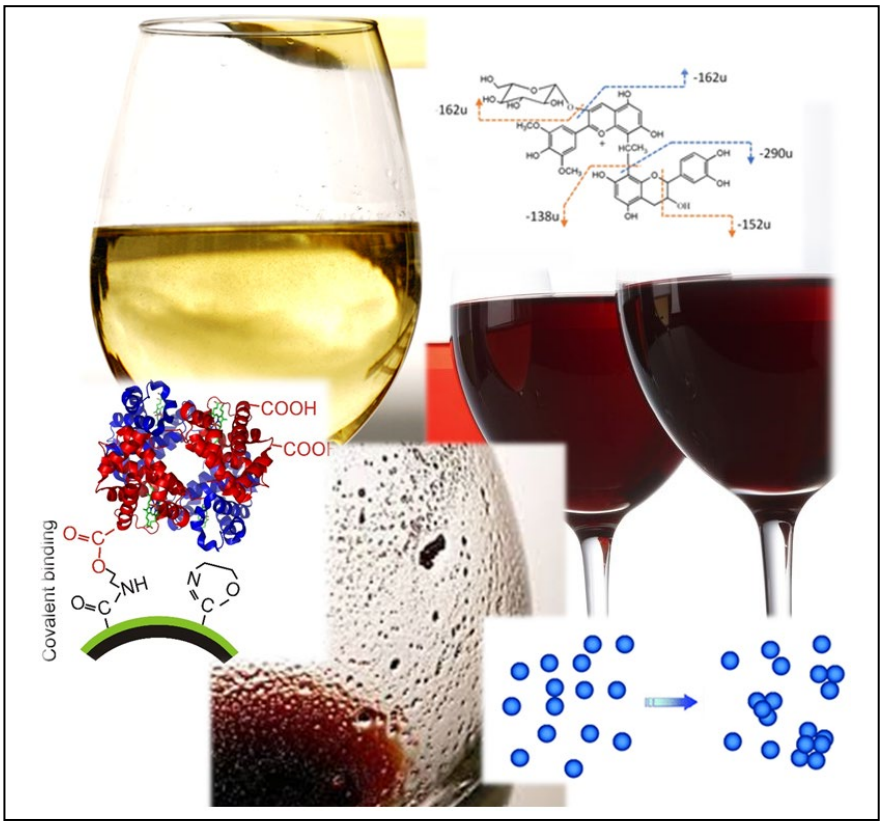
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AWRI Australian Wine Research Institute

Managing wine extraction, retention, clarity and stability for defined styles and efficient production



FINAL REPORT TO WINE AUSTRALIA

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Research Organisation: **Australian Wine Research Institute**

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Table of contents

1. Abstract 4
2. Executive summary 5
3. Acknowledgements 6
4. Background 7
5. Project aims 9
6. Methods 15
7. Results and discussion 23
8. Outcomes and conclusions 62
9. Recommendations 65
10. Appendix 1: Communication 67
11. Appendix 2: Intellectual property 71
12. Appendix 3: References 72
13. Appendix 4: Staff 75
14. Appendix 5: Other supporting material (including raw data, if appropriate) 76

1. Abstract

This project provided fundamental knowledge and practical approaches to manage the extraction and stability of wine macromolecules. Key areas addressed were protein stability, cold stability and maceration techniques. New techniques for wine macromolecule analysis were developed and alternative approaches to stabilisation were demonstrated. Natural zeolites can confer both protein and cold stability in a single application. Protein and cold stability could be achieved using plasma polymerised coatings, applied as nanoparticles or functionalised surfaces. Monomeric anthocyanin was found to significantly inhibit tartrate crystallisation, while polysaccharides did not. Red wine maceration approaches were studied, identifying levers to modify wine phenolic composition and style.

2. Executive summary

The key stakeholders in this project are wine producers. At the outset of the project, its goal was to generate new knowledge and tools to allow winemakers to more objectively manage and improve extraction processes and address stability, clarity, filterability and production efficiency issues during winemaking.

Alternative strategies for achieving protein stability and cold stability were evaluated, deploying novel additives or processing techniques. Regeneration protocols were developed for magnetic nanoparticles (MNPs), previously shown to successfully remove haze-forming proteins. MNPs could be regenerated and reused with a water wash, which would be a low-cost process.

For the first time, natural zeolites were shown to have dual functionality to heat- and cold-stabilise white wines in a single treatment. This could have a positive impact on wine production in terms of cost and energy efficiency. Importantly, compared to bentonites, zeolites cause much less wine loss and can be potentially reused as soil amendments in agriculture. Zeolites could also be completely regenerated through electrochemical methods or simply by treatment with salt solution (10%). Further research is needed to understand potential aroma scalping or deposition of aluminium before regulatory approval is sought.

A further alternative cold stabilisation approach was implemented, which harnessed the use of plasma-polymerised surfaces for the first time. In this work, the ability of plasma-polymerised surfaces to bind potassium bitartrate (KHT) through electrostatic interactions was used to initiate and progress crystallisation at the functionalised surface. After crystallisation was complete, the surface could be removed from the wine and regenerated. Importantly, this approach was found to be effective for different white wine samples at both lower and cellar temperatures. This work has therefore shown the promising potential for cold stabilisation to be conducted more sustainably, without the need for a chilling step.

A further area of research aimed to better support the management of cold stabilisation by being able to more effectively predict if it will occur. A new method to assess cold stability was developed which uses KHT crystal recovery and quantification following a three-day cold test. Using this, and other traditional analytical approaches, it was found for white wine that lower wine tartaric acid and high potassium largely explained KHT crystal formation, and cold stability was not clearly influenced by macromolecules such as polysaccharides, proteins and phenolics. This led to the conclusion that for the development of more cost-effective and sustainable approaches to white wine cold stabilisation, potassium removal needs to be considered as a primary strategy. For red wines, it was found that monomeric, ionised anthocyanins were able to confer cold stability at even low concentrations (250 mg/L), while as for white wine, other macromolecules had little, or no influence. This indicates that the maintenance of a fraction of ionised anthocyanins in red wines may be able to confer protection against KHT crystallisation, but more knowledge is required to better control pigment conversion and loss during wine processing and ageing.

Work on macromolecule extraction, retention and stability continued in this project. A number of new analytical methods were developed, validated and implemented in wine matrices. A rapid fluorescence-based method to detect haze-forming proteins in white wines was developed. The method is selective for haze-forming proteins, providing a linear detection range and with a low detection limit. It offers winemakers an alternative to the commonly used heat test, and could offer faster, cheaper and more accurate screening of protein-unstable white wines. Noting that in certain grape cultivars, protein can cause tannin loss, a new method for measuring red wine protein was validated. A study was undertaken to attempt to decouple protein-tannin interactions during fermentation by using a proteolytic enzyme (proctase). It was found that adding proctase alone could increase tannin extraction, but did not alter protein concentration. This could be a simple approach to enhance tannin extraction in low phenolic cultivars, sites or regions.

A range of methods for the study of wine colloids and macromolecular interactions were applied in this project. A pioneering study aimed to monitor the interactions between wine proteins, polysaccharides and tannins using fluorescence correlation spectroscopy (FCS). The study provided strong experimental evidence that macromolecular interactions could be characterised with FCS. Other techniques such as nanoparticle tracking analysis (NTA) and isothermal titration calorimetry (ITC) were also applied in this project when required to characterise wine macromolecular interactions. A key outcome was to show that wine acetaldehyde may react to form unstable adducts between tannin and anthocyanin. NTA revealed that these unstable pigments undergo extensive aggregation before precipitating. Further research should seek to understand the extent of acetaldehyde-mediated precipitation and loss of pigments during fermentation, in particular in aerated ferments.

Three AWRI-led trials were completed which investigated outcomes of maceration techniques on macromolecule extraction and retention in red winemaking. A study was undertaken on water addition to Shiraz musts, which showed that although losses in phenolics could be expected with water addition, it was nonetheless an effective way to manage high sugar concentrations associated with very ripe fruit.

A second trial studied whole bunch fermentation in Pinot Noir and Shiraz, with whole bunch inclusions ranging from 25% to 100% relative to crushed fruit. For Pinot Noir, differences in phenolic extraction with whole bunch inclusion were minor, with greater effects observed for aroma and flavour (reported in the Final Report for Project 3.1.1). For Shiraz, increases in extraction of tannin from stems with whole bunch inclusion were substantial, and improvements in colour were found even with the lowest level of whole bunch addition. Whole bunch inclusion also increased wine astringency. Although a small level of whole bunch inclusion showed some benefits for tannin extraction and colour improvement in Shiraz, a cautionary approach to including higher levels of whole bunches in ferments is advised, due to the introduction of harsh astringency and ‘green capsicum’ attributes.

A final maceration trial aimed to investigate the relative impact of maceration time and the use of Della Toffola’s maceration accelerator (DTMA) on wine phenolics. A further objective was to understand the importance of grape ‘phenolic potential’ on maceration outcomes. Vineyard phenolics were monitored by comparing the trend in total and extractable tannin and colour in grapes during ripening, showing for the first time that extractable tannin and colour consistently increased. The application of DTMA to high- and low-phenolic grapes showed that DTMA with a short (four day) maceration increased tannin extraction for both grape batches. However, as maceration time was extended, the DTMA-treated ferments from the high-phenolic grape batch were not different from the control, while for the low-phenolic musts, DTMA enhanced both tannin and colour. These measures were improved relative to both the control and DTMA treatments of the high-phenolic grapes, a remarkable outcome. Based on these findings, the DTMA technique holds the potential to improve phenolic outcomes in low phenolic varieties, or regions that may struggle to meet optimal grape colour or tannin targets, such as warm, irrigated regions.

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4. Background

The texture and appearance (colour, clarity) of wine is an important element of product differentiation, both in terms of wine style and market value. The capacity to optimise these wine characteristics, while maintaining profitability is a major challenge for the wine industry. Therefore, developing fundamental knowledge of the role of macromolecules such as polysaccharides, tannins, pigments, proteins and their aggregates on wine texture, stability and clarity are key to improving the efficiency and profitability of winemaking.

Haze prevention in white wine relies on removing pathogenesis-related (PR) proteins before packaging. Protein removal relies primarily on bentonite, which presents a significant cost to the wine industry as it cannot be reused. Historically, AWRI research has identified a number of potential bentonite alternatives (Marangon et al. 2011, Van Sluyter et al. 2015). Unpublished results from AWRI research also revealed that in certain white musts, pasteurisation could completely remove PR proteins, and it was therefore of interest to further explore its use as a stand-alone approach to heat stabilisation. Plasma polymer-coated MNPs (Mierczynska-Vasilev et al. 2017) have also been applied to remove PR proteins, but further research was required to be able to regenerate and reuse them. Further to this, natural zeolites were identified as a potential alternative to bentonite due to their cation exchange capacity, and a research gap was identified to study their application as protein fining agents.

In order to achieve cold stabilisation, it is necessary to induce precipitation of excess KHT by cooling wine to below 0°C for up to a few months, and this represents one of the most costly steps in the winemaking process. It therefore remains relevant to the Australian industry to develop cost-effective, efficient and sustainable alternative cold stabilisation processes. A knowledge gap was identified regarding the role that endogenous wine macromolecules and wine matrix composition may play in the inhibition of KHT crystallisation and precipitation. A further important goal was to identify novel techniques or processes that could produce cold-stable wines with minimal intervention, or the need for additives.

At the outset of this project, a body of pioneering research had been completed at AWRI which made headway into the application of new and existing techniques for the study of macromolecules, their interactions and colloid formation in wine (McRae et al. 2010, Bindon et al. 2016, Mierczynska-Vasilev and Smith 2016). It was recognised that a new method, or sensor, which could be deployed

rapidly and without sample preparation for the analysis of white wine PR proteins would be beneficial for winery laboratories. A further area identified for new research was to assess red wine protein in particular, since traditional analysis methods for protein do not perform effectively in red wines due to interference from phenolic compounds (Springer et al. 2016).

An opportunity was also identified to use fluorescence correlation spectroscopy (FCS) to study colloids in wine without disturbing the wine matrix, since fluorescently labelled molecules of interest could be added to the wine. Further, it was recognised that a greater understanding of acetaldehyde-mediated pigment formation on colour stability was required, to study pigment aggregation and precipitation, using nanoparticle tracking analysis (NTA). A knowledge gap was also identified with respect to the impact of aeration on tannin (and pigment) colloidal properties and interactions, to further characterise these using NTA and isothermal titration calorimetry (ITC).

In terms of practical applications, at the outset of this project, AWRI had a well-established portfolio of macromolecule analysis techniques for grape phenolic extractability, wine tannin, polysaccharide and colour composition (Smith et al. 2015). Prior to this project, these techniques had been used to understand the factors that may enhance or limit the extraction and retention of macromolecules in wine, and also to characterise the outcomes of different maceration techniques. Within the current project, this analysis platform provided continuing opportunities for collaborations within and outside of AWRI, to study water addition to high sugar must, whole bunch inclusion and maceration acceleration.

5. Project aims

The project investigated:

- the role of macromolecules such as tannins, polysaccharides, proteins and their aggregate colloids, and their impact on stability, clarity, filtration and fouling
- the impact of other wine matrix components on macromolecule extraction, retention and function
- the source of these molecules or their precursors in grapes and yeast, and the impact of winemaking processes such as clarification, flotation, vinification and filtration on their retention and/or transformation
- the impact of fouling of surfaces by macromolecules leading to production inefficiencies
- alternative strategies for achieving protein stability and cold stability, for example, through use of novel additives and/or processing techniques
- practical methods for wineries to determine likely extractability of macromolecules during winemaking and the factors that affect extraction and retention (e.g. enzymes, water additions and heat treatments).

Project deliverables and activities are listed below.

Deliverables and activities for 2017/18:

Deliverables	Activities	Target date
A. Improved methods to assess protein stability (2018).	1. Finalise correlations between haze status at 1 year and the time 0 results of the modified rapid heat test. Develop extension material. 2. Develop a gold nanoparticle (AuNP)-based colourimetric sensor assay for protein detection in wine. Synthesise and modify AuNPs for selective adsorption of pathogenesis-related (PR) proteins, optimise assay conditions and colourimetric response.	June 2018
B. Improved methods to assess cold stability, improved knowledge of the roles that matrix composition (especially macromolecules) play in increasing the cold stability of wines (2019).	**1.** Review the current cold stability methods used within industry and assess the influencing factors (such as time and temperature) on establishing cold stability in a test compared with actual wine stability. 2. Design and execute reconstitution experiments in wines using different concentrations of grape and wine macromolecules (such as phenolics, polysaccharides and proteins) and assess the influence of each on wine cold stability. 3. Conduct a preliminary survey of surface coatings that may promote potassium bitartrate crystal nucleation and growth.	June 2018
C. Novel or more efficient approaches to achieving protein stabilisation (2020).	**1.** Investigate regeneration (cleaning) methods for magnetic nanoparticles with proteins bound to them. 2. Investigate application of natural zeolites in separation of PR proteins from wines.	June 2018

	3. Apply pasteurisation of juice for protein stabilisation during vintage trials. Assess whether there are any impacts of heating juice on wine sensory properties (specifically), plus volatile profiles and macromolecules. 4. Develop and assess other surface functional groups (e.g. amine and oxazoline), on magnetic particles, for binding of PR protein molecules.	
D. Improved knowledge of the roles that matrix composition (especially macromolecules) play in modulating the protein stability of wines (2020).	**1.** Develop new techniques to study wine macromolecule interactions and colloid diffusion in wine environments, through external collaborations. Focus initially on fluorescence correlation spectroscopy (FCS) to develop protocols to monitor conformational changes in PR wine proteins and their interactions with other macromolecules.	June 2018
E. Knowledge of how to modulate extraction and retention of key compounds for given wine styles (2020).	**1.** Purify tannin fractions and malvidin-3-glucoside to investigate the role of tannin composition (sub-unit composition, degree of polymerisation) on polymeric pigment formation in model ferments. 2. Purify wine polysaccharide fractions for a study of their interactions with tannin in model wine solution. 3. Draft manuscripts from 2014 (fit for purpose yeast) and 2015 (yeast, enzyme and extended maceration interactions) vintage trials. 4. Conduct chemical analysis of wines (27) from the vintage 2017 trial on the effect of alcohol reduction on macromolecule extraction and retention in wines made using sequential harvest versus must dilution and schedule sensory analysis. 5. Design and implement a vintage 2018 winemaking trial involving one or a combination of the following treatments: co-fermentation (two grape varieties), oxygen application (collaboration with Project 3.1.5), crushing/maceration techniques to modulate extraction (collaboration with Project 4.1.1).	June 2018

Deliverables and activities for 2018/19:

Deliverables	Activities	Target date
A. Improved methods to assess protein stability (2018).	1. No research planned as this deliverable has been completed. 2. Submit a paper on an improved method for heat	June 2019

	stability testing and prepare a *Technical Review* article on the subject. 3. Prepare poster and/or workshop for the AWITC in 2019.	
B. Improved methods to assess cold stability, improved knowledge of the roles that matrix composition (especially macromolecules) play in increasing the cold stability of wines (2019).	1. Apply mini-contact test using micronised KHT to determine whether this is suitable to test wine tartrate stability and compare with the brine test. 2. Perform an experiment to determine how changes in tannin structure associated with wine ageing may affect red wine cold stability to address the common observation that young red wines are cold stable and may lose this stability by the time of bottling. 3. Continue work on surface coatings that may promote potassium bitartrate crystal nucleation and growth.	June 2019
C. Novel or more efficient approaches to achieving protein stabilisation (2020).	1. Draft a manuscript on the application of natural zeolites to separate PR proteins from wines. 2. Draft a manuscript on the use of surface functional groups (e.g. amine and oxazoline) on magnetic particles to bind pathogenesis-related (PR) protein molecules. 3. Complete the chemical analyses of wines from the vintage 2018 juice pasteurisation trials. Assess wine sensory properties and compare to untreated and bentonite-fined control wines. 4. Prepare poster and/or workshop for the AWITC in 2019.	June 2019
D. Improved knowledge of the roles that matrix composition (especially macromolecules) play in modulating the protein stability of wines (2020).	1. Develop new techniques to study wine macromolecule interactions and colloid diffusion in wine environments, through external collaborations. Focus initially on fluorescence correlation spectroscopy (FCS) to develop protocols to monitor conformational changes in PR wine proteins and their interactions with other macromolecules. 2. Develop and validate a method to measure red wine protein to determine whether there is potential for the development of unstable protein-tannin complexes in red winemaking. 3. Conduct a survey of protein concentration in a range of unfiltered and commercial red wines.	June 2019
E. Knowledge of how to modulate extraction and retention of key compounds for given wine styles (2020).	1. Complete red wine polymeric pigment formation and stability study. 2. Conduct sensory analysis of the vintage 2017 wines comparing harvest time and dilution. 3. Conduct chemical analysis of wines from the vintage 2018 trial on the effect of whole bunch	June 2019

	fermentation on macromolecule extraction and retention in wines and schedule sensory analysis. 4. Conduct chemical analysis of wines from experiments on oxygen dose and timing during fermentation in Project 3.1.5. 5. Conduct a preliminary investigation of how juice macromolecule composition and zeta potential affect the flotation process for clarification. 6. Design and implement a winemaking trial which draws from key results of the 2017-18 studies to determine the impact of key winemaking intervention(s) on macromolecule extraction, retention and/or stability. 7. Prepare poster and/or workshop for the AWITC in 2019 and update roadshow presentation if required.	

Deliverables and activities for 2019/20

Deliverables	Activities	Target date
A. Improved methods to assess protein stability (2018).	1. Prepare a presentation on protein stability testing for delivery at the 17th AWITC in July 2019. 2. Apply gold nanoparticle (AuNP)-based colourimetric sensor and fluorescence-based dye to determine the optimum approach for further development of rapid detection of protein in wine.	June 2020
B. Improved methods to assess cold stability, improved knowledge of the roles that matrix composition (especially macromolecules) plays in increasing the cold stability of wines (2019).	1. Conduct a comparative survey of cold stability tests (three-day test, conductivity and saturation temperature) on a range of red and white wines, including compositional data on wine metals, ionic strength and macromolecules. Store wines for an ageing study. 2. Design and execute an experiment based on 2018/19 results to investigate the role of macromolecules (e.g. tannin, polysaccharide, protein) on cold stability. 3. Summarise preliminary results of potassium removal from wines with zeolites of different sizes and observed effects on cold stability. 4. Compare surface coatings that may promote potassium bitartrate crystal nucleation and growth.	June 2020
C. Novel or more efficient approaches to achieving protein stabilisation (2020).	1. Finalise the results of the white wine pasteurisation trial and prepare a manuscript for a peer-reviewed journal, as well as a poster or presentation for the 17th AWITC. 2. Perform a scale-up study on two protein unstable white wines, to assess zeolite effects on wine stability and sensory attributes. 3. Investigate effect of nanoparticle monodispersity	June 2020

	on protein removal in white wine using synthesised (iron oxide) nanoparticles.	
D. Improved knowledge of the roles that matrix composition (especially macromolecules) play in modulating the protein stability of wines (2020).	1. Finalise the results of the fluorescence correlation spectroscopy work and prepare a manuscript for a peer-reviewed journal. 2. Finalise work on the red wine protein analysis method, in particular to investigate conditions which cause tannin-protein precipitation during fermentation and ageing. Source protein from red wines and juices containing high protein concentrations to include in the final method validation.	June 2020
E. Knowledge of how to modulate extraction and retention of key compounds for given wine styles (2020).	1. Prepare manuscripts from completed projects for peer-reviewed journals. 2. Conduct chemical analysis for collaborative projects on extraction in 3.1.5 (oxygen dose effects), 3.1.1 /4.1.1 (berry shrivel effects). 3. Initiate collaboration with Project 3.2.1 to compare macromolecule extraction across up to 100 yeast strains, using micro-scale (red grape) fermentations. 4. Obtain data after the application of a selected grape maceration technique (e.g. DTMA/Della Toffola) on macromolecule extraction and retention.	June 2020

Deliverables and activities for 2020/21:

Deliverables	Activities	Target date
A. Novel or more efficient approaches to achieving cold stability	1. Finalise the results of the experiment to determine the role of wine macromolecules in tartrate crystal formation. 2. Continue long-term monitoring of red and white wine cold stability in wines of known macromolecular composition to determine method performance and reasons for failure. 3. Conduct experiments to determine the impact of potassium removal using adsorbents (zeolite, acrylic acid) in wines with different compositional characteristics on cold stability. Include an experiment using ion exchange membranes for potassium removal. 4. Identify surfaces to promote potassium bitartrate crystallisation and validate in real wine. Evaluate performance under different temperature conditions to determine if there is potential for reduced energy input to cold stabilisation.	June 2021
B. Knowledge of surface interactions that affect the efficiency of production and	1. Design and implement experiments to investigate the role of wine macromolecules in fouling and/or colloidal instability that may affect the performance of membrane filtration. 2. Conduct experiments to determine the functional properties of the best-performing zeolites for the	June 2021

practical, improved production processes based on that knowledge	adsorption of wine proteins, including benchmarking of zeolites.	
C. Knowledge of how vineyard and winery production processes influence factors affecting extraction and retention of key compounds, wine clarity, stability and production efficiency.	1. Conduct sensory analysis and compositional analysis of the 2020 vintage trial wines to determine the role of DTMA maceration on red wine macromolecules and if relevant, volatiles. 2. Finalise experiment on the use of grape homogenate and wine-like extracts to determine the relative changes in total versus extractable phenolics in the ripening of four commercial Shiraz vineyards. 3. Finalise the results of the red wine protein survey for publication. 4. Complete an experiment to determine the impact of (grape) protein-tannin interactions which may lead to instabilities during fermentation and winemaking processes. 5. Analyse oxygen-treated and control wines from project 3.1.5. using targeted chemical analysis and mass spectrometry to determine the structural modification of proanthocyanidins by macro-oxygenation, with a view to understanding their pigmentation properties and stability. 6. Complete analysis of bentonite-treated unstable wines with fluorescent dye as indicator to quantify white wine protein. Evaluate protein concentration and heat test results to determine if there is a threshold for protein concentration below which wines are heat stable. Finalise the research with fluorescent dyes for publication.	June 2021
D. Practical advice and extension materials for winemakers (ongoing).	1. Continue publication of completed studies in peer-reviewed and/or industry journals. 2. Revise roadshow presentations on protein stability and phenolic extraction for use in other extension activities 3. Work with the AWRI Business Development Manager and Project 2.1.1 to identify potential pathways for the effective adoption of alternative heat and cold stabilisation strategies.	June 2021

Deliverables and activities for 2021/22:

Deliverables	Activities	Target date
A. Novel or more efficient approaches to achieving cold stability	1. Finalise the data on the role of wine macromolecules in cold stability for publication. 2. Conduct an experiment to optimise white wine cold stabilisation with zeolite addition as part of C2. 3. Analyse juice and wine composition of selected Shiraz samples from Project 3.3.1 to assess compositional and viticultural factors that may	June 2022

	contribute to cold instability.	
B. Knowledge of surface interactions that affect the efficiency of production and practical, improved production processes based on that knowledge	1. Compare the performance of ultrafiltration membranes on red and/or white wine fractionation. 2. Initiate experimentation to determine the role of surface interactions in membrane fouling. 3. Conduct further work on the development of surfaces that induce potassium bitartrate crystallisation to investigate their effectiveness in different wines.	June 2022
C. Knowledge of how vineyard and winery production processes influence factors affecting extraction and retention of key compounds, wine clarity, stability and production efficiency.	1. Benchmark zeolites for the selection of materials that combine improved heat and cold stabilisation features with reduced flavour scalping using GC-MS profiling. 2. Based on the outcome of C1, scale up selected zeolite for use during or post-fermentation in a proof-of-concept winemaking trial; compare zeolite results with bentonite fining. 3. Determine the impact of macro-oxygenation conducted in Project 3.1.5 on wine phenolic composition and associated protein-phenolic interactions using isothermal titration calorimetry. 4. Provide support for projects requiring analysis of non-volatile targets (3.1.1 blending study and 'jammy' Shiraz) 5. Conduct analysis of polysaccharide, colour and tannin for Project 3.2.1 (hybrid yeast). 6. Improve the sensitivity and reduce the time required for the red wine protein analysis method, using fluorescent dye and/or fluorescence-HPLC.	June 2022
D. Practical advice and extension materials for winemakers (ongoing).	**1.** Support Projects 2.1.1 (Extension) and 2.1.2 (Communication and awareness-raising activities) through identifying topics and preparing materials for awareness-raising and practice change activities. 2. Continue publication of completed studies in peer-reviewed and/or industry journals. 3. Continue to update extension presentations on protein stability/cold stability/phenolic extraction. 4. Benchmark the cost of production using existing methods for heat and/or cold stabilisation against alternative methods. 5. Prepare extension materials for a workshop summarising the outcomes of the accelerated extraction winemaking trials conducted in 2020.	June 2022

6. Methods

6.1 Analysis of musts and grapes

Musts from the vintage trials were analysed for YAN, pH and TA. Juice and wine pH and TA were determined using a pH meter and combination electrode. The TA was determined by titrating with 0.33 M sodium hydroxide solution to a pH end-point of 8.2 and expressed in g/L of tartaric acid equivalents. Ammonia concentration in fresh juices was determined using the glutamate dehydrogenase enzymatic bioanalysis UV-method test (Roche, Mannheim, Germany). Juice free amino acid nitrogen was determined by the o-phthaldehyde/N-acetyl-L-cysteine spectrophotometric assay, which excludes proline (Bindon et al. 2013). Juice YAN was calculated by adding the nitrogen present in ammonium to the YAN concentration. Three replicate 50 g berry samples were subject to a 'wine-like' extraction from the 2017 and 2019 trials on Shiraz grapes to determine extractable tannin and anthocyanin according to the method described by Bindon et al. (2014a). Briefly, 50 g of berries were transferred into zip-lock plastic storage bags and crushed by hand. The must (solids and juice) obtained was transferred into 70 mL plastic screw-cap containers, using a spatula to ensure all berry contents were removed from each bag. Aqueous ethanol (15 mL of 40% adjusted to pH 3.4 with a 10 g/L aqueous tartaric acid solution) was added and the capped containers were gently shaken for 40 hours at room temperature. Afterwards, the extracts were pressed through a sieve with a 1 mm^2 pore mesh, and the extract volume was recorded. The extracts were transferred into 50 mL tubes, centrifuged, and the supernatants were analysed for tannin and anthocyanin concentration as described below.

6.2 Micro-scale fermentations

Triplicate 250 g fermentations were performed on fresh Shiraz grape samples sourced from two blocks in the McLaren Vale region and Pinot Noir from the Adelaide Hills region in the 2020 season. Fruit (approximately 10 kg) was destemmed, mixed and subdivided into 250 g batches for small-scale fermentations. Grapes were gently crushed by hand in a snap-lock plastic bag and transferred to a 500 mL plastic container equipped with a screw-cap lid, fitted with a rubber seal and airlock. To the crushed grapes, 12.5 mg (as a 250 µL aliquot in water) of potassium metabisulfite was added. Preliminary trials on spare fruit were conducted to optimise microwave control and temperature of must for heat treated samples. Proctase (80 mg in 20 mL) was added directly to an aliquot of the corresponding juice and then subsequently added to the respective treatments. Heated treatments were microwaved in the plastic container for 2 min (Power 80) to reach 75°C, 1 mL of juice or juice containing proctase added, stirred for 1 minute then cooled to 45°C and left to return to room temperature. Non-heated samples received 1 mL of either juice or juice containing proctase, and were stirred for 1 minute after addition. An addition of 100 mg (as a 1 mL aliquot in water) of hydrated *S. cerevisiae* PDM (Maurivin, Sydney, Australia) and 75 mg (as a 200 µL aliquot in water) diammonium phosphate was made, and mixed well using a metal spatula. A plastic cylinder covered with fine plastic mesh was inserted into each container, designed to keep the cap submerged during fermentation. Ferments were transferred to a platform shaker in a temperature-controlled room and fermented at 23°C for seven days with a 3 mL aliquot removed on Day 0, 2, 5 and 7 and frozen at -20°C. Thereafter, ferments were pressed through fine plastic mesh, and the residual glucose was tested using Keto Diabur 5000 test strips (Roche Diagnostics, NSW, Australia). If the result was >2% glucose, fermentation was continued at room temperature in a Schott bottle fitted with a rubber seal and airlock. Once dry for glucose, 26 mg of potassium metabisulfite was added to a 130 mL aliquot of wine, which was then decanted into a 120 mL container with no ullage and settled at 4°C for one month. Wines were racked off gross lees, pH adjusted to ≈ 3.5 using tartaric acid in water (50% w/w) and bottled in 100 mL glass bottles with no ullage, and stored at 4°C until analysed for protein, tannin and colour (within one month).

6.3 Juice pasteurisation and winemaking

Batches of 2018 vintage Semillon and Sauvignon Blanc juice from the Riverland, SA were heated using a commercially available pasteurising unit at 75°C for periods of 1 and 2 minutes. The flow rates for the 1- and 2-minute heat treatments were approximately 1,900 L/h and 900 L/h

respectively. Juice was heated from 4°C and maintained at 75°C for the required time for each treatment. Juice exiting the unit was 12°C, indicating an overall increase in temperature of 8°C between input and output. Heated and unheated batches of juice (200 L each) were stored at 0°C for two days, before being fermented using a standard white winemaking protocol, and cold stabilised. Control wines were also made from juice with no heat treatment. Prior to cold stabilisation, control wines were split into two batches, with half being fined with bentonite and half left unfined.

6.4 Small-scale red winemaking

In the 2017 season, a pre-fermentation water addition trial was performed on Shiraz grapes (reported in detail in a previously submitted final report for project AWR1701-4.1.1 and published in Teng et al. 2020a). Briefly, Shiraz fruit was hand-harvested from a vineyard near Nuriootpa, in the Barossa Valley on three occasions, targeting 13.5, 14.5 and 15.5°Baumé. Triplicate 45 kg batches of fruit harvested at 13.5 and 14.5°Baumé were fermented normally according to a standard WIC Winemaking Services protocol (Teng et al. 2020a), as was a portion of the fruit harvested at 15.5°Baumé. The balance of the 15.5°Baumé harvest was diluted either by direct addition of potable rainwater, or by a run-off/replace approach with the same water to achieve targeted Baumé levels of 14.5 ° or 13.5°. The ferments were inoculated with a commercial yeast (EC1118) and lactic acid bacteria (VP41) on the second and third day after crushing, respectively. All ferments were maintained on skins for nine days in a 20°C room from crushing to pressing, and sequentially acid-adjusted to target a titratable acidity of approximately 5 g/L, and bottled as described (Teng et al. 2020a).

In the 2018 season, Pinot Noir and Shiraz grapes were hand-harvested from premium vineyards in the Adelaide Hills and small-lot replicated winemaking proceeded via a published small-scale WIC Winemaking Services protocol (Teng et al. 2020a) with the following modifications. Bunches were randomly separated into five treatments: no whole bunch inclusion (control, all destemmed and crushed fruit) and inclusion of crushed whole bunches at 25%, 50%, 75% and 100%. Whole bunches (WB) were placed at the bottom of a 50 kg fermenter together with a small amount of inoculated must, with destemmed and crushed fruit then added in the required proportions. All treatments received hand-plunging twice a day during fermentation. After pressing, the wines went through malolactic fermentation and received no oak treatment. They were then bottled and stored for approximately nine months, after which time sensory and chemical analyses were completed.

In the 2020 season, Shiraz grapes were harvested from two commercial vineyards (Coriole Wines), selected for their relative grape phenolic concentration. Grapes were hand-harvested and then crushed in a commercial crusher. In order to capture the heterogeneity of the vineyard, the crusher was connected to the standard outlet or the DTMA maceration accelerator outlet at approximately 15 min intervals. Three 100 L fermenters were filled either from the crusher or DTMA outlets at staggered 30 minute time points. Fermenters were transported back to the WIC Winemaking Services facility and standard winemaking was applied as described previously (Teng et al. 2020a). Treatments applied were to press after four days or seven days, and were otherwise the same as described. Wines were analysed at approximately twelve months post-fermentation.

6.5 Wine basic compositional analysis

Wines were analysed for their basic composition using a Foss WineScan FT 120 as described by the manufacturer (Foss, Hillerød, Denmark).

6.6 Phenolics, colour and tannin in extracts, musts and wines

Wine and must samples were centrifuged for 5 minutes at 16.1 *g* prior to analysis. Tannin and colour was analysed in grape extracts and wines using a standard high-throughput method (Mercurio et al. 2007). For white wines, centrifuged wine was diluted 1 in 5 with 1 M HCL and the absorbance determined at 280 nm in a 1 cm pathlength quartz cuvette (Cary 300, Varian, Australia) to estimate differences in wine total phenolics. To compare the phenolic content of white wines before and after

treatments, absorbance in the spectral range of 280 to 420 nm was measured using a Cary 60 UV-vis spectrometer (Agilent Technologies). For model studies, grape and wine tannin were extracted using affinity chromatography as described previously (McRae and Kennedy 2010, Bindon and Kennedy 2011). Anthocyanin was prepared from a commercial concentrate (GrapEx, Tarac Technologies, N. Adelaide, SA, Australia) either by MLCCC (Teng et al. 2019) or by phase separation with isoamyl alcohol (Somers 1971).

6.7 Tannin composition in wines

Tannin was purified from wine samples using the modified solid phase extraction described by Kassara and Kennedy (2011). Recovered tannin material was reconstituted in pure methanol to approximate a concentration of 10 g/L (by MCP) and stored at -20 °C until analysed. Gel permeation chromatography (GPC) analysis was conducted following the method of Kennedy and Taylor (2003) with the modifications reported previously (Bindon and Kennedy 2011). The purified tannin fraction was also analysed by phloroglucinolysis (Kennedy and Jones 2001) with the HPLC modifications described previously (Kennedy and Taylor 2003). For phloroglucinolysis, (-)-epicatechin (Sigma Aldrich, St. Louis, MO, USA) was the quantitative standard.

6.8 Wine volatiles

Yeast volatile fermentation products were analysed using HS-SPME–GCMS, with SIDA as described previously (Siebert et al. 2005). Twenty-seven compounds, including ethyl and acetate esters, higher alcohols and volatile acids were quantified.

6.9 Wine polysaccharide concentration and composition

Wine polysaccharides were analysed according to the methods described in Bindon et al. (2019) and Kassara et al. (2019). Monosaccharide derivatives were identified and quantified using commercial standards (Sigma-Aldrich, St. Louis, MO, USA). Polysaccharide was quantified as the sum of the monosaccharides.

6.10 White wine protein

The composition and concentration of haze-forming proteins in must and wines were assessed using high performance liquid chromatography (HPLC) performed as previously described (Culbert et al. 2017). For protein purification, an unfined Chardonnay wine was adjusted to pH 3 and sterile filtered using a three-stage system (2/0.65/0.22 μm). Protein isolation by ion exchange was conducted at 4°C. A Pharmacia column (XK50), packed with 300 mL SCX resin was equilibrated with 1.5 L Buffer A (30 mM sodium citrate adjusted to pH 3.0) An 18 L sample of wine was loaded using a peristaltic pump overnight, then the column was washed with 0.5 L Buffer A, to rinse out wine components other than protein. Proteins were eluted using 1.25 L 30 mM MES (adjusted to pH 6.0). The eluate was added 777 g NH_4SO_4 to bring it to 90% saturation and stirred at 4°C for 4 hours. The eluate was centrifuged using a Beckmann centrifuge. The pellet was dissolved in 150 mL water and dialysed against ultrafiltered water using SnakeSkin 3500 MWCO dialysis tubes, for three days, changing the water twice daily (10 L beaker). Afterwards, the protein concentration of the solution was determined by HPLC as described above, and frozen at -80°C until used.

6.11 Red wine protein

Analysis of red wine protein was performed according to the methods outlined in Kassara et al. (2022).

6.12 Heat test

White wines were heated for two hours and red wines for six hours at 80°C. After heating, the samples were removed from the water bath and cooled on ice for two hours. After equilibration to room temperature, the haze in wines was measured by calculating the difference between a heated and unheated sample in nephelometry turbidity units (NTU) using a nephelometer. The test was considered passed with differences in NTU < 2 for white wines and NTU < 5 for red wines.

6.13 Cold stability measurements

The mini-contact test involved chilling a stirred 200 mL sample of wine to 0°C, before adding 2g of micronised KHT. The conductivity of the wine was measured before the addition of the KHT and 4, 20 and 30 minutes afterwards. A wine is assessed as stable if its conductivity drops by less than 5% over the duration of the test[i] , though some prefer a criteria of less than 3% for red table wines.

For the refrigeration/brine test, or three-day cold test, 100 mL of filtered wine was chilled to a temperature of -4°C and held there for 72 hours. After returning to ambient temperature, the samples were visually assessed for the presence of crystalline deposit. A sample was determined to have passed if no crystals were detected. A sample with crystals present was assigned a fail level of 1 – 3, depending on the quantity observed.

After this assessment, samples were filtered through a 0.45 µm PES membrane, which was collected and washed with dilute hydrochloric acid, to dissolve any tartrate crystals present. This solution was then analysed for tartaric acid by HPLC as described below. It became necessary in some applications to reduce the volume used for the three-day cold test. To this end, 10 mL of filtered wine was chilled. After visual assessment, the sample was centrifuged to ensure all loose crystals were collected at the bottom of the tube. The supernatant was decanted and the remaining sample washed with 1 mL of absolute ethanol. After further centrifugation, the supernatant was again decanted and the sample was left to dry. Dilute hydrochloric acid was then added to dissolve any tartrate crystals before the solution was analysed for tartaric acid by HPLC. This quantitative analysis of samples after a three-day cold test, for both 10 and 100 mL volumes, is referred to as 'Crystal ID' in the remainder of this report.

The saturation temperature (T_{sat}) of each wine was determined by following the Erbslöh EasyKrista method. The conductivity (C_1) of approximately 30 mL of wine sample was measured in µS/cm. Approximately 2 g of KHT was added and the sample agitated. After five minutes the conductivity (C_2) and temperature (T) of the sample were measured again.

6.14 Wine organic acid concentration

The concentration of organic acids (citric, tartaric, malic, succinic, and lactic) was determined by HPLC using an Aminex column fitted on an Agilent 1200 series quaternary pump LC (Agilent Technologies). The column was eluted at 65°C in isocratic mode with 5 mM H_2SO_4 at a flow rate of 0.5 mL/min. Samples were diluted five-fold to a final content of 1.25% (v/v) isopropanol and 1 mM sulfuric acid and centrifuged at 13,000 x *g* for 15 minutes at 4°C. Injection volumes were 10 mL. Organic acids were detected by absorbance at 210 nm using an Agilent 1200 series diode-array detector. For tartaric acid analysis for cold stability studies, wine samples were diluted with water and injected onto an Agilent Technologies Hi-Plex H column (300 mm x 7.7 mm). Separation was achieved with a solvent system of isocratic 10 mM sulfuric acid/water at a flow rate of 0.6 mL/min. Tartaric acid was detected at 210 nm, quantification being performed against a standard curve of known concentration of tartaric acid. For the Crystal ID method, dry recovered crystals were reconstituted in dilute hydrochloric acid and analysed for tartaric acid by HPLC, as above. Quantification was performed against a tartaric acid standard curve.

6.15 Settling experiments

Natural zeolite and bentonite suspensions were prepared by mixing the mineral slurry with white wine for three hours. The suspensions were then transferred to 100 cm^3 graduated glass cylinders and allowed to settle for 48 hours. After this time the volume of lees created in the graduated measuring cylinders was measured using a caliper.

6.16 Wine fractionation

Prior to fractionation a 50 mL aliquot of wine was evaporated to dealcoholise the wine under vacuum at 30 °C. A 0.1 M NaOH solution was used to neutralise the wine to pH 7.0. The fractionation

procedure applied to each wine has been previously described by Sun et al (2006). For fractionation, a Chromabon vacuum manifold system was used. An empty 60 mL fritted solid-phase extraction (SPE) cartridge (Supelco, Sigma-Aldrich) was filled with 10 g of Lichroprep RP 18 (25-40 µm) suspended in methanol or 30 mL Toyopearl 40 (F). Both columns were pre-treated with 30 mL of methanol and washed with 100 mL of ultra-pure water. The neutralised, dealcoholised wine was then loaded to the column and the first fraction (F1, phenolic acids) was collected with 100 mL of diluted (1/8) phosphate buffer, pH 7 with the flow rate maintained at 2 mL/min. The column was washed again with 100 mL of distilled water and briefly dried under vacuum. Two further fractions were obtained by the successive application of 100 mL of ethyl acetate (monomeric and oligomeric flavan-3-ols) followed by 100 mL of methanol containing 0.1 formic acid (anthocyanins, polymeric proanthocyanidins and other pigmented complexes). Both of these fractions were dried under vacuum and reconstituted in ultra-pure water. The Lichroprep RP 18 column was then washed with ultra-pure water and the reconstituted ethyl acetate fraction was re-applied. In order to separate monomeric flavan3-ols from oligomers, 100 mL of diethyl ether, was applied to obtain Fraction 2 (F2, monomeric flavan-3-ols), followed by 100 mL of methanol to obtain Fraction 3 (F3, flavan-3-ol oligomers). In parallel, the second Toyopearl 40 (F) column was pre-treated with methanol followed by ultra-pure water. The reconstituted methanolic fraction was applied to the column and eluted successively with 100 mL of methanol and 100 mL of 75% acetone containing 0.1% HCl to respectively obtain Fraction 4 (F4, non-acylated and acylated anthocyanins with their derivatives), and Fraction 5 (F5, polymeric proanthocyanidins and pigmented complexes).

6.17 Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) of samples was performed as described previously (Bindon et al. 2016).

6.18 Isothermal titration calorimetry (ITC)

A Malvern MicroCal PEAQ-ITC was used to measure the binding affinity and the thermodynamic parameters created by molecular interaction. The interaction of each wine fraction with poly-L-proline (PLP) of molecular weight 10 kDa (Sigma Aldrich) was determined. The reference cell was filled with MiiliQ water and the titrant was a solution of each fraction between 0.5 mM to 2 mM, reconstituted in a 10 mM ammonium acetate buffer containing 5% v/v ethanol, pH 4.0. The molar concentration was calculated using the average molecular mass of each fraction determined by gel permeation chromatography (GPC). The sample cell contained a 20 mM PLP solution prepared in the same ethanolic buffer. The titration method was based on 23 × 0.5 µL injections for four seconds with 70 seconds between each injection. During titration, continuous stirring at 750 rpm was applied, and the cells retained at room temperature. A buffer titration control was injected each time to verify that the heat exchange was negligible. To treat the ITC data, MicroCal PEAQ-ITC analysis software was used. After subtraction of baseline data obtained from the blank titration, the data were fitted by a one-site model to obtain the thermodynamic parameters, namely: the association constant (Ka), the enthalpy of interaction (ΔH), the Gibbs free energy (ΔG) and the entropy (ΔS). Each sample was injected three times.

6.19 Tannin activity

Tannin activity of purified tannins from selected wines was determined using a previously described RP-HPLC method (Watrelot et al. 2016). This method is based upon the thermodynamics of interaction between injected isolated tannin dissolved in model wine (5 g/L tartaric acid, pH 3.5 and 12% ethanol) and polystyrene divinylbenzene HPLC column. Enthalpies of interaction were then determined according to Watrelot et al. (2016). Analyses were carried out in duplicate.

6.20 Protein modeling

Structures of chitinase (CHI) and thaumatin-like protein (TLP) was modelled using Swiss-Model. The structure and the surface of the protein were presented using UCSF Chimera 1.13.

6.21 Metal analysis

Metals content in wine was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) performed by AWRI Commercial Services (now Affinity Labs).

6.22 Plasma deposition

Plasma polymerisation of different plasma precursors was carried out in a custom-built reactor with a 13.56 MHz plasma generator described elsewhere (Robinson et. al 2008). Allylamine, acrylic acid, and 2-methyl-2-oxazoline were used as precursors for plasma deposition to generate thin films rich in amine, carboxyl, and oxazoline chemical groups, respectively. Clean silicon wafers and 100-mesh stainless steel sheets were used as substrates. Before plasma deposition, the chamber was evacuated to a base pressure of 1×10^{-3} mbar. Allylamine or acrylic acid vapour was introduced at 0.13 and 0.15 mbar, and plasma was applied at 40 W and 10 W, respectively. Deposition of 2-methyl-2-oxazoline was carried out at 0.08 mbar and power of 50 W. In all three cases, the plasma deposition time was two minutes.

6.23 X-ray photoelectron spectroscopy (XPS)

To determine the surface composition of materials used, XPS analysis was performed. The XPS spectra were obtained with a Specs SAGE XPS spectrometer using an Al Ka radiation source (hm = 1253.6 eV) at 10 kV and 20 mA. Elements present on the sample surface were identified from the survey spectrum recorded in the energy range 0-1,000 eV with a pass energy of 100 eV and a resolution of 0.5 eV. The areas under the selected photoelectronic peaks in a broad scan spectrum were used to calculate the percentage of atomic concentrations. High-energy-resolution spectra (0.1 eV) were then recorded for the relevant photoelectronic peaks at passing energy of 20 eV to identify the possible chemical bonding environments for each element. All binding energies were referred to the neutral carbon C1s peak at 285 eV to compensate for the effect of surface charge. Data processing and curve fitting were performed with the Casa XPS software.

6.24 Thickness measurements

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

6.25 Fourier transform infrared spectroscopy (FTIR)

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

6.26 Scanning Electron Microscopy/ Energy Dispersive X-Ray (SEM/EDX)

The SEM was used to determine the morphology of the natural zeolite samples, while the elemental composition of the zeolites was determined with EDX. An FEI Quanta 450 FEG-ESEM with an EDAX Apollo X Energy Dispersive X-Ray (EDX) spectrometer was used for the analysis.

6.27 Optical microscopy

Optical microscopy images were acquired with an Eclipse Ni-U upright microscope equipped with a Nikon DS-U3 camera and an 8MP USB Handheld Microscope. Images were analysed using Image J software. For calculating % surface coverage we prepared three samples per coating. These samples were analysed by taking three images per sample.

6.28 X-ray diffraction (XRD)

Powder X-ray diffraction patterns were recorded at room temperature using a PANalytical X'Pert Pro MPD diffractometer in the Bragg-Brentano reflection configuration. Copper Cu Kα radiation from a

sealed tube was used. Data were collected in the 2θ range of 5 - 90° with a step of 0.0167° and exposure per step of 27 s. Since the raw diffraction data contained some noise, the background during the analysis was subtracted using the algorithm of Sonneveld and Visser (1975). The data were then smoothed with a cubic polynomial function.

6.29 Specific surface area and porosity

The specific surface area of the natural zeolite samples was determined using the Brunauer, Emmett, and Teller adsorption technique (BET). Pore size distribution was determined using the BJH method of Barrett, Joyner, and Halendam. Measurements were performed on the Micromeritics ASAP 2020 adsorption instrument (Surface Area and Porosity Analyzer, Micromeritics Instrument Corporation, Norcross, GA, USA).

6.30 Zeta potential measurements

The electrophoretic mobility of bare and plasma-coated magnetic nanoparticles was determined using Zetasizer Nano ZS (Malvern, UK) and transformed into zeta potential using the Smoluchowsky equation for recognition. Measurements were carried out at 22 °C in 10^{-3} M NaCl.

6.31 Fluorescence correlation spectroscopy (FCS)

FCS experiments were carried out on a commercial set-up (LSM 880, Carl Zeiss, Jena, Germany). The excitation of the Alexa 488 labelled species was conducted by an argon ion laser (488 nm) focused into the studied solution by a high numerical aperture water immersion objective (C-Apochromat 40x/1.2 W, Carl Zeiss, Jena, Germany). The emitted fluorescence light was collected with the same objective after passing through a confocal pinhole directed to a spectral Quasar, Carl Zeiss detection unit. The emission of Alexa 488 was detected in the spectral range 500 - 550 nm. These arrangements resulted in the formation of a very small (< 1fL) confocal observation volume (Vobs) in the studied sample. Eight-well polystyrene chambered cover glasses purchased from Nunc, Thermo Fisher Scientific, Waltham, MA, USA were used as sample cells for the studied solutions. For each solution, a series of 20 measurements with a total duration of 200 s were performed at room temperature (23°C).

6.32 Synthesis of a fluorescent dye

A 4-(1,1,2-Trimethyl-*1H*-benzo[*e*]indol-3-ium-3-yl)butane-1-sulfonate was prepared according to a modified protocol (Ioffe et al. 2007) based on a previously published method (Li et. al 1997), the squaric acid (2,3-dihyroxy-3-cyclobutene-1,2-dione) was commercially available and was used as supplied. In a 50 mL round bottom flask, equipped with an electromagnetic stirrer, silicone bath, reflux condenser, connected to a Dean-Stark trap, under an argon atmosphere 0.5 g (0.0015 mol) 4-(1,1,2-trimethyl-*1H*-benzo[*e*]indol-3-ium-3-yl)butane-1-sulfonate, 0.083 g (0.0007 mol) 2,3-dihyroxy-3-cyclobutene-1,2-dione and 15 ml toluene, 15 mL n-butanol and 10 µL pyridine were loaded. The mixture was heated to 130 °C (the temperature of the silicone bath) for two hours. After cooling, the precipitate formed was suctioned, filtered, and air dried, and the yield was 0.34 g (63%). The chemical structure and purity of the synthesised dye were confirmed by NMR spectroscopy. The synthesised squarylium dye had a molecular weight of 768.94 Da, and teal colour. All solvents used in the NMR spectroscopy were commercially available (HPLC grade). NMR spectra were obtained on a Bruker Avance III 500 DRX 600 MHz spectrometer in $CDCl_3$.

6.33 Preparation of stock solutions, standards and fluorescence measurement

The stock solution of the fluorescent dye was prepared by dissolving the dye in DMSO (Sigma Aldrich, St. Louis, MO, USA). The solution was heated to 60 °C and sonicated for five minutes with a concentration of 1 x 10^{-3} M. To explore the photophysical properties of the dye, the absorbance and fluorescence were measured in Tris (hydroxymethyl) aminomethane-ethylenediaminetetraacetic acid buffer (Tris-EDTA buffer, TE buffer) pH 8 and model wine solution (12% ethanol, 4 g/L of tartaric acid, pH 3.5). The absorption spectra of the dye were measured at a concentration of 1 x 10^{-5} M and the fluorescence emission spectra at 1 x 10^{-6} M. Thaumatin was used as the standard (Sigma Aldrich,

St. Louis, MO, USA). For calibration purposes, fluorescence was measured in a model wine solution at pH 3.5 in the absence and presence of thaumatin at different concentrations (0 mg/L and 700 mg/L). As for the actual wine experiments, no dilution was required. Fluorescence was also measured with total polysaccharides isolated from red and white wine (200 mg/L), as well as in white wine polysaccharide solution clarified with bentonite to remove proteins. The selectivity of the dye towards chitinases was also investigated. For this, the fluorescence was measured in a model wine solution containing 20 mg/L of chitinase O3 protein. The O3 is Class IV chitinase (*Vitis vinifera*) with a molecular weight of 31833 D, isolated and purified from the Sauvignon Blanc wine by ion-exchange chromatography according to the previously published method (Van Sluyter et al 2009). Fluorescence intensity was measured on Agilent Cary Eclipse Fluorescence Spectrometer using a 600 nm excitation wavelength. All fluorescence measurements were carried out under ambient conditions.

7. Results and discussion

7.1 Year 3 deliverable. Improved methods to assess protein stability

Many existing methods to predict protein haze potential in wine are time-consuming and come with a risk of false positive results. Currently, the heat test is the most widely used method in the industry to assess the heat stability of white wines and determine the amount of bentonite additions. This method was recently optimised to be shorter and more reproducible. However, it still takes a very long overall time of five hours (two hours of heating and three hours of cooling) (McRae et al. 2018). There is a strong need to establish rapid analytical methods for quantifying the haze protein, which would result in a reduction of the amount of bentonite used and number of heat tests performed, with consequent cost and quality benefits. In addition, establishing the level of haze-forming proteins among the total protein level in wine is expensive as it requires the use of techniques such as high-performance liquid chromatography (HPLC), mass spectroscopy (MS) or sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE).

The possibility of using gold nanoparticles as a calorimetric sensor for haze-forming protein (HFP) detection was investigated. Following initial positive findings, unexpected aggregation at wine pH led to the selection of a fluorescence-based dye as a more promising alternative approach to detect HFPs in wine. Although a method for protein detection using fluorescence did already exist and had been used in wine studies (Marangon et al. 2013), it was time-consuming, could not be used directly in wine, and its selectivity for haze-forming proteins was unknown.

A new efficient and selective fluorescence-based sensing technology was therefore developed to detect haze-forming proteins in white wine. The method is simple, provides results rapidly, and is accurate. A schematic illustration of the interaction of the fluorescent dye with wine proteins is presented in Figure 1. As shown in this figure, when the dye binds to protein, a shift in the maximum fluorescence intensity from 668 nm to 682 nm occurs.

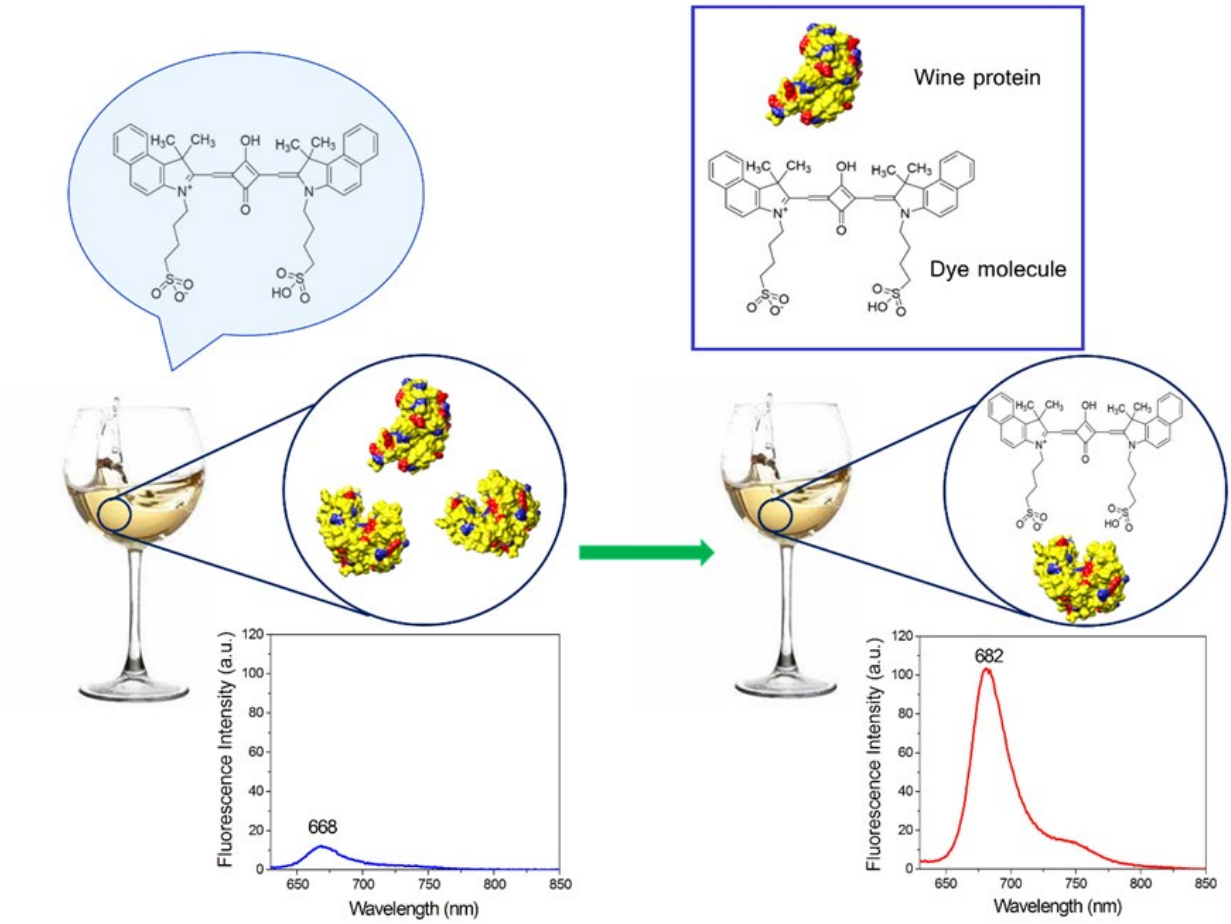


Figure 1. Schematic illustration of the interaction of the fluorescent dye with wine proteins

The developed method was found to be selective for haze-forming proteins, provided a linear detection range and had a low detection limit of 2 mg/L. It was validated using a range of white wines, including Sauvignon Blanc, Chardonnay, Semillon, Muscat Gordo, Pinot Grigio, Colombard, Elbling, Riesling and Gewürztraminer. Using this method, the baseline level for protein below which haze in white wine did not occur was determined to be 12 mg/L for this diverse sample set. Given that certain wines may in fact be heat stable at substantially higher protein concentrations (McRae et al. 2018), the protein detection approach cannot immediately replace the existing heat test, as it presents a risk of over-fining if extremely low levels of protein are targeted. Hence, it is suggested that rapid protein detection could be used as an initial screen to determine if the protein concentration of a wine is above the threshold for instability, with wines then to be analysed using the heat test only if required.

To the best of the authors' knowledge, this represents the first example of a fluorescence-based sensing approach for detection of haze-forming proteins in wine. More information on this study can be found in Mierczynska-Vasilev et al. (2022).

7.2 Year 3 deliverable. Improved methods to assess cold stability, improved knowledge of the roles that matrix composition (especially macromolecules) play in increasing the cold stability of wines

7.2.1. Unravelling the role of white and red wine macromolecules in conferring cold stability

The earliest stage of the cold stability project involved a literature review to identify approaches to improve the assessment of cold instability in white and red wine. Although it is accepted that wineries apply a variety of cold stability tests, each is considered to have its flaws. The three-day (brine) test is the most widely used within Australia, and involves chilling to -4°C for 72 hours, without seeding. After the chill period, the wine is brought to room temperature, and any soluble tartaric acid is reconstituted. Crystal formation, as potassium hydrogen tartrate, is then assessed visually. This test is considered to be overly harsh, as wines that may otherwise remain cold stable in the long term could throw crystals under these conditions. However, for research purposes this test was considered critical to any comparative study, given its widespread use in Australia. Further, the conductivity test (mini-contact test) is widely used in the USA and Europe, but has seen little uptake in Australian wineries. Despite this, the test can be considered to be simple and easy to apply, and involves seeding of a wine with 10 g/L KHT, chilling to 0°C with stirring, and monitoring the change in conductivity over a period of 4 to 30 min (Bosso et al. 2016). Inaccuracies are thought to arise as a result of the short period in over which crystallisation occurs relative to the longer three-day test, but also due to a high level of variability in the particle size distribution of KHT crystals used for seeding.

The literature review indicated that Bosso et al. (2016) had validated an improved version of the mini-contact test for cold stability using micronised KHT, allowing for a reduced test period of only four minutes. The decision was therefore taken to incorporate the improved version of the mini-contact test into the AWRI study, but to compare different time periods over which the conductivity change was observed, 4, 20 and 30 minutes.

It was also recognised that the current three-day cold test only provides an indication of a categorical fail (class 1-3), which is not quantifiable for statistical purposes in research. Therefore, a new, quantitative test (crystal ID) was developed as part of the cold stability project to accurately quantify and recover KHT crystals induced from the three-day test, and to measure these by HPLC. This was a change from other studies which have instead analysed changes in tartaric acid in the wine itself during the three-day test (Bosso et al. 2016 and references therein). It was an early observation that the absolute change in tartaric acid in a wine might be small, even while the formation of visible crystal deposits was evident. It was therefore preferable to develop a method to quantify crystal formation as opposed to losses in tartaric acid. In the course of the study, both the crystal ID test and the mini-contact test were reduced to 10 mL and 30 mL formats respectively, allowing for smaller sample sizes to be analysed (e.g. for reconstitution studies).

Following an investigation of cold stability tests, a long-term assessment of white and red wines was performed, starting in 2019. A comparison of new and existing cold stability tests was made for 40 red and white wines over two vintages (2019 and 2020), with detailed compositional analysis also performed in order to understand the role of macromolecules and/or other compositional factors in conferring cold stability, if any. The improved format for the mini-contact test using micronised KHT was implemented, as well as the new crystal ID test, the standard AWRI three-day (categorical fail level 1 to 3) and saturation temperature (Tsat) tests. Wines were also cold-stabilised for three months at 0°C, and the crystals measured visually and by recovery as tartaric acid. Repeat analyses were done at 18 months.

The results for the % change in conductivity related well to the absolute conductivity of a wine (R^2 >0.9), independent of grape variety or vintage. This indicated that the micronised mini-contact test may have overcome some observed limitations of the standard conductivity test, as this correlation has previously been observed to be poor (pers. comm. Eric Wilkes, E. & J. Gallo data). The crystal ID test was also shown to have a strong correlation with the micronised mini-contact and Tsat test, and was then further used for response surface area experiments to identify the key compositional attributes of white and red wines which lead to cold instability. Wine macromolecular targets identified by this preliminary study will be explored in the new AWRI 2.4.1 'Non-volatiles' project, to better understand their role in improving cold stability.

From the compositional study, red wines were generally more cold stable than whites, and had higher overall polysaccharide, tannin and potassium concentrations, as well as a higher potassium:tartaric acid ratio. The data were modelled using PLS to predict cold stability, determined either by analytical methods, or the actual cold instability determined following the recovery of crystals from three months of storage at 0°C. Results for the weighted regression coefficients of factors significant in the PLS model developed for the prediction of crystals formed after three months of storage at 0°C are shown in Figure 2. For unfined white wines, it was found all three predictive methods used (Tsat, mini-contact and Crystal ID) were correlated with one another, and were strongly associated with the actual cold instability measured as crystals formed after three months of storage at 0°C. The absolute conductivity was found to be a strong positive coefficient in the model, and was related to the potassium concentration as well as high concentrations of a number of other metals, as would be expected. The cold instability was related to the potassium:tartaric acid ratio (Figure 2A), with a lower wine tartaric acid (negative regression coefficient) and high potassium concentration largely explaining KHT crystal formation. Importantly, white wine cold instability was not clearly influenced by macromolecules such as polysaccharides, proteins and phenolics, as indicated by the fact that these measures were not significant in the PLS model.

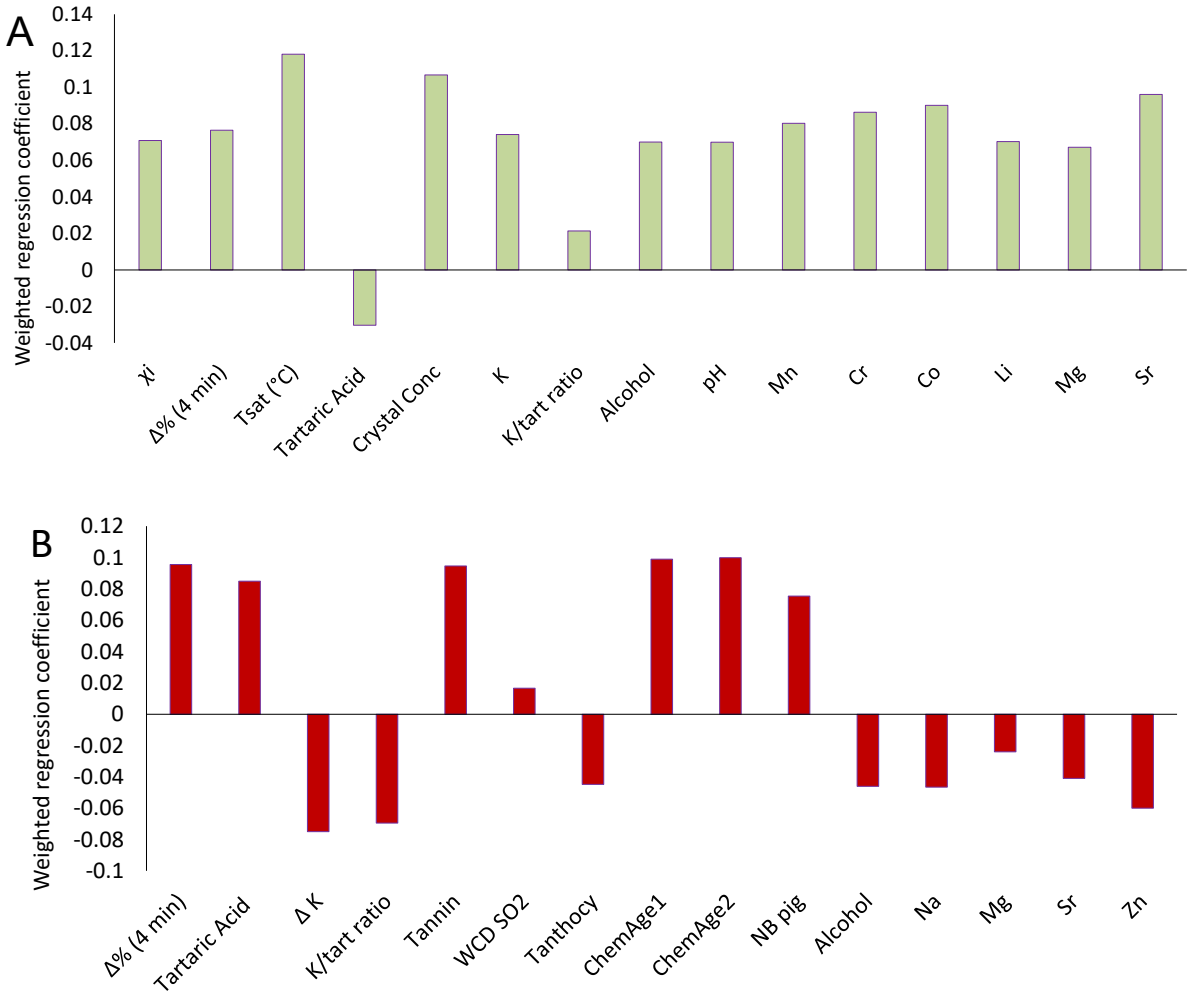


Figure 2. Weighted regression coefficients derived following partial least squares analysis for the prediction of crystal formation following a three-month storage period at 0°C for **A.** white wines from 2019 and 2020, **B.** red wines for 2019 and 2020 (ci, conductivity; Δ% (4 min), percentage change in conductivity following the mini-contact test over four minutes; crystal conc, crystal concentration recovered using the crystal ID test; K, potassium; ΔK, change in potassium; K/tart ratio, potassium/tartaric acid ratio; WCD SO2, wine colour density corrected for SO_2 concentration; Tanthocy, total anthocyanin; ChemAge, chemical age measurement 1 or 2; NB pig, non-bleachable pigments.

The assay of unfined red wines over two seasons produced some interesting results (Figure 2B). Firstly, it was found that the mini-contact test results were most strongly related to the crystals formed after three months of storage at 0°C, with a high, positive regression coefficient in the PLS model. For red wine, tartaric acid was also important in the model, but unlike for white wines, it was positively associated with cold instability. Total potassium was not significant in the model, but the change in potassium during the mini-contact test, as well as the potassium/tartaric acid ratio both had high, negative, regression coefficients. Various metals also had negative regression coefficients in the model. This was contrary to what was observed for white wines. Therefore, for red wines, higher tartaric acid was an important predictor of cold instability, with the relevance of the potassium/tartaric acid ratio likely being driven by the tartaric acid concentration.

As for white wine, polysaccharides were not an important inhibiting factor in KHT crystallisation in red wines, which again was unexpected. Most interestingly, higher tannin concentrations in red wine were invariably associated with increased cold instability. This was unexpected, as preliminary work

where wine tannin was added to a bentonite-treated white wine was found to improve cold stability (data not shown). To account for this observation, it was important to note that wine colour density, chemical age measures 1 and 2, as well as polymeric pigments were significant in the PLS model, and were positively loaded, while total anthocyanin had a negative regression coefficient. The chemical age measurements relate to non-bleachable pigments (which are resistant to SO_2 bleaching) and the total anthocyanin measure. Chemical age 2 describes how much of total red pigment at low pH is imparted by non-bleachable pigment as a percentage, while chemical age 1 reflects the proportional recovery of the colour of non-bleachable pigment by acetaldehyde. The relevance of these measures in the PLS model indicates that the relative proportion of non-bleachable forms of anthocyanin to total (monomeric, ionised) anthocyanin were important predictors of cold instability. Tannin concentration is important to non-bleachable pigment formation, in that it forms pigmented complexes with anthocyanin.

To attempt to further unravel these results, response surface area experiments were conducted to understand the specific role of wine macromolecules in tartrate crystal formation. Firstly, a response surface study was performed to develop a cold-unstable model wine, capable of precipitating crystals within the range reported for white and red wines. Follow-up response surface studies then investigated key macromolecule categories found in white wine (polysaccharide, protein, monomeric phenolics) or red wine (tannin, anthocyanin, polysaccharide). To this end, purified fractions of the principal macromolecules in white and red wine were prepared. For white wine phenolics, commercial standards of flavonoids and hydroxycinnamic acids were added at wine-like concentrations.

In the white wine response surface models, it was found that protein and phenolics could slightly reduce KHT crystal formation either independently or in combination (Figure 3A). White wine polysaccharides were found to have no impact on KHT crystal formation, contrary to expectation (Figure 3B). The inhibition of KHT crystallisation by phenolics was removed when polysaccharide was present. However when protein was combined at higher concentrations with polysaccharide, a small drop in KHT crystal formation was found, but not to the extent found for protein alone. These results confirmed the results of the compositional survey and demonstrate why for white wines, which invariably have a substantial concentration of polysaccharides, the potentially protective influence of other macromolecules has little to no influence on KHT crystallisation.

Further investigation of the key red wine macromolecule categories (tannin, anthocyanin and polysaccharide and their interactions) was conducted for the range of concentrations expected in red wine. Contrary to what was expected from the wine compositional survey, tannin was found to introduce some protection against KHT crystallisation when applied in the higher concentration range (Figure 3 C). Red wine polysaccharides, which are present at higher concentrations in red wines than in whites, also showed no protective effect against KHT crystallisation. Red wine polysaccharides also reduced the inhibiting effect of tannin on crystal formation when in combination. Surprisingly, anthocyanin was found to bring the model wine close to cold stable, at the lowest applied concentration, and this effect was exerted even when tannin or polysaccharide were present (Figure 3 C and D). It was found that the addition of anthocyanin to a bentonite-fined white wine or model wine was able to cold stabilise the wine to the same extent as a commercial dose of potassium polyaspartate. Although the potential role of anthocyanin in limiting KHT instability has long been known, these results potentially explain why the compositional study revealed a positive association of wine tannin and KHT crystal formation. Wines with higher tannin concentration may more rapidly undergo polymerisation reactions with anthocyanin, losing the protective monomeric structural forms. This indicates that the measurement of anthocyanin, and its expected conversion (hence loss) to other forms with time may provide a useful prediction of cold instabilities developing during red wine maturation.

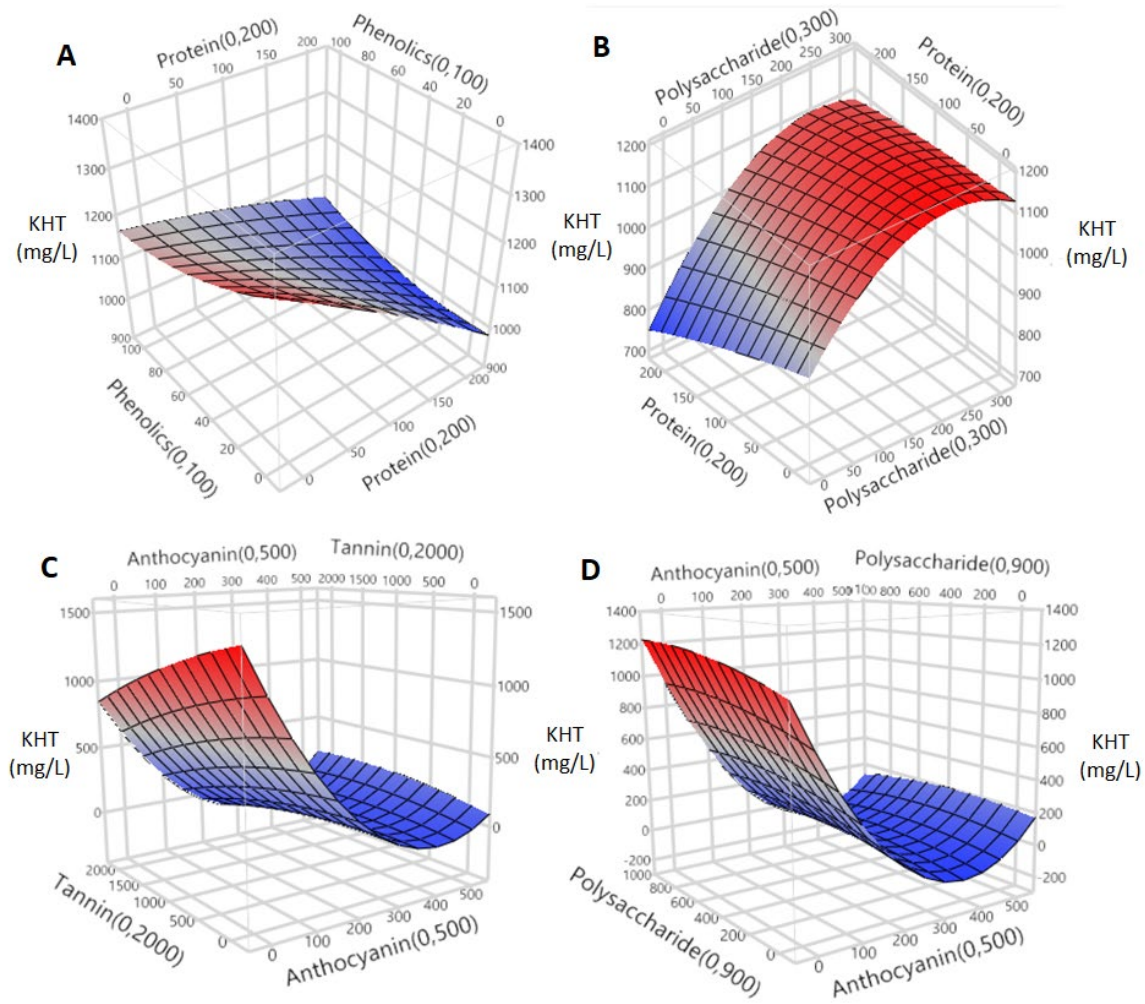


Figure 3. Response surface experiments comparing the influence of various wine macromolecules and their interactions on the formation of potassium bitartrate (KHT) crystals in model wine during a 3-day cold test showing **A.** White wine model, protein and phenolics; **B.** White wine model, protein and polysaccharide; **C.** Red wine model, tannin and anthocyanins; **D.** Red wine model, polysaccharide and anthocyanin.

In a subsequent study, young red Shiraz wines (36) from the 2021 season were analysed prior to cold stabilisation. Young red wines were found to be invariably stable, despite a wide range of potassium (0.9 – 1.6 g/L) and anthocyanin (0.7 – 1.1 g/L) concentrations. Only one of the wines analysed was highly cold unstable, and this wine had a low degree of anthocyanin ionisation, although not the lowest value recorded in the set. Since all wines went through cold stabilisation as part of the winemaking process, further experimentation on the progression of cold stability through pigment polymerisation and ageing was not achieved. However, further studies will be performed in the new 'Non-volatiles' project.

7.2.2 Alternative cold stabilisation processes

The cold stability work also progressed to investigate the development of surfaces with defined functional groups to induce crystallisation. Five different plasma polymerised coatings (allylamine, acrylic acid, 2-methyl-2-oxazoline, ethanol and 1,7-octadiene) with unique functional groups were produced on silicon wafers. The thicknesses of the deposited plasma polymers were determined using spectroscopic ellipsometry and were respectively 35, 30, 25, 20 and 22 nm for allylamine, acrylic acid, 2-methyl-2-oxazoline, ethanol and 1,7-octadiene. Except for they hydrophobic octadiene coating with a water contact angle of 86^{O}, all other coatings were hydrophilic with water contact

angles of 46^{O}, 53^{O}, and 62^{O} for the acrylic acid, 2-methyl-2-oxazoline, and allylamine coatings, respectively. Surfaces were introduced to highly cold-unstable Muscat Gordo wine and their ability to promote crystal nucleation and growth was investigated. Three out of five plasma-coated surfaces (amine, oxazoline and acrylic acid) were identified as having the ability to promote crystal nucleation and growth at 0°C and 4°C. This suggested that crystallisation might be able to proceed at higher temperatures in which would reduce energy inputs required for cold stabilisation.

The potential ability of natural zeolites to cold stabilise white wines had been identified based on previous observations when using this material for protein stabilisation of white wines (Mierczynska-Vasilev et al. 2019). Wyss and Cuénat (2005) demonstrated that the selective reduction of potassium ions in wines (i.e. 10-30% of the initial potassium amount) likely inhibits the precipitation of potassium bitartrate salts. It was found that after zeolite treatment, the potassium ion concentration in the treated wines was significantly reduced by more than 30% in all cases. Therefore, work was undertaken to evaluate the ability of natural zeolites to cold stabilise white wines. Application of zeolites to finished white wines was identified as a possible alternative approach to introduce cold stability at a warmer temperature of 15°C, without the requirement for a cooling step to introduce crystallisation.

Crystallisation was spontaneously initiated following zeolite addition in wines made from three white grape varieties. In wines with less intrinsic cold stability, crystallisation was initiated immediately and was complete within a few days. In wines with greater intrinsic cold stability, crystallisation proceeded at a slower pace, and was achieved at four months, based on mini-contact test results (Figure 4). Minimal changes in wine pH indicated that despite losses in tartaric acid during crystallisation, substantial additional acid additions would not be required, further reducing input costs.

Cold-unstable wines which were treated with zeolite had improved cold stability relative to the control post-bottling, which improved further up to four months. Between four and nine months post-bottling further improvements in cold stability were minor.

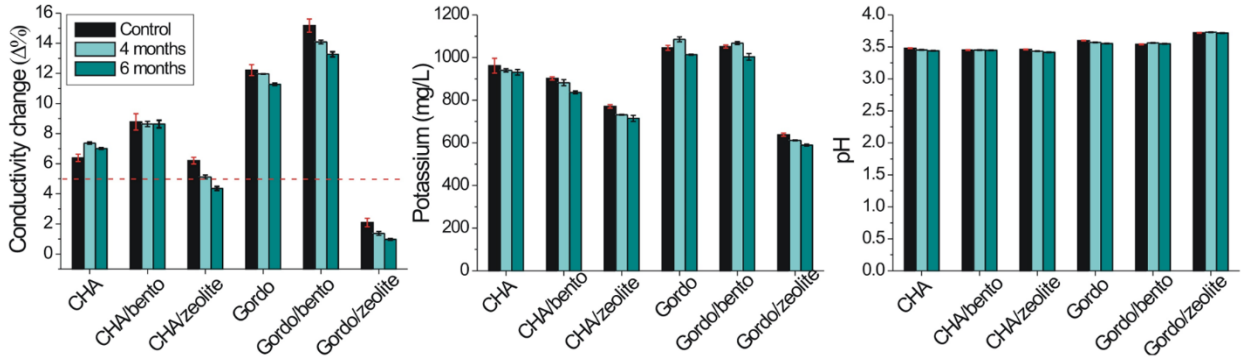


Figure 4. Conductivity change in the mini contact test, potassium concentration and pH of Chardonnay (CHA) and Muscat Gordo (Gordo) wines before and after treatment with Pluxcompact bentonite (bento) and Indonesian zeolite. The red line represents a change in conductivity of 5% in the mini-contact test, which is typically considered the level below which wines are considered cold stable.

7.3 Year 3 deliverable. Novel or more efficient approaches to achieving protein stabilisation.

7.3.1. Pasteurisation of must for the reduction of haze-forming proteins

Previous trials indicated that heating juice for one minute can reduce protein concentration, and therefore the amount of bentonite required for heat stability (Marangon et al. 2011). To further

explore this concept, batches of 2018 vintage Semillon and Sauvignon Blanc juice from the Riverland, SA were heated using a commercially available pasteurising unit at 75°C for periods of one and two minutes. Heated and control juices were then fermented and cold stabilised. Control wines were separated into two treatments, with one batch left unstabilised and one batch receiving bentonite fining. It was found that the unheated, control musts took longer to complete primary fermentation (56 and 120 days for Semillon and Sauvignon Blanc, respectively) than the heat-treated ferments (44 and 70 days for Semillon and Sauvignon Blanc, respectively). A further key difference between the ferments of the unheated and heat-treated juice was that the unheated controls went through spontaneous malolactic fermentation whereas the heat-treated juice ferments did not. This may be to be due to the heat treatment destroying some of the nutrients required for malolactic fermentation, or directly sterilising the juice by killing any native lactic acid bacteria.

The goal of pasteurising the grape juice was to reduce the concentration of the wine proteins that cause haze, and therefore reduce the amount of bentonite required to heat stabilise the wine. For the juices, heat treatment for one minute reduced the protein concentration by approximately 58% and 65% for Semillon and Sauvignon Blanc, respectively (Figure 5). Heating was most effective in removing chitinases, which are less stable than thaumatin-like proteins and therefore more likely to form a haze (Van Sluyter et al. 2015). Heating juice for an additional minute did not substantially change the protein concentration.

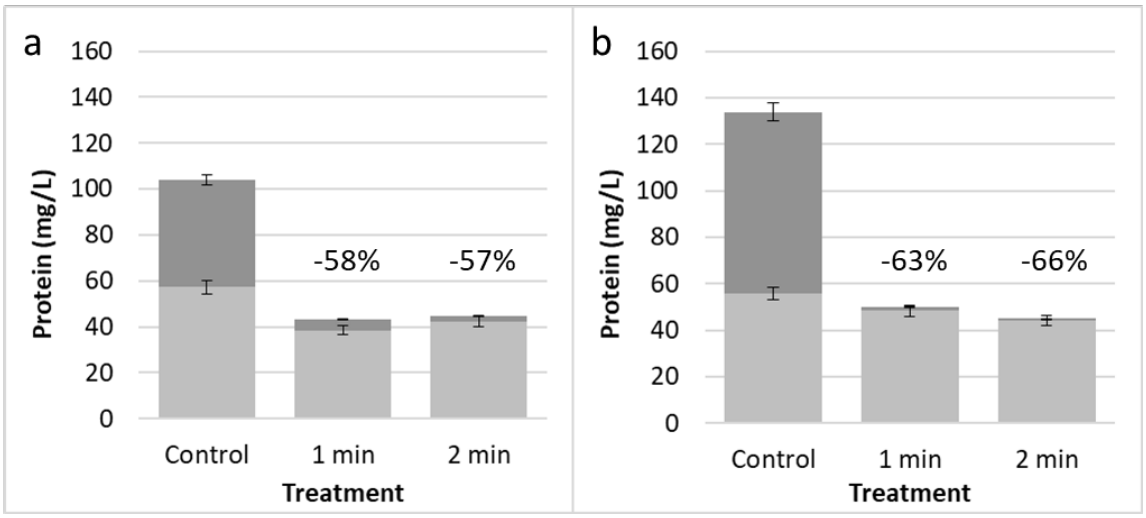


Figure 5. Concentration of total haze-forming proteins, showing proportions of thaumatin-like proteins (light grey) and chitinases (dark grey) in unheated juice and juice heated for 1 and 2 minutes at 75°C for a) Semillon and b) Sauvignon Blanc, including the per cent reduction in total protein concentration of treated juice compared to the unheated control.

However, when looking at the protein concentrations of the wines made in this trial, it was found that the wines made from unheated juice experienced a natural loss of wine protein during fermentation, filtration and packaging, whereas for the wines made from pasteurised juice, the protein concentration did not reduce further during the winemaking process. This natural loss of proteins is something commonly observed during fermentation (Vicenzi et al. 2011) and during the long-term storage of white wines that have not been heat stabilised (unpublished data). As a result, in this trial, the wines made from heated and unheated juices had similar protein concentrations at bottling, with both sets of wines heat unstable (>17 NTU turbidity difference observed in the heat test). These results suggest that where bentonite is to be added early in the winemaking process (i.e. during fermentation), heating juice may significantly reduce the bentonite dose required, in this case by 79% (from 1.2 g/L to 0.25 g/L for both Semillon and Sauvignon Blanc). However, in cases where bentonite is to be added later in the life of the wine, reductions in bentonite dose from juice heating may not be realised, since a certain proportion of haze-forming proteins may naturally be lost during the winemaking process.

A critical concern when heat-treating grape juice is any potential effect on wine flavour and aroma. Triangle tests were performed using a panel of approximately 30 wine consumers to identify if there were any differences between wines made from the juice that had been heated for one minute and the two different control wines (made from unheated juice and either unfined or bentonite fined). Sensory analyses were conducted on the wines six months after bottling. No significant differences were found ($p < 0.05$) between wines produced from heated juice and either of the control wines for both Semillon and Sauvignon Blanc (Table 1). This is likely to be because the juice was heated to 75°C for only a short amount of time and without the presence of oxygen, minimising any potential loss of volatile aroma compounds. Many aroma compounds are also only produced by yeast during fermentation, and so these would not have been affected by heating juice. These results suggested that juice can be heated for one minute at 75°C without causing a significant change to the wine sensory profile.

Table 1. Results of the triangle tests used to assess any sensory differences between wine produced from juice heated for 1 minute at 75°C and either of the control wines made from unheated juice (unfined or bentonite-fined).

Test	Comparison	Number of responses	Correct answers	Significance
1	Unfined control wine (made from unheated juice) vs wine made from juice heated for 1 minute (Sauvignon Blanc)	30	10	ns
2	Bentonite-fined control wine (made from unheated juice) vs wine made from juice heated for 1 minute (Sauvignon Blanc)	30	10	ns
3	Unfined control wine (made from unheated juice) vs wine made from juice heated for 1 minute (Semillon)	31	10	ns
4	Bentonite-fined control wine (made from unheated juice) vs wine made from juice heated for 1 minute (Semillon)	31	14	ns

ns = not significant at $p<0.05$

Heating juice had no significant sensory effect on the wine, but did induce some changes in wine chemistry, particularly polysaccharide composition. Wines produced from heated juice contained a lower proportion of high molecular weight polysaccharides, largely attributed to yeast-derived mannoproteins, and higher proportions of mid- and low-molecular weight grape-derived polysaccharides (Figure 6). As high molecular weight mannoproteins are produced by yeast during fermentation, this change is unlikely to be due to a degradation of juice polysaccharides during the heat treatment and may relate to changes in yeast nutrients that limit mannoprotein production. There were no significant differences in colour parameters between bentonite-fined control wines and wines produced from pasteurised juice, as measured by the overall difference in colour from the unfined control wines, (Figure 7), indicating that the wines did not suffer browning.

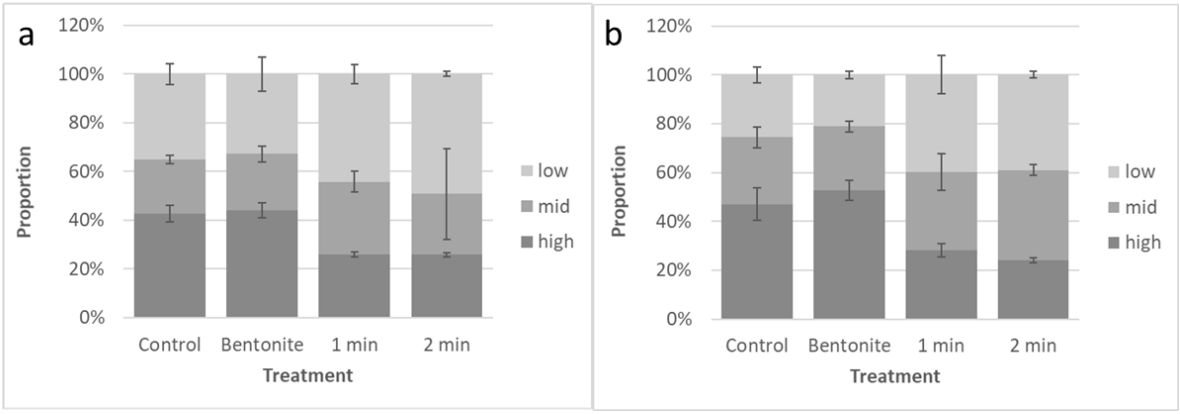


Figure 6. Proportion of low-, mid- and high-molecular weight polysaccharides in a) Semillon and b) Sauvignon Blanc wines produced from heated juice (1 and 2 minutes at 75°C), unfined control wines and bentonite-fined control wines. Results are shown as the average of three replicate ferments ± one standard deviation.

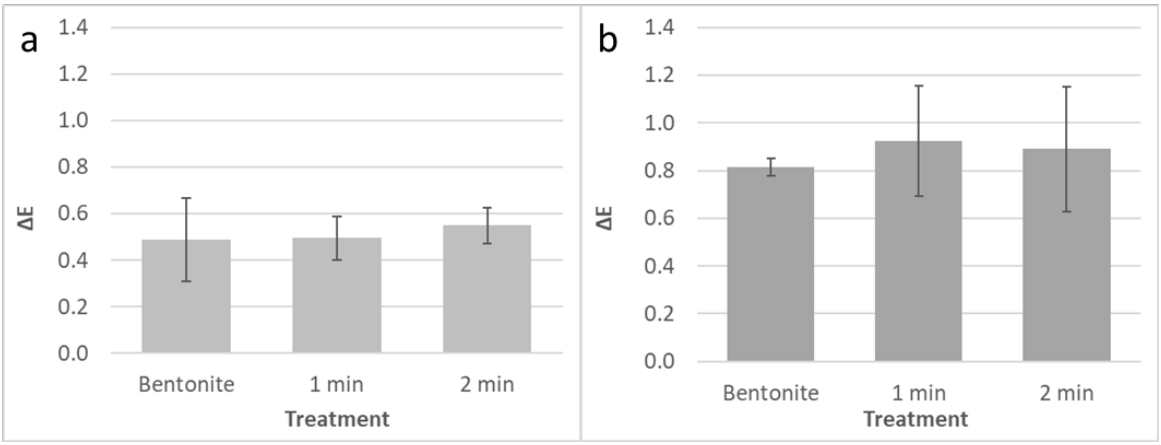


Figure 7. Change in wine colour (ΔE) compared to the unfined control wines for a) Semillon and b) Sauvignon Blanc wines produced from heated juice (1 and 2 minutes at 75°C) and bentonite-fined control wines. Results are shown as the average of three replicate ferments ± one standard deviation.

Phenolic concentrations in the wines (as measured by absorbance at 280 nm) were consistently lower in bentonite-treated wines than the untreated controls (Figure 8). For the Semillon wines the phenolic concentrations for both heat treatments were similar to those of the bentonite-treated control wine (Figure 8A). The Sauvignon Blanc wines had slightly higher phenolic concentrations than the Semillon wines, and in this case only the wine made from juice heated for two minutes had reduced phenolics concentrations, similar to the bentonite-fined wine (Figure 8B). These results show that in some cases treatments which remove protein may also remove some phenolic compounds from the wine; however, the absolute differences were minor.

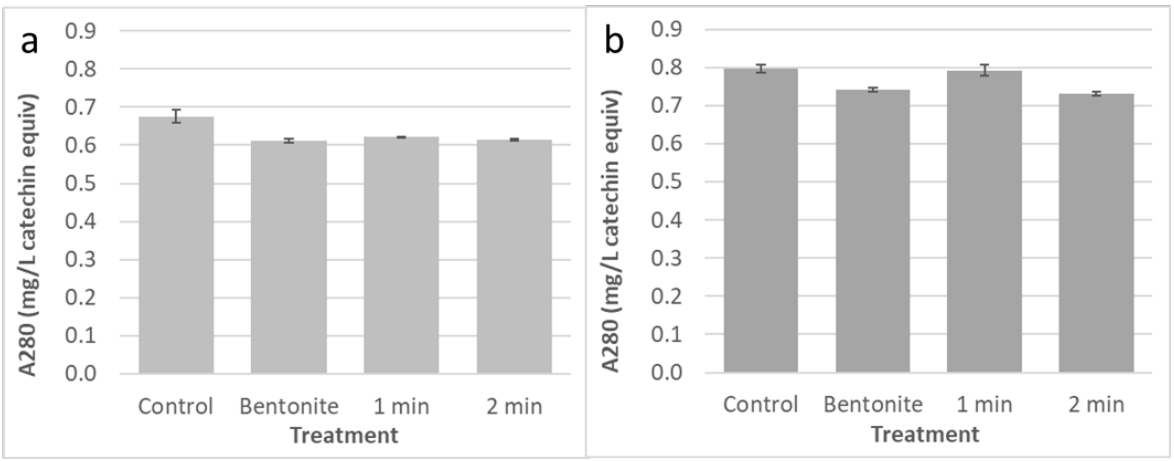


Figure 8. Total phenolics content (as measured by absorbance at 280 nm) for a) Semillon and b) Sauvignon Blanc wines produced from heated juice (1 and 2 minutes at 75°C) and bentonite-fined control wines. Results are shown as the average of three replicate ferments ± one standard deviation.

In this trial, heating grape juice at 75°C for one minute removed most of the haze-forming proteins and substantially reduced the amount of bentonite required to heat stabilise the wine if bentonite was to be applied during fermentation. When bentonite is to be applied later in the winemaking process, the benefits of heating juice may not necessarily be realised due to a likely natural loss of haze-forming proteins. Heating of juice did not produce significant changes to the sensory profile of the Semillon or Sauvignon Blanc wines in the trial, or sensorially important changes to wine composition, suggesting that quality loss would not result from application of this technique during winemaking.

7.3.2. Natural zeolites and magnetic nanoparticles as bentonite alternatives.

Natural zeolites and magnetic nanoparticles (MNP) were studied as possible alternatives to bentonite for wine heat stabilisation. Indonesian zeolite was found to effectively reduce concentrations of haze-forming proteins in wine, to the same levels achievable with bentonite. It was determined that zeolite particle size in the range of 20-50 microns and a treatment time of three hours were sufficient to achieve complete heat stability. Semillon was fully stabilised by applying 4 g/L of zeolite, whereas Sauvignon Blanc and Chardonnay wines were fully stabilised at 6 g/L dosage of zeolite. Although the gravimetric dose was higher with zeolite (4-6 g/L) than bentonite (0.8-1.8 g/L), zeolite does not exhibit shrink-swell behaviour, enabling higher wine recovery, and could also be regenerated. Therefore, wine volume recovery was more efficient with zeolite than bentonite.
More information on this study can be found in Mierczynska-Vasilev et al. (2019).

A comparative study of zeolites was carried out which, in addition to the Indonesian zeolite, included four natural zeolites sourced from Australian zeolite distributors. During the benchmarking experiments, all Australian zeolites were able to protein stabilise white wines. However, Indonesian zeolite showed differences in the removal of proteins from wine (e.g. 33 and 86 % of HFP was removed from Muscat Gordo using two Indonesian zeolite samples). Based on structure-activity studies, it was found that natural zeolites with a low calcite content had a better ability to remove haze-forming proteins in wines, making these zeolites more attractive for use as they induce both heat and cold stability in a single treatment (e.g. natural zeolite with a Ca content of 28% removed 33% of haze-forming proteins (HFP), while natural zeolite with a Ca content of 6% removed 86 %). In addition, the zeolite with a very high Si/Al ratio (8.4) was less effective than the zeolite with a lower Si/Al ratio (6.5). A manuscript on this study has been prepared for publication in the *Australian Journal of Grape and Wine Research.*

Following previous work showing successful heat stabilisation with acrylic acid plasma-coated magnetic nanoparticles, and considering the practical implications, the influence of surface functionalities on removal efficiency of haze-forming proteins from wines and the possibility to regenerate and reuse MNPs were examined. Surface coatings that have positive and negative surface charges and provide different interaction mechanisms with proteins were employed. An important selection criterion for the coatings was that they must be sufficiently hydrophilic to provide good dispersibility of the nanoparticles. Allylamine, acrylic acid, and 2-methyl-2-oxazoline monomers were used to produce plasma coatings rich in amine, carboxyl and oxazoline functional groups. The results showed that in the case of the protein attachment/binding process all three coatings were capable of adsorbing wine proteins, with the efficiency for protein removal as follows: $COOH > POx > NH_2$. The additional practical benefit of magnetic separation in wine processing is that MNPs can be used as a quick and simple method for the efficient and reliable capture and removal of proteins from wine. Furthermore, such surface functionalised magnetic nanoparticles may be suitable for other applications in the production of beverages where selective, effective and facile

separation of undesirable components and processing aids is required (e.g. membrane fouling). More information on this study can be found in Mierczynska-Vasilev et al. (2019a).

Regeneration studies were carried out using bare and acrylic acid plasma-coated MNPs and verified that acrylic acid plasma-coated MNPs and surface-active maghemite nanoparticles (SAMNs) exhibited a high HFP removal capacity and good reusability. Bare MNPs were not effective in removing pathogenesis-related proteins from wines and could not be successfully regenerated via the three solvents selected for this study. For coated MNPs, 10% sodium dodecyl sulfate (SDS)/water and pure water performed very well for successive wash and re-use steps, retaining a very high HFP removal efficiency (Figure 9). Analysis of other important wine constituents such as metals, organic acids and phenolics was also conducted in order to determine the impact of the MNP technology on the key attributes relevant to wine quality. For SAMNs, 0.5% SDS was an effective cleaning agent. Both forms of nanoparticles also show capacity for regeneration and re-use, presenting potential for commercialisation. More information on this study can be found in Mierczynska-Vasilev et al. (2019b).

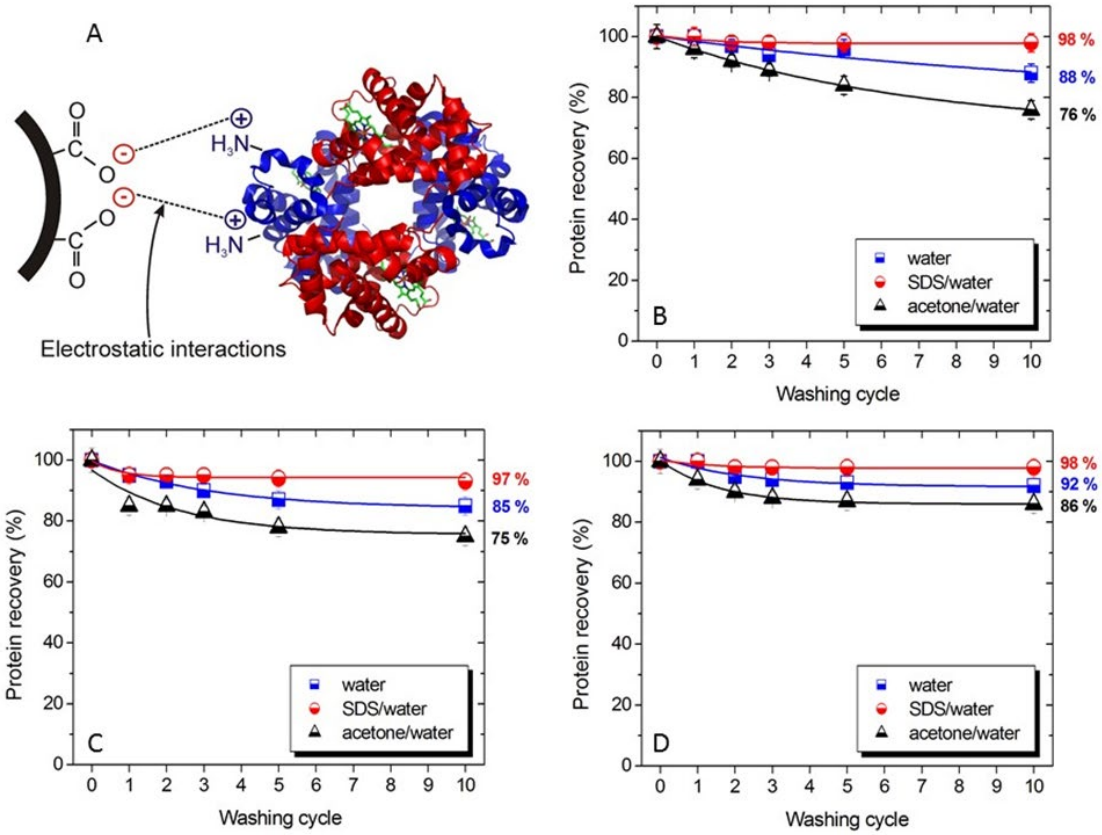


Figure 9A. Schematic of a possible mechanism of protein adsorption on acrylic acid plasma coated magnetic nanoparticle surface. Cleaning efficiency of Milli-Q water, 10% SDS/water and acetone/water for B. Sauvignon Blanc (SAB), C. Semillon (SEM) and D. Chardonnay (CHA) wine treated with acrylic-acid plasma coated magnetic nanoparticles (AcrA-MNPs). Figure reproduced from Mierczynska-Vasilev et al. (2019c).

7.4 Year 3 deliverable. Improved knowledge of the roles that matrix composition (especially macromolecules) play in modulating the protein stability of wines

7.4.1. Application of fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy FCS has been demonstrated to be an innovative approach to study the complex interactions between proteins, polysaccharides, and tannins in both model or real wines *in situ,* with high sensitivity and selectivity. Despite being accepted as an invaluable tool other fields of study, FCS had not yet been used for the study of wine colloids. It was proposed that the application of FCS to wine carbohydrates and tannins using a range of proteins including BSA, proline-rich proteins and salivary mucins could be a very important tool to better understand the texture of wine, especially with respect to astringency perception. In addition, FCS might be a useful tool for the study of other factors influencing protein-tannin interactions which may lead to instabilities, such as alcohol, pH and ionic strength.

Using this technique, it was found that an increased hydrodynamic radius of BSA eventuated in water-ethanol mixtures, caused by unfolding of the BSA and, to a lesser degree, could be also related to partial aggregation of the BSA in the presence of ethanol. Under wine-like conditions (solutions also containing 4 g/L of tartaric acid, 20 mM NaCl and adjusted to wine pH 3.5) BSA collapsed into a more compact globular structure with no signs of aggregation (Figure 10). The effect of wine polysaccharides on protein aggregation was very pronounced, with an RGII-enriched fraction inducing aggregation, while an MP-enriched fraction exhibited a protective effect. Low and medium concentration of RGI-enriched fraction exhibited a protective effect against protein aggregation while high RGI concentration encouraged aggregation (effect comparable to RGII).

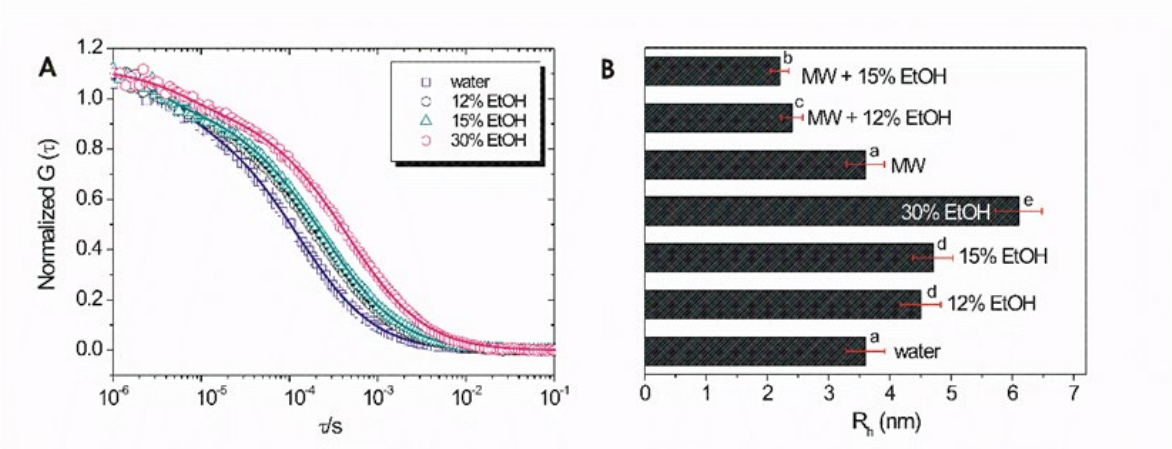


Figure 10. A) Normalised experimental autocorrelation curves (symbols) of Alexa Fluor 488-labeled BSA diffusing in the solutions of water and ethanol. B) Hydrodynamic radius (R_h) of Alexa Fluor 488-labeled BSA diffusing in water and model wine solution with and without ethanol. Significant differences between hydrodynamic radius were assessed by Student's *t* test at $p < 0.05$. Mean values with a different letter indicate significant differences. Figure is reproduced from Mierczynska-Vasilev et al. (2021) with permission from Elsevier.

The interaction between proteins and tannins was strong, as expected, leading to precipitation of protein-tannin complexes. The presence of polysaccharides partially prevented this precipitation as BSA 'encapsulation' in well-dispersed aggregates was observed. The aggregate size increased with increasing tannin concentration. In addition to demonstrating the utility of FCS to provide valuable information about protein conformational changes and dynamics of the interaction with various wine macromolecules, the project team also demonstrated the usefulness of the technique to

characterise protein haze formation in real wines (Figure 11). Heat-induced aggregation in white wine can be monitored *in situ* without the need of dilution and filtering the wine sample. This study points to further opportunities provided by FCS to more accurately and selectively study the complex phenomena occurring in wines in a novel way. Furthermore, this work also points to the potential of the FCS technique to address scientific challenges in other complex liquid matrices that pertain to food and beverage research. More information on this study can be found in Mierczynska-Vasilev et al. (2021).

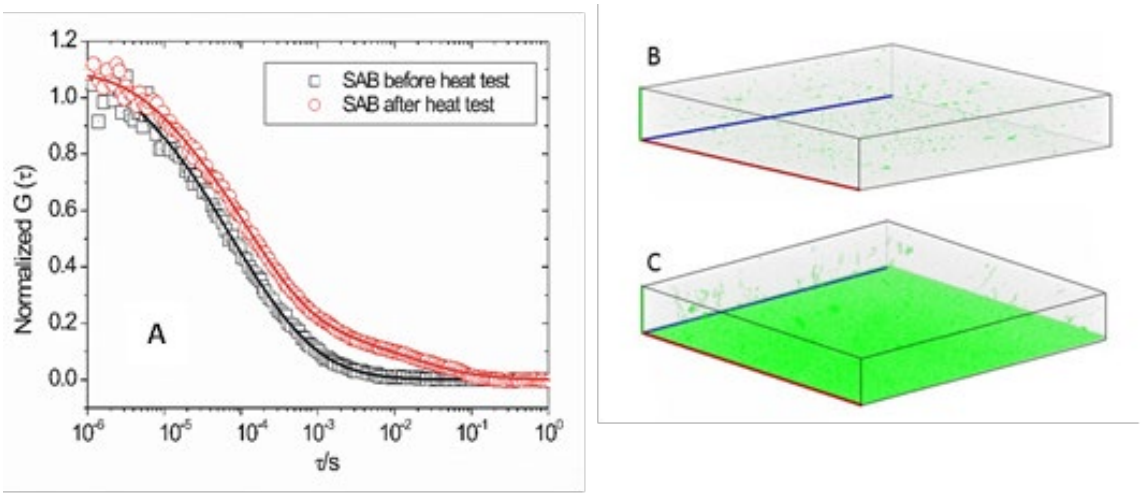


Figure 11. A) Normalised experimental autocorrelation curves of Alexa Fluor 488-labelled BSA diffusing in unfined Sauvignon Blanc (SAB) wine before and after heat test. The solid lines represent fits with eq.1. B) A 500x500x80µm confocal 3D image of Alexa Fluor 488-labelled BSA diffusing in unfined SAB wine before heat test and C) after heat test. Figure reproduced from Mierczynska-Vasilev et al. (2021)with permission from Elsevier.

7.4.2 Red wine protein

Wine protein is most commonly considered in relation to white and rosé wines, since they are the wines most susceptible to the development of visible protein haze. In red wine, protein is often thought to be absent, being removed from the wine during the early stages of winemaking due to precipitation of tannin-protein complexes. Earlier research at the AWRI revealed that in red grapes, protein is present in sufficient quantities to precipitate a substantial amount of tannin that would otherwise be extracted into wine (Bindon et al. 2016). Furthermore, in cold-hardy hybrid (non-*vinifera*) grape varieties that contain higher levels of protein than *Vitis vinifera* grapes, high concentrations of residual protein remain in wine and can precipitate oenological tannins added during winemaking (Springer et al. 2016). Based on these observations, it was of interest to understand how important protein is in modifying macromolecule extraction and stability in red wines.

Initially, existing methods for protein analysis were used, but they were found to be unsuitable for red wine due to interference from wine tannins. A new method was developed and validated specifically for protein quantification in red wines, and more than 100 red wines, including wines made from non-*vinifera* varieties and their interspecific hybrids sourced from the USA were analysed. Protein concentrations in Australian *Vitis vinifera* red wines ranged from 16 mg/L to 113 mg/L with a median of around 50 mg/L (Figure 12). Protein concentration was found not to vary according to grape variety in *Vitis vinifera* red wines. USA wines made from non-*vinifera* varieties and their interspecific hybrids had a wide range of protein concentrations from as low as 13 mg/L up to 350 mg/L, with a median of 70 mg/L. It was noteworthy that the cold-hardy hybrid varieties had the highest protein concentrations, with a median of 124 mg/L.

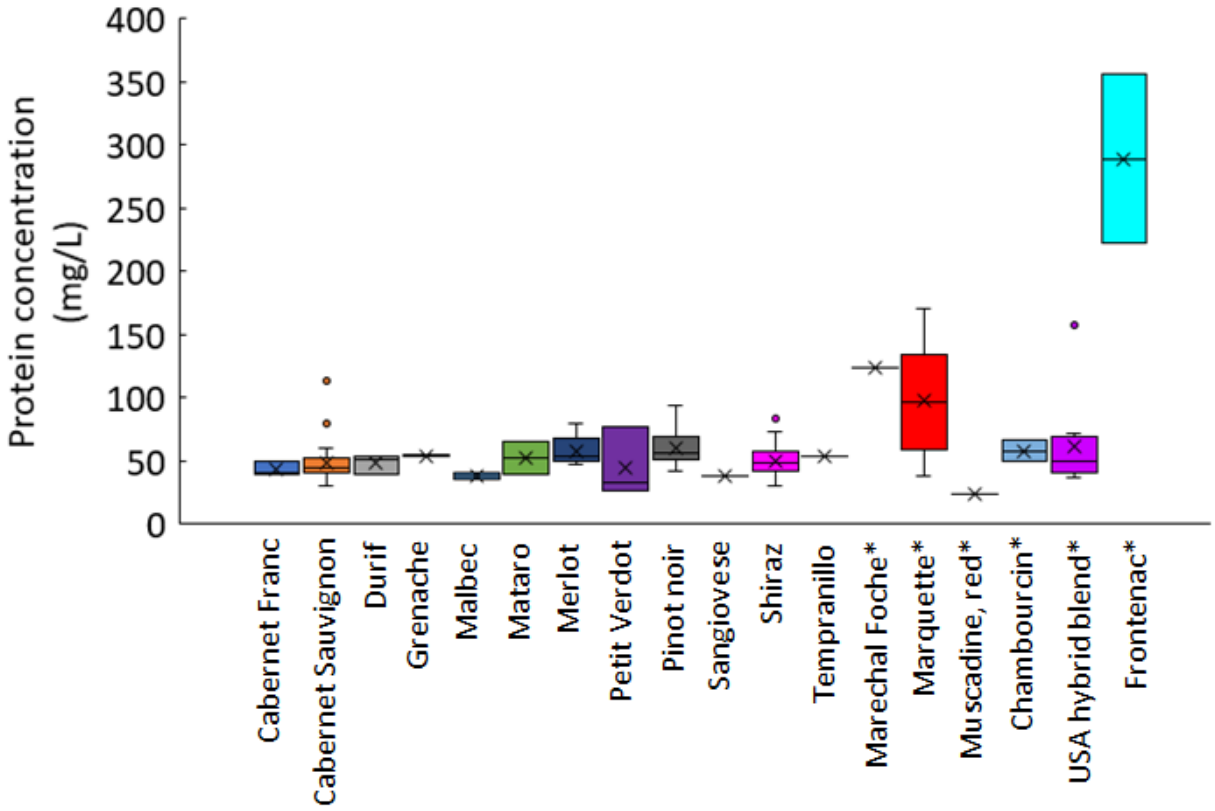


Figure 12. Protein concentrations detected in a survey of Australian and USA wines, prepared from *V. vinifera* and non-*vinifera* (*) grape varieties (including native and interspecific hybrids) showing the mean (X), median, distribution of data into quartiles (2nd and 3rd = colored box; 1st and 4th = whiskers) where single points outside of the whiskers indicate single-sample outliers and —X— indicates n = 1. Figure is reproduced from Kassara et al. (2022) with permission from Elsevier.

Given the relevance of protein to the development of haze in white wine, it was considered that high-protein red wines might in fact be heat-unstable, and potentially present difficulties with filtration, or develop deposits following addition of oenotannin or during ageing. This was particularly relevant, given that red wines could contain protein concentrations at levels that would result in haze in white wines. It was found that while most red wines (both *vinifera* and non-*vinifera* varieties) were not heat stable according to a red wine heat test, the extent of heat instability did not increase with increasing protein concentration. It was also found that unfiltered wines did not necessarily have higher protein concentration than filtered wines. More information on this study can be found in Kassara et al. (2022).

It was further considered that protein might be enzymatically broken down during fermentation, and could be manipulated by the use of proteolytic enzymes such as proctase (Marangon et al. 2012). It was also considered that by uncoupling protein-tannin interactions during fermentation, wine tannin concentration might be improved in low-tannin grape varieties, such as Pinot Noir. An experiment compared wine protein and phenolic composition following fermentation of grapes sourced from two Shiraz (Galaxia, Clonal) and one Pinot Noir vineyards. These ferments were performed at room temperature, heated to 75°C (heat) with the addition of proctase enzyme with heat (heat proctase) or without heat or enzyme (control), or without heat and with proctase (proctase). The results for wine composition are shown in Table 2.

Table 2. Composition of finished wines prepared from Shiraz (2 vineyards, Clonal and Galaxia) and Pinot Noir grapes treated with heat alone, heat and aspergillopepsin enzyme (proctase), enzyme alone, or untreated.

Treatment[†]	Anthocyanin (mg/L)	Non-bleachable pigment (A.U.)	Wine colour density[§] (A.U.)	Total phenolics (A.U.)	Hue (no units)	Tannin[¶] (mg/L)	MM[††] (g/mol)	mDP[††] (no units)	Protein (mg/L)	Poly-saccharide (mg/L)
Clonal[‡] control	1243 ± 15b	2.71 ± 0.04c	28.06 ± 0.17b	27.93 ± 0.39b	0.42 ± 0.00b	1677 ± 12c	3906 ± 70	12.9 ± 0.24	41 ± 2.6c	220 ± 74
Clonal heat	1421 ± 44a	3.68 ± 0.06a	36.02 ± 1.35a	33.99 ± 1.22a	0.47 ± 0.00a	2544 ± 96a	3731 ± 231	12.3 ± 0.76	91 ± 6.7a	250 ± 19
Clonal proctase	1195 ± 12b	2.36 ± 0.07d	26.95 ± 0.22b	27.23 ± 0.25b	0.42 ± 0.00b	1623 ± 85c	4414 ± 137	14.6 ± 0.45	39 ± 1.9c	259 ± 29
Clonal heat + proctase	1419 ± 36a	3.27 ± 0.15b	34.41 ± 0.72a	33.77 ± 1.08a	0.45 ± 0.01a	2064 ± 84b	3638 ± 212	12.0 ± 0.71	70 ± 6.7b	226 ± 8
ANOVA (*P*-value)	<0.001	<0.0001	<0.0001	<0.001	<0.001	<0.0001	ns	ns	<0.001	ns
Galaxia[‡] control	1223 ± 11c	2.63 ± 0.05b	26.57 ± 0.14c	27.36 ± 0.37b	0.45 ± 0.00b	1501 ± 71c	3695 ± 100b	12.2 ± 0.33b	65 ± 2.9b	413 ± 12a
Galaxia heat	1497 ± 22a	4.20 ± 0.18a	35.18 ± 1.20a	36.23 ± 1.15a	0.52 ± 0.00a	2353 ± 73a	3246 ± 148c	10.7 ± 0.50c	141 ± 14.2a	197 ± 60bc
Galaxia proctase	1205 ± 15c	2.64 ± 0.07b	26.20 ± 0.59c	28.20 ± 0.44b	0.45 ± 0.00b	1809 ± 86b	4094 ± 91a	13.5 ± 0.29a	67 ± 7.3b	325 ± 64ab
Galaxia heat + proctase	1417 ± 24b	3.25 ± 0.35b	30.37 ± 1.49b	33.57 ± 1.18a	0.47 ± 0.02b	1927 ± 112b	3355 ± 98bc	11.1 ± 0.32bc	65 ± 5.4b	161 ± 25c
ANOVA (*P*-value)	<0.0001	<0.01	<0.001	<0.001	<0.01	<0.001	<0.01	<0.01	<0.001	<0.05
Pinot control	270 ± 6c	0.51 ± 0.03b	4.97 ± 0.18c	7.56 ± 0.43b	0.71 ± 0.02b	235 ± 23c	1454 ± 34bc	4.9 ± 0.11bc	41 ± 0.1c	267 ± 94
Pinot heat	367 ± 7a	1.25 ± 0.07a	8.35 ± 0.19a	11.52 ± 0.51a	0.93 ± 0.00a	752 ± 28a	1293 ± 39c	4.3 ± 0.14c	130 ± 4.9a	302 ± 9
Pinot proctase	254 ± 2c	0.55 ± 0.08b	5.50 ± 0.13c	8.28 ± 0.32b	0.70 ± 0.03b	611 ± 31b	1776 ± 22c	5.9 ± 0.07a	52 ± 6.6bc	419 ± 35
Pinot heat + proctase	329 ± 3b	0.70 ± 0.11b	7.09 ± 0.55b	10.34 ± 0.40a	0.71 ± 0.04b	558 ± 54b	1529 ± 89b	5.1 ± 0.29b	64 ± 8.9b	445 ± 21
ANOVA (*P*-value)	<0.0001	<0.001	<0.001	<0.001	<0.001	<0.0001	<0.01	<0.01	<0.0001	ns

[†]Data presented show mean values ± standard error; treatments were compared using one-way ANOVA, n=3, and differences between treatments were determined by a post-hoc Student's T-test with significance indicated by different letters; [‡]Shiraz vineyards designated by name as 'Clonal' and 'Galaxia'; [§]Wine colour density corrected for bisulfite bleaching; [¶]Tannin concentration determined by methyl cellulose precipitation as epicatechin units; [††]Tannin molecular mass (MM) and mean degree of polymerisation (mDP) determined using subunit composition from phloroglucinolysis.

For two of the treatments, Pinot Noir and the Galaxia Shiraz, the addition of proctase alone, without heat, substantially increased tannin concentration and molecular mass, but did not change protein concentration in the wine. In each treatment, heating the must was found to increase tannin concentration, and it was observed that protein concentration was also up to three times higher in the finished wines. Interestingly, when heat treatment and proctase were applied together in these musts, tannin concentration dropped from that observed for heat treatments alone, and protein concentration was decreased. In each case, heat treatment was found to increase wine colour density and non-bleachable pigments, but proctase had no effect on wine colour properties. All of the wine treatments were found to have similar particle size and concentration using nanoparticle tracking analysis (data not shown), but heated wines had a higher turbidity. Interestingly, despite protein concentrations up to 140 mg/L in the heat treatments, all the wines in this study were found to be heat stable according to the red wine heat test. It therefore appears that tannin and protein may exist in a stable colloidal state in red wine, even up to high concentrations.

In order to better understand the impact of heating and proctase on wine protein and tannin concentration, analysis of one of the experiments (Shiraz, Galaxia) was performed over the course of fermentation. It was found that protein declined in all of the treatments during fermentation (Figure 13) but was higher in heat-treated wine which did not have added proctase at the end of fermentation. Heated musts that received proctase had similar protein concentrations to the other treatments. It is possible that heating may have denatured native proteolytic enzymes in the must, which was overcome by the addition of proctase. However, the reduction in protein over the course of fermentation in the wines that received heat treatment alone may indicate that some precipitation may have occurred. Alternatively, yeast may have partially degraded the protein present.

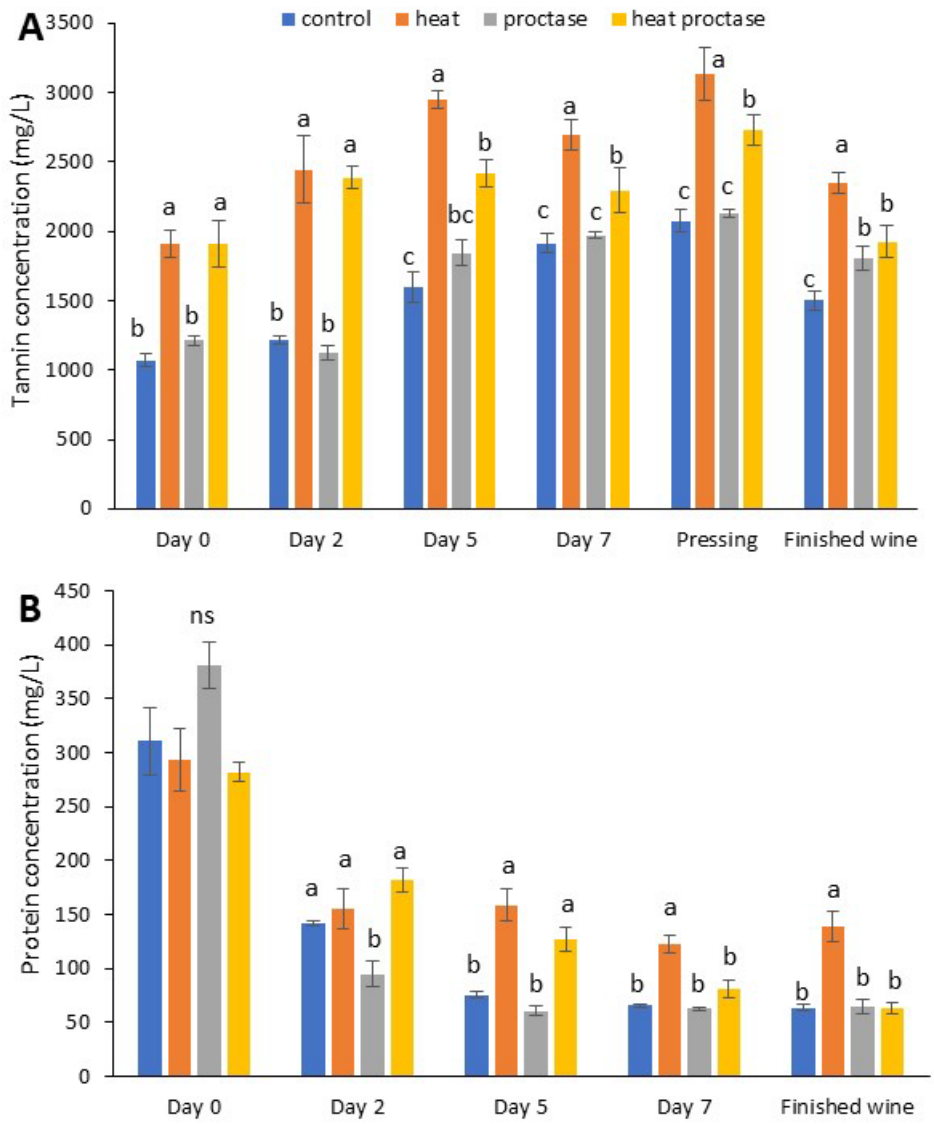


Figure 13. Tannin (A) and protein (B) concentration determined during fermentation of Shiraz (Galaxia vineyard) grapes treated with heat and proctase

This study demonstrated that adding a proteolytic enzyme during fermentation, without heating, may have some benefit for the extraction of tannin, in particular for a low phenolic variety such as Pinot Noir. It was not possible to ascertain the mechanism by which proctase enhanced tannin extraction since protein concentration did not differ from the controls. Further, the results indicate that proteolysis may occur during red wine fermentation, but its specificity for the target proteins is unknown. For example, grape PR proteins are expected to be resistant to proteolysis unless they are in the unfolded state. The origin of the enzyme activity is also unknown, and could derive from the grape or associated wild microorganisms.

7.5 Year 3 deliverable. Knowledge of how to modulate extraction and retention of key compounds for given wine styles

7.5.1. The effect of water addition on phenolic extraction in Shiraz

A study that investigated the mode and quantity of water addition to Shiraz must was completed in collaboration with Project 4.1.1 and has been reported previously in the final report for that project. This work was initiated due a change in FSANZ regulations that allowed water addition to high-sugar grape must to a minimum of 13.5°Baumé. Shiraz grapes were harvested at 13.4, 14.4 and 15.6°Baumé. Water was added via direct addition or substitution to the 15.6°Baumé must to match the sugar concentrations of the earlier harvests. Both water addition treatments significantly

decreased the concentration of phenolic compounds throughout the winemaking process. In finished wines, total phenolic concentration, tannin concentration, tannin molecular mass, non-bleachable pigments and colour density were lower in the water addition treatments in comparison with the control, but in all other respects were higher than wines made from earlier harvest dates. Interestingly, where the extent of alcohol reduction by water addition was equivalent, no differences in any of the measured wine attributes were found between the two modes of water addition. Ultimately, it was found that water addition to the latest harvest led to higher concentrations of wine tannin, colour and non-bleachable pigment than harvesting at a lower sugar concentration. More information on this study can be found in Teng et al. (2020a) and Teng et al. (2020b).

To further address the observation that water addition to Shiraz grapes reduced wine phenolics, irrespective of the mode of addition, a follow-up study was initiated in collaboration with The University of Adelaide. This trial also assessed the use of Della Toffola's maceration accelerator (DTMA), a commercialised version of the technique known as 'Accentuated Cut Edges' (ACE) maceration developed by the Tasmanian Institute of Agriculture (Sparrow et al. 2016). This maceration technique was developed for low-phenolic varieties such as Pinot Noir and aims to gently cut the skins of the grape berry, facilitating the extraction of tannin and colour, without damaging the seeds (Sparrow et al. 2016). The goal of the collaborative study was to apply DTMA to Shiraz in combination with pre-fermentation water addition, to explore whether the technique might overcome losses in phenolic extraction (Kang et al. 2020).

Three treatments were investigated for both DTMA (ACE) and conventional maceration (NOACE) in this study, whereby three-day maceration (Short) was compared with a six-day maceration (Long), and a six-day maceration with pre-fermentative water addition to 13.5 °Baumé (Long_Dil). Analysis of basic wine composition, wine colour, phenolic and polysaccharide composition was performed. Neither water addition, maceration time nor DTMA/ACE were found to affect Shiraz wine colour in this study. Further, water addition did not reduce tannin and phenolics as had been found in the earlier study (Teng et al. 2020a). In all treatments, DTMA/ACE significantly enhanced the concentration of total tannin and phenolics (Figure 14). No impact of DTMA/ACE was found on tannin composition (data not shown), with the primary outcome of the study being that tannin molecular mass was reduced as maceration time increased, due to enhanced tannin extraction from the seeds, associated with an increase in the proportion of epicatechin gallate. Polysaccharide concentration in the wines was mainly influenced by maceration time rather than the DTMA/ACE technique, and a small increase was observed from three to six days on skins (data not shown). Although the results of this study did not indicate that losses in phenolics could consistently be expected with pre-fermentative water addition in Shiraz winemaking, it was evident that any potential losses in phenolics might be overcome by using DTMA. It was therefore decided to further explore the impact of DTMA maceration on vineyards of different phenolic potential (see section 7.8). More detail on these studies can be found in Teng et al. (2020a), Teng et al (2020b) and Kang et al. (2020).

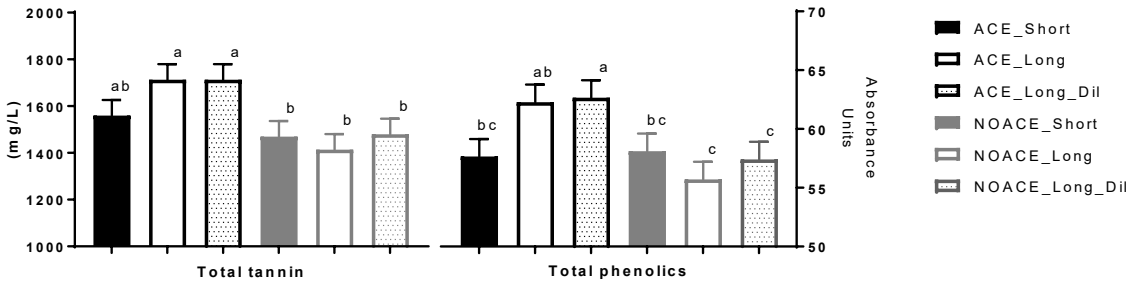


Figure 14. Mean total tannin concentrations and total phenolics (± standard error) of wines in the DTMA/water addition study. The different superscript letters indicate significant differences ($p < 0.05$) analysed by LSD.

7.5.2. The effect of whole bunch inclusion on tannin, polysaccharide and colour composition in Pinot Noir and Shiraz

In collaboration with Project 3.1.1, a single-season project was conducted investigating the inclusion of whole bunches in Pinot Noir and Shiraz fermentations. Results regarding volatile composition, aroma and flavour from this work are reported in the Final Report for Project 3.1.1. Different outcomes in terms of non-volatiles were found with whole bunch inclusion between the two varieties.

For Shiraz, as whole bunch inclusion increased from 100% crushed to 100% whole bunches (WB), tannin extraction from stems occurred, increasing tannin concentration, size, and astringency in finished wines (Table 3). The increase in astringency was not linearly related to the increase in tannin concentration (Figure 15), with no change in astringency observed for a substantial increase in tannin between 100% crushed to 25% WB. Increases in astringency were found from 25% WB to 75% WB, and thereafter plateaued to 100% WB, in this case for only a small change in tannin concentration (Figure 15). On the other hand, tannin size by GPC increased consistently, as did the proportion of galloylation in the extension units (indicative of stem tannin extraction). It is therefore likely that changes in tannin composition contributed more significantly to the observed astringency differences. Further, in the Shiraz study, wine colour density was found to increase with any level of whole bunch inclusion and was not different between the different whole bunch treatments. This effect appeared to be driven by higher levels of anthocyanin, as non-bleachable pigments and hue were not affected. Only small changes in polysaccharide concentration were observed, decreasing with whole bunch inclusion.

Table 3. Compositional outcomes of whole bunch (WB) inclusion in Shiraz and Pinot Noir fermentations for wine colour, tannin and polysaccharide.

Treatment[†]	Anthocyanin (mg/L)	Non-bleachable pigment (A.U.)	Wine colour density[§] (A.U.)	Hue (no units)	Tannin[¶] (mg/L)	MM subunit[††] (g/mol)	MM GPC[‡‡] (g/mol)	mDP[††] (no units)	Tri-OH[‡] (%)	Gall/Ext[‡] (%)	Poly-saccharide (mg/L)
Shiraz											
Control	388 ± 4b	1.70 ± 0.06	8.87 ± 0.1b	0.63 ± 0.00	596 ± 16d	1967 ± 65b	1473 ± 11e	6.58 ± 0.22b	25.9 ± 0.4a	4.7 ± 0.1d	467 ± 23a
25% WB[‡]	442 ± 9a	1.80 ± 0.03	9.95 ± 0.2a	0.63 ± 0.00	973 ± 100c	2131 ± 44ab	1581 ± 25d	7.11 ± 0.15ab	24.9 ± 0.3a	5.6 ± 0.1c	440 ± 16a
50% WB[‡]	430 ± 12a	1.83 ± 0.04	10.2 ± 0.3a	0.64 ± 0.00	1114 ± 81c	2252 ± 67a	1667 ± 23c	7.52 ± 0.22a	21.2 ± 0.6b	6.1 ± 0.1b	413 ± 23ab
75% WB[‡]	427 ± 10a	1.83 ± 0.04	9.91 ± 0.2a	0.65 ± 0.00	1184 ± 35b	2220 ± 18a	1742 ± 34b	7.40 ± 0.06a	20.4 ± 0.4b	6.7 ± 0.1a	428 ± 29a
100% WB[‡]	426 ± 9a	1.86 ± 0.04	10.1 ± 0.1a	0.65 ± 0.02	1359 ± 26a	2189 ± 55a	1829 ± 8a	7.30 ± 0.18a	21.5 ± 0.6b	6.4 ± 0.1b	349 ± 3b
ANOVA (*P*-value)	<0.05	ns	<0.01	ns	<0.0001	<0.05	<0.0001	<0.05	<0.0001	<0.0001	<0.05
Pinot Noir											
Control	131 ± 7bc	0.73 ± 0.02a	3.62 ± 0.10a	1.08 ± 0.02a	207 ± 10a	759 ± 18c	1212 ± 6ab	2.59 ± 0.06c	6.56 ± 0.27c	2.65 ± 0.08	776 ± 29a
25% WB[‡]	119 ± 3c	0.58 ± 0.02b	3.08 ± 0.05c	1.06 ± 0.01a	150 ± 4c	687 ± 9d	1187 ± 6b	2.35 ± 0.03d	4.27 ± 0.21d	2.55 ± 0.27	698 ± 31b
50% WB[‡]	137 ± 1ab	0.59 ± 0.02b	3.35 ± 0.07b	1.00 ± 0.02b	186 ± 17ab	767 ± 17bc	1218 ± 14ab	2.62 ± 0.06bc	5.81 ± 0.17c	2.55 ± 0.28	654 ± 8b
75% WB[‡]	133 ± 0.4b	0.58 ± 0.01b	3.22 ± 0.05bc	0.96 ± 0.01bc	215 ± 9a	821 ± 22ab	1245 ± 26a	2.80 ± 0.07ab	9.00 ± 0.55a	2.46 ± 0.13	571 ± 24b
100% WB[‡]	148 ± 5a	0.54 ± 0.00b	3.17 ± 0.02bc	0.92 ± 0.01c	169 ± 7c	842 ± 17a	1252 ± 4a	2.87 ± 0.06a	7.59 ± 0.12b	2.52 ± 0.12	519 ± 5c
ANOVA (*P*-value)	<0.01	<0.0001	<0.001	<0.0001	<0.01	<0.001	<0.05	<0.001	<0.0001	ns	<0.0001

[†]Data presented show mean values ± standard error; treatments were compared using one-way ANOVA, n=3, and differences between treatments were determined by a post-hoc Student's T-test with significance indicated by different letters; [‡]WB, whole bunch addition as percentage of total; [§]Wine colour density corrected for bisulfite bleaching; [¶]Tannin concentration determined by methyl cellulose precipitation as epicatechin units; [††]Tannin molecular mass (MM), mean degree of polymerisation (mDP), percentage epigallocatechin (% Tri-OH) and percentage galloylated extension units (% Gall/Ext) determined using subunit composition from phloroglucinolysis; [‡‡]Tannin MM determined by gel permeation chromatography (GPC).

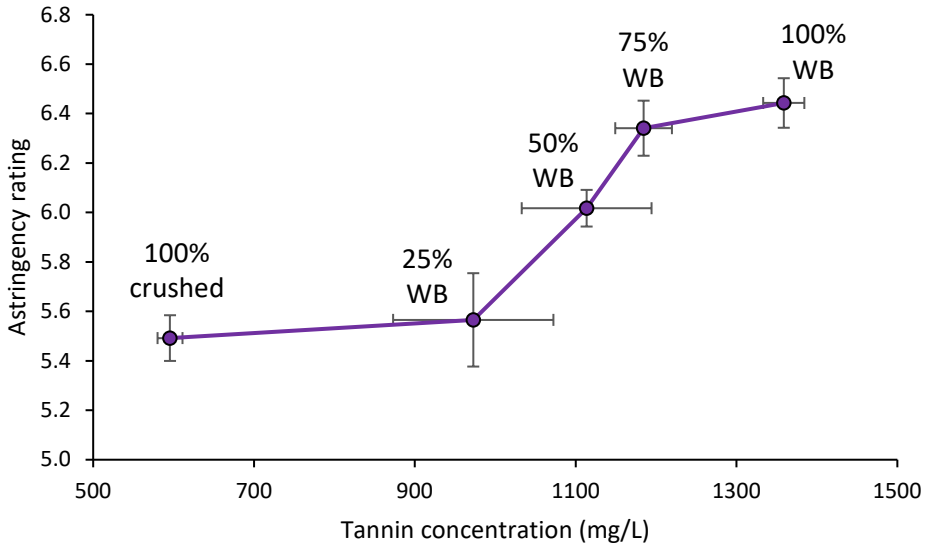


Figure 15. Relationship between tannin concentration and astringency rating following whole bunch (WB) inclusion in Shiraz fermentations

In the study of whole bunch inclusion in Pinot Noir, very different outcomes for non-volatile composition were observed. Tannin concentration was variable in response to whole bunch addition, with no clear treatment effect (Table 3). Generally, changes in tannin composition followed the observed differences in tannin concentration, where higher concentrations were associated with higher tannin molecular mass, and % trihydroxylation. Since there was no difference observed for tannin galloylation in response to whole bunch inclusion, it could be concluded that differences in tannin concentration between treatments were not driven by extraction from the stems. Other studies on Pinot Noir incorporating up to 20% whole bunch addition (Casassa et al. 2016) also found minor or no effect on tannin composition. Similar to the Shiraz study, in Pinot Noir the polysaccharide concentration decreased as whole bunch inclusion increased.

A small decrease in wine colour density and non-bleachable pigments was observed between the control treatment and all of the whole bunch treatments (Table 3.). Interestingly, total monomeric anthocyanin increased from 25% WB to 100% WB, while hue decreased. In terms of the sensory outcomes for the wines, the perceived colour changed from a browner to a red tint as whole bunch inclusion increased (Figure 16). Anecdotally, since the wines were very pale due to the nature of the variety, this gave the wines which had higher whole bunch inclusion a more vibrant red appearance. While overall colour density was higher in the control wine, this incorporated both higher spectral measures for 420 nm (brown) and 520 nm (red), and also a higher hue value. The decrease in hue with whole bunch inclusion may reflect a proportional increase in red colour relative to brown, accounting for the optical differences between the wines.

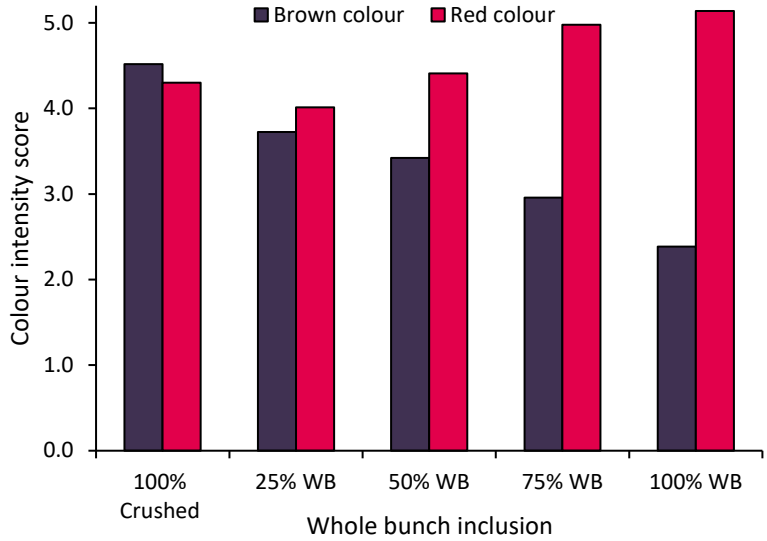


Figure 16. Relationship between whole bunch (WB) inclusion in Pinot Noir fermentations and colour intensity rating

7.5.3. Kinetics of polymeric pigment formation and loss

The incorporation of nanoparticle tracking analysis into the AWRI laboratory allowed for a number of new areas to be studied. A key area for development was to further explore polymeric pigment formation, in particular that which proceeds via ethyl linkages between grape tannins and anthocyanin, since these are considered important to the development of stable red wine colour. Within this work, the aim was to explore instabilities which might arise following the formation of ethyl-linked tannin-anthocyanin pigments in red wines, including the role that aeration during fermentation might play.

A preliminary study was completed investigating the role of tannin origin (skin versus seed) and size on the subsequent stability of pigments formed via reaction with anthocyanin and acetaldehyde (ethyl-linked) under two different sets of experimental conditions. Tannin was prepared from the skins and seeds of white (mutant) Cabernet Sauvignon grapes and fractionated into discrete size categories with average polymerisation between 4 and 43 units. Anthocyanin was purified from a commercial anthocyanin-rich grape skin extract, GskinEX (Tarac Technologies, Australia) to a malvidin-3-glucoside (M3G) enriched fraction by using multi-layer coil counter-current chromatography (MLCCC).

In the first experiment, the role of tannin structure on the stability and colour properties of ethyl-linked polymeric pigments was investigated, with these pigments formed from tannin fractions of different sizes combined with M3G in model wine containing excess acetaldehyde. As tannin molecular mass increased, the reaction rate with M3G increased. Compared with skin tannins of comparable molecular mass, seed tannins reacted more rapidly with M3G, but were prone to precipitation. This resulted in a loss of polymeric pigments formed from seed tannins, with greater pigment loss observed as tannin molecular mass increased. It was found that substantial aggregation occurred following reaction of seed tannin with M3G in comparison with seed tannin alone (Figures 17 A and B), and this was concomitant with precipitation. The aggregation-precipitation phenomenon was not observed for skin tannin-derived pigments which had a similar aggregation pattern to pure skin tannin (Figures 17 C and D). Initially, this led to the conclusion that pigmented polymers derived from skin tannins may have a greater stability in wine-like solution than those formed from seed tannins.

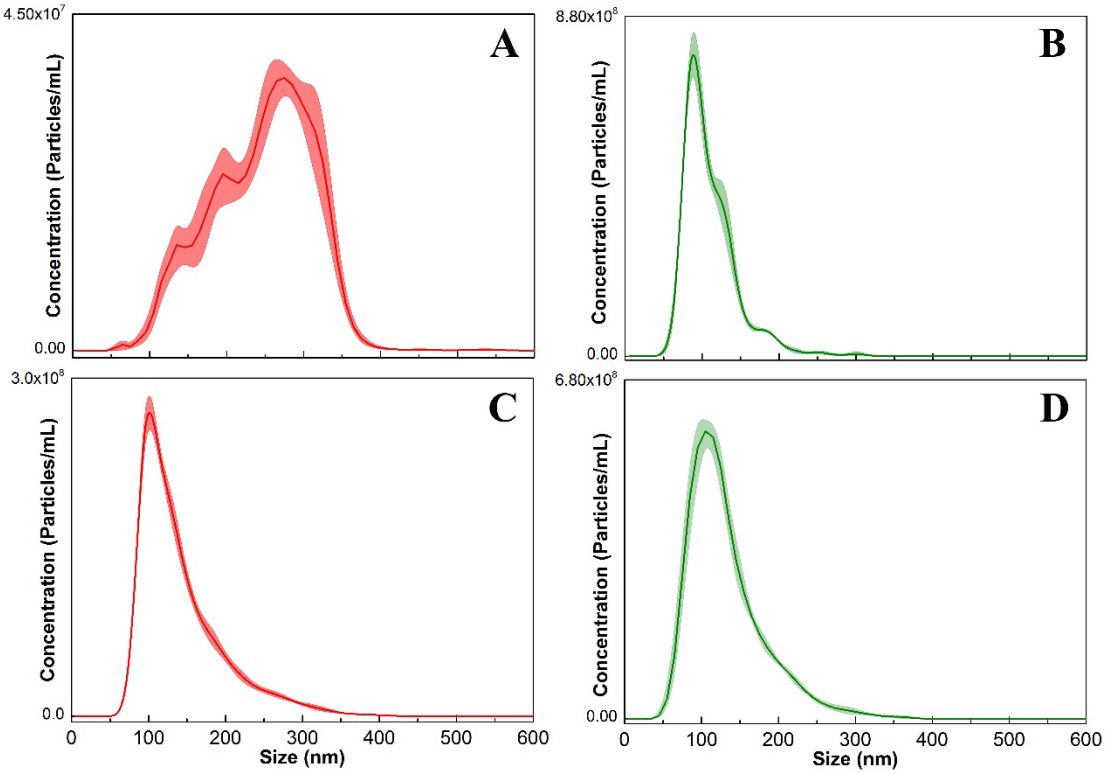


Figure 17. Particle size distribution of tannin-M3G and tannin-only reactions at day 15 A) Seed tannin with anthocyanin and acetaldehyde, B) Seed tannin alone, C) Skin tannin with anthocyanin and acetaldehyde, D) Skin tannin alone. Figure reproduced from Teng et al. (2019) with permission from the publisher, ACS.

Follow-up experiments were performed using wines prepared from chemically defined musts (CDM) with or without added acetaldehyde (CDMA) at a wine-like range. For comparison, experiments were also prepared in model wine without acetaldehyde. The same grape skin and seed tannin fractions of defined molecular mass used previously were reacted with M3G for 120 days. The study aimed to explore the role of tannin composition and size on the formation and stability of polymeric pigments. Tannins of a defined size range were combined with M3G in three different media: wine made from chemically defined must (CDM), wine made from CDM with added acetaldehyde (CDMA) and model wine made up of acidified 15% ethanol (MW). While the first experiment (7.5.3.1) had limited the concentration of M3G relative to tannin, and retained acetaldehyde in excess, this experiment aimed to combine tannin, M3G and acetaldehyde at wine-like concentrations. In this study, only minimal precipitation was observed in model wines. In all experiments conducted in CDM, precipitation was observed after the reaction period and this increased in response to both higher tannin molecular mass and higher acetaldehyde concentration. As a result, in the model wine, average particle sizes of the tannins ranged from 80 to 140 nm but far larger particle sizes were found in CDM and CDMA wines (Figure 18). This suggested that the principal mechanism for tannin and colour precipitation was the increased aggregation mediated by acetaldehyde, and this might partially explain the lack of large tannins observed in red wine. Unlike the preliminary kinetic study where anthocyanin was limiting, these findings indicated that precipitation could occur for both skin and seed tannins when the acetaldehyde-mediated reaction with M3G was able to continue. Despite substantial losses of tannin due to precipitation, the resulting non-bleachable pigment concentration and colour intensity were higher in wine media containing elevated acetaldehyde, when each tannin size category was considered independently. However, it also indicated that the development of non-bleachable pigments from larger tannins may be limited when acetaldehyde-mediated condensation with anthocyanin predominates in wine, concomitant with precipitation. More detail can be found in Teng et al (2019) and Teng et al. 2021.

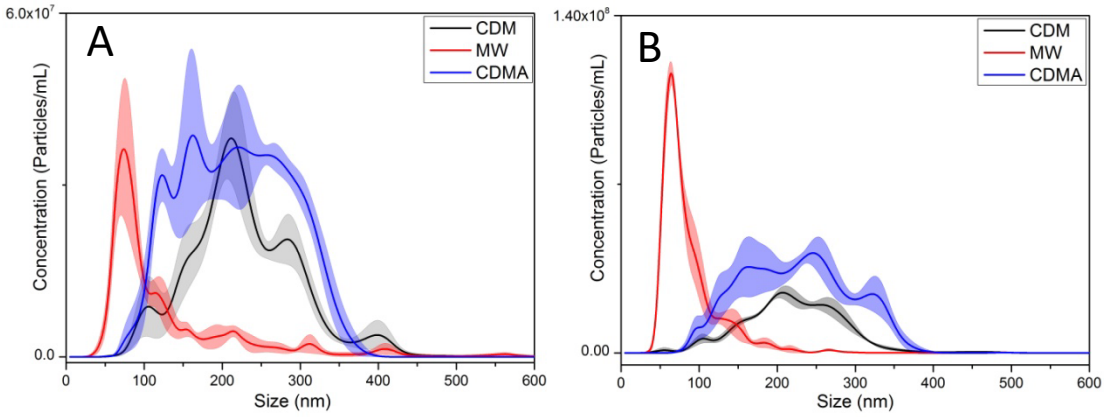


Figure 18. Particle size distribution of large molecular mass seed (A) and skin (B) tannins combined with anthocyanin in chemically-defined wine media (CDM), CDM with acetaldehyde (CDMA) and model wine (MW). Figure reproduced from Teng et al. (2021) with permission from the publisher (ACS)

7.5.4 Aeration during fermentation alters the structural properties and resulting interactions of wine tannin

As an extension of studies completed in Project 3.1.5, work was conducted to better understand the impact of macro-oxygenation during fermentation on pigmented tannin structure, properties and interactions. A discrete study was completed using purified tannin fractions isolated at the end of fermentation, and after three years of ageing from a macro-oxygenation trial completed in 2012 (McRae et al. 2015).

In order to characterise the effect of aeration on tannin structure and reactivity with poly-L-proline, tannins were purified from red wines that had been injected with nitrogen or oxygen during fermentation. Tannin was purified from wine immediately after fermentation and after three years of bottle storage. Tannins from nitrogen-treated wine had a lower percentage of galloylation and were less pigmented than tannin from oxygen-exposed wine. Self-aggregation of tannin was measured by nanoparticle tracking analysis and a larger particle size was observed for the oxygen-exposed treatment (Table 4). The interaction of tannin and poly-L-proline was measured by isothermal titration calorimetry, and involved more hydrogen bonding than hydrophobic interactions in the case of nitrogen-treated wine tannin. Conversely, oxidised tannin was more hydrophobic and the association with poly-L-proline was entropy-driven due to a change of solvation. The results show meaningful changes in the structure and reactivity of tannin as a result of oxygen exposure during winemaking, which may affect astringency perception. More details on this study can be found in Watrelot et al. (2019).

Table 4. Thermodynamic parameters for the interaction of tannins treated with nitrogen (TN) and oxygen (TO) at the end of fermentation (EF) and after three years of ageing (3Y) (2 mmol/L) and poly-L-proline (0.05 mmol/L) identified by ITC, as well as enthalpy for the interactions of tannins with a hydrophobic surface by HPLC-DAD. Particle size and concentration of wine tannins in model wine were determined by nanoparticle tracking analysis (NTA). Table reproduced from Watrelot et al. (2019) with permission from Elsevier.

	ITC					HPLC-DAD	Particle size (nm) and concentration by NTA					
	n	Ka (1/M) (10^6)	ΔH (kJ/mol)	ΔG (kJ/mol)	-TΔS (kJ/mol)	ΔH (kJ/mol)	D10	D50	D90	mean	SD	Particle conc. (10^8particles/mL)
TNEF	4.0^{c}	0.6^{a}	-28.3^{b}	-32.7^{a}	-4.4^{a}	-9.1^{c}	114.0^{b}	203.3^{b}	356.3^{c}	225.7^{b}	94.8^{c}	11.9^{a}
TOEF	5.3^{ab}	0.6^{a}	-22.5^{a}	-32.5^{a}	-10.0^{ab}	-6.9^{b}	161.4^{a}	282.5^{a}	454.8^{a}	300.1^{a}	106.1^{ab}	11.8^{a}
TN3Y	5.1^{b}	2.0^{a}	-24.5^{a}	-35.0^{ab}	-10.5^{ab}	-5.2^{a}	44.8^{c}	107.4^{c}	276.6^{d}	144.5^{c}	96.9^{bc}	3.1^{b}
TO3Y	6.3^{a}	26.0^{a}	-22.0^{a}	-38.0^{b}	-15.9^{b}	-5.9^{a}	102.5^{b}	202.2^{b}	392.0^{b}	232.4^{b}	111.0^{a}	10.9^{a}

ITC measurements conducted five times, HPLC-DAD measurements conducted in duplicate and NTA measurements conducted in triplicate. The superscript letters correspond to the significant differences identified per column ($p < 0.05$). n, stoichiometry; Ka, association constant; ΔH, enthalpy; ΔG, free enthalpy; ΔS, entropy; particle size at the 10th (D10), 50th (D50), 90th (D90) percentiles of the distribution.

7.5.5. Changes in polysaccharide structure introduced by pectolytic enzyme alter their aggregation under wine-like conditions

To explore the effect of pectolytic enzyme application on polysaccharide size and colloidal interactions, rosé wines were prepared from pressed Shiraz grape juice, and red wines were made from the same juice by the addition of skins and seeds from fresh pressings. A pectolytic enzyme preparation containing polygalacturonase and arabinase activities was used. Enzyme treatment enhanced the contribution of high molecular weight (≈ 200 kDa) polysaccharides rich in mannose and glucose (mannoproteins) and removed arabinose-rich polysaccharides of intermediate (≈ 40 kDa) size. Enzyme application reduced the molecular weight average of a class of smaller polysaccharides of approximately 6 kDa (rhamnogalacturonan II). All wines had similar particle sizes, but enzyme treatment markedly reduced particle concentration in wines, particularly in rosé wines. Wine polysaccharides were purified and when reconstituted in model wine, showed reduced particle concentration in response to enzyme treatment (Table 5). Aggregation of polysaccharide in the presence of seed tannin was also markedly reduced by enzyme treatment. The results indicated that changes in structure introduced by the enzyme affected wine colloidal properties, potentially increasing polysaccharide solubility. This may have important implications for filtration, since it shows that pectolytic enzyme may improve filterability not due to a reduction of polysaccharide concentration but rather due to a change in polysaccharide structure and aggregation. More information can be found in Kassara et al. (2019).

Table 5. Particle size and concentration in rosé wines produced from free-run Shiraz juice prepared with or without commercial pectolytic enzyme, and reconstituted polysaccharide isolated from these treatments (0.5 mg/mL) determined using nanoparticle tracking analysis. Data shown are mean ± standard error where differences between means were determined by one-way ANOVA. Table reproduced from Kassara et al. (2019) with permission from Elsevier.

Particle concentration and size	Enzyme-treated	Control	ANOVA *P* value
Whole wine			
Concentration (particles/mLx10^6)	656.0 ± 177	7836.7 ± 1347	<0.01
Particle size average (nm)	240.9 ± 11.1	264.0 ± 12.88	ns
Polysaccharide			
Concentration (particles/mLx10^6)	195.3 ± 61	1019 ± 106	<0.01
Particle size average (nm)	160.7 ± 5.2	141.5 ± 0.6	<0.05

7.5.6 Collaborative studies on macromolecule extraction and composition

7.5.6.1 Aeration in red winemaking

A collaboration with Project 3.1.5 was performed over two seasons exploring aeration during fermentation of Shiraz. Detailed results are provided in the Final Report for that project. In brief, increasing aeration during fermentation was found to generally reduce tannin concentration, wine colour density and opacity (sensory). A manuscript summarising the results of four consecutive seasons of work on aeration in Shiraz was published by Day et al. (2021).

7.5.6.2 Amelioration of low molecular weight volatile sulfur compounds

A further project was conducted in collaboration with Project 3.4.2 to investigate winemaking remediation strategies to address the formation of low molecular weight volatile sulfur compounds (VSCs) and is described in the Final Report for that project. This work affirmed that aeration during fermentation could reduce colour and tannin but was an effective remediation strategy for the removal of reductive attributes in wine. This work was published by Bekker et al. (2021).

7.5.6.3 Effect of hybrid yeast on macromolecule extraction

A collaboration with Project 3.2.1 was initiated to study the effects of different hybrid yeasts (*S. cerevisiae* parent strain) on wine macromolecules and is described in the Final Report for that project. Initial results identified that a wide range of tannin and polysaccharide concentrations were achievable simply by using different hybrid yeast strains.

7.5.6.4 Effect of advanced Shiraz shrivel on phenolic extraction

A collaboration with Project 3.1.1 was conducted for two seasons to explore the impact of advanced grape shrivel in Shiraz on phenolic composition and extraction. Results are provided in the Final Report for that project. Briefly, it was found that despite the substantial decrease in berry weight during shrivel (hence an expected increase in the surface area to volume ratio), extractable tannin and colour did not increase on a per gram berry weight basis. However, when the concentration of extractable tannin and colour was expressed on a per berry basis, or the content per berry, there was a close to significant decrease in both measures in the later harvested grapes relative to the control. This could indicate that losses in extractable phenolics may have begun to occur with excessive hang time. These results indicate that after the onset of excessive shrivel, reduced extractability may occur either due to degradation of phenolic compounds or may result from a limitation in extraction due to other phenomena (e.g. cell wall degradation and increased adsorption to grape solids).

7.6 Year 5 deliverable. Novel or more efficient approaches to achieving cold stability

7.6.1 Functionalised surfaces

As mentioned in section 7.2.2, three plasma-coated surfaces (amine, oxazoline and acrylic acid) were previously identified as having the ability to promote crystal nucleation and growth. These surfaces were placed in cold-unstable Muscat Gordo wines for periods of one week and one month at three different temperatures, 0°C (fridge), 4°C (cold room) and 15°C (cellar). Control and treated wines stored at 0°C and 4°C all became cold stable after one month of wine storage. Excitingly, the application of POX, AA and AcrA-plasma modified surfaces reduced KHT crystal formation by 98, 96 and 93%, respectively at 15°C, indicating cold stabilisation was achieved.

Further studies were conducted to investigate the capacity of the plasma polymer coated surfaces to achieve cold stability in cold-unstable Sauvignon Blanc, Chardonnay and Pinot Grigio white wines after three months of storage at 15°C. The mini-contact test was performed to assess the cold stability of wines. Results showed that all control wines were unstable after three months of storage. Sauvignon Blanc and Chardonnay wines treated with modified surfaces were cold stable regardless of the coating used. Pinot Grigio wine came close to cold stability after treatment with amine and POx functionalised surfaces. There was a clear differentiation between coatings used for the treatment of Pinot Grigio wine. While allylamine and oxazoline-modified surfaces were quite effective with wines reaching an almost 5% stability threshold, the acrylic acid-modified surfaces were not as effective. The data indicated that surface chemistry modulates the KHT crystals' binding mechanism, with the binding capacity following the order AA>POX>AcrA for cold- and heat-unstable wines. The main advantage of this method is that it can be easily applied to wine and does not require cooling facilities. Another major benefit of using plasma polymer coated surfaces in wine

processing is that functionalised surfaces are classified as processing aids rather than additives. This study demonstrated that using collecting surfaces with carefully designed chemical functionalities can induce cold stabilisation at higher temperatures, reducing the need for cooling facilities, saving energy, and improving cost-effectiveness. A manuscript on this study is currently under internal review.

7.6.2 Zeolites for cold stabilisation

Six different zeolites were selected for benchmarking. Zeolites were sourced from Australian zeolite distributors such as ZeoNatural, Orku, and Nikita Naturals (Aussie Zeolite) and an Indonesian mining company (CV Mountain Stones) in micronised powder form. One zeolite was purchased from Sigma Aldrich (NaY Zeolite) also in micronised powder form. The sixth zeolite sample came from Enfield Produce in a granular form and was micronized by Bureau Veritas Minerals, referred to in the text as Grind zeolite. Zeolite samples were used after three hours of rehydrating in Milli-Q water at a zeolite:water ratio of 1:10. Three cold-unstable Muscat Gordo, Sauvignon Blanc and Chardonnay wines were treated with zeolites for three hours. To reduce aluminium content in the zeolites, they were also subjected to a baking process. During baking, the zeolite was placed in the oven at 400^{O}C for two hours. In addition, the Indonesian zeolite samples GK del and PN del were dealuminated using 3N HCl by soaking for 24 hours, followed by neutralisation by washing several times in water and finally drying at 100^{O}C.

Following treatment with all zeolites tested, the potassium content in the Muscat Gordo wine was reduced by 47-60%, leading to crystallisation and precipitation of KHT. At the same time, the sodium concentration in wine increased to an average of 130 mg/L. In Sauvignon Blanc, the initial potassium concentration in the wine was approximately 750 mg/L and decreased to 650 and 515 mg/L, representing a 13 to 30% decrease in potassium concentration after treatment. The Chardonnay control wine had a higher potassium concentration of 920 mg/L. When treated with natural zeolites, this concentration decreased by 18 to 28%. The sodium concentration in these wines increased to similar levels after treatment, with a maximum sodium concentration of 70 mg/L. Although the sodium content of all three wines increased as the potassium content decreased, the concentration of these metals was within the range common for wines.

Metal analysis of the treated wines revealed that the aluminium concentration was drastically increased, particularly, after treatment with Australian zeolites. The concentration of aluminium in the control Muscat Gordo wine was 0.2 mg/L. After treatment, the aluminium concentration increased to 9 mg/L for Aussie, Orku and Natural zeolite, to 11 for Grind zeolite and to 2 mg/L for Indonesian zeolite. After six months of wine storage in the cellar, these aluminium concentrations dropped to around 7 mg/L for all Australian zeolites. However, the levels were still very high.

A high aluminium concentration was also found in Sauvignon Blanc and Chardonnay wines after treatment with Australian zeolites (S4G and H). Initially, the aluminium content was 16 mg/L for Grind zeolite. After three and six months of storage, the aluminium content decreased significantly but remained high, between 5 and 10 mg/L after treatment with Australian zeolites. A moderate increase was observed for all treated wines after treatment with Indonesian zeolite (~ 1.5-1.6 mg/L). The increase in aluminium content was no surprise since it is well documented that, for example, the addition of aluminosilicate minerals such as bentonite contributes to the aluminium content in wines. What was surprising was the magnitude of this increase, which was twenty-five and thirty-five-fold. After the addition of bentonite, the aluminium content typically increases about two-fold. Two processes were used to reduce aluminium concentration, zeolite dealumination and baking. While the dealumination process was not entirely successful in reducing the aluminium concentration, zeolite baking resulted in a substantial decrease in aluminium, as shown in Figure 19 for Muscat Gordo and Pinot Grigio wines.

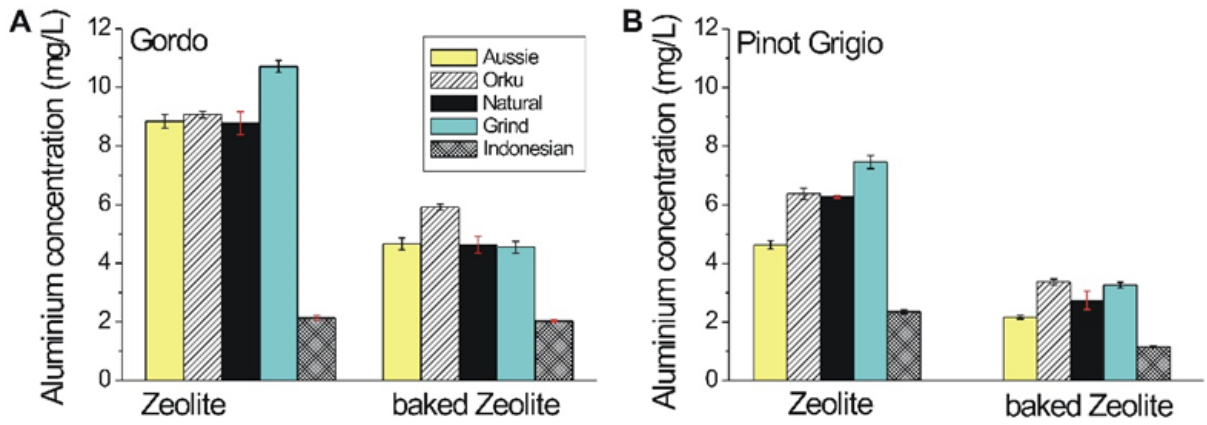


Figure 19 The concentration of aluminium in **A)** Muscat Gordo and **B)** Pinot Grigio wines after treatment with as received zeolites and zeolites baked in the oven for 2 hours at 400°C

The cold stability was measured by the Crystal ID test in wine before and after zeolite addition, either immediately after zeolite treatment or after three and six months of wine storage in the cellar at 15^{O}C. Indonesian zeolite was found to be the most effective in removing tartaric acid crystals from the wine. This zeolite reduced the concentration of tartaric acid crystals in the wine by 99.4, 99.8, and 100% after treatment and after three and six months of wine storage. Australian zeolites were also highly effective. These zeolites reduced the concentration of tartaric acid crystals by an average of 95.5, 98, and 99 % after treatment and after three and six months of wine storage. At the same time, tartaric acid concentrations in Muscat Gordo wine decreased after treatment with Australian zeolites due to KHT crystallisation and precipitation. However, the pH value of the wine changed only minimally.

The situation was different when Muscat Gordo wine was treated with Indonesian zeolite. In this case, the tartaric acid concentration dropped so drastically after treatment that the pH of the wine increased from 3.6 to 5.2. The same high pH was measured for Sauvignon Blanc and Chardonnay wines treated with Indonesian zeolite. No pH change was observed in these wines after treatment with Australian zeolites. An 8% reduction in tartaric acid was observed for Sauvignon Blanc and Chardonnay wines when Australian zeolites were used for treatment. However, when Sauvignon Blanc and Chardonnay were treated with Indonesian zeolite, the tartaric acid was almost completely removed (only 2% left), which explains why the pH of these wines increased after treatment.

Sauvignon Blanc was initially cold unstable and remained unstable up to three months of storage in the cellar. After treatment with natural zeolite, the concentration of tartaric acid crystals in this wine initially decreased by an average of 92% and by 98% after three months of storage in the cellar. After six months of storage, the wine was almost cold stable even without zeolite addition. Chardonnay was cold stable almost from the beginning, indicating that no treatment was required to cold stabilise the wine.

A manuscript on this study is currently under internal review and will be submitted to the *Australian Journal of Grape and Wine Research*.

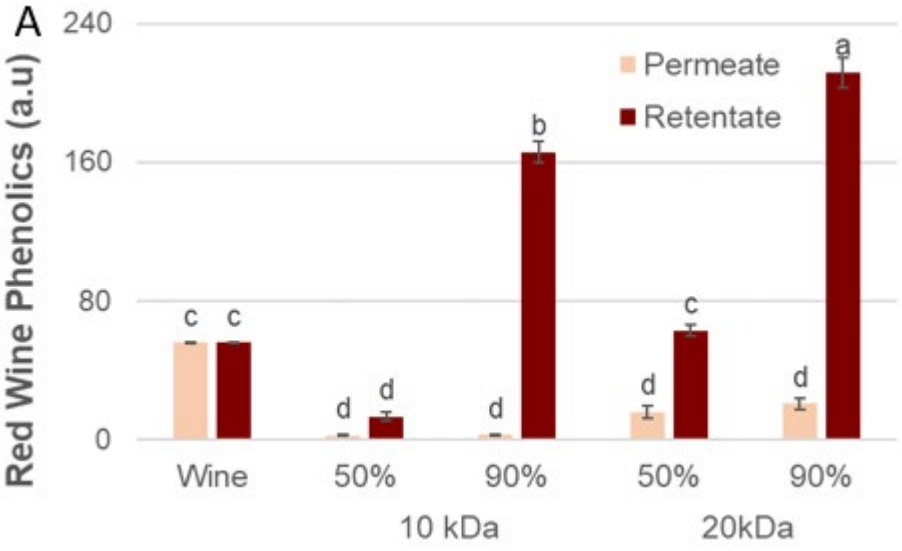
7.7 Year 5 deliverable. Knowledge of surface interactions that affect the efficiency of production (e.g. fouling) and practical, improved production processes based on that knowledge

7.7.1 Ultrafiltration

Membrane ultrafiltration (UF) a type of separation technology which is seeing increased use in a wide range of food production applications, including in wine (Sui et al. 2021). UF primarily fractionates wine molecules based on their molecular weight, and this is dependent upon the molecular weight cut-off (MWCO) specifications of the membrane used. In this process, larger molecules tend to be retained (retentate), while low molecular weight substances are able to pass through the membrane (permeate). However, it is found that surface interactions with the membranes can occur, such that smaller molecules which should pass through the membrane are in fact retained. One example of this is wine phenolics.

This project aimed to explore UF applications to ameliorate excessive phenolics in heavy-pressed or laccase-affected white wines, or to concentrate red wine macromolecules to improve wine composition/quality. In this work, permeate and retentate were obtained following pilot-scale fractionation of a phenolic-rich white wine, and a commercial red wine. To achieve this, 10 and 20 kDa spiral ultrafiltration membranes were used, and the wine was subjected to different degrees of permeation (50, 80, 90 and 95%). It was found for both wine types that the wine alcohol, pH and viscosity were not significantly different from the initial wine in either permeate or retentate following fractionation. In white wine, titratable acidity, proteins, polysaccharides and phenolic compounds were concentrated in the retentate, depending upon the specific MWCO of the membrane used, together with the degree of permeation (Figure 20B).

The selective removal of white wine phenolics demonstrated a unique opportunity to employ UF to remediate oxidised or excessively phenolic white wine. Also, the concomitant removal of white wine proteins, which are predominantly haze-forming, indicated a dual fining effect which could also be used to improve heat stability in a single step. In red wine, ultrafiltration had a negligible effect on wine alcohol and pH, but tannin and polysaccharide were sequestered in the retentate, irrespective of the pore size of the membrane or the permeation degree. Anthocyanin and phenolics (Figure 20A) were also strongly retained by the ultrafiltration process, in particular for the 10 kDa membrane, producing a concentrated retentate with an intense colour. Since phenolics such as tannin and anthocyanin are in fact far smaller than the nominal molecular weight cut-off of the membranes, this points to interactions (including fouling) at the membrane surface preventing permeation. Future work will continue to investigate the fouling phenomenon as well as the potential application of ultrafiltration to modulate phenolics in commercial winemaking.



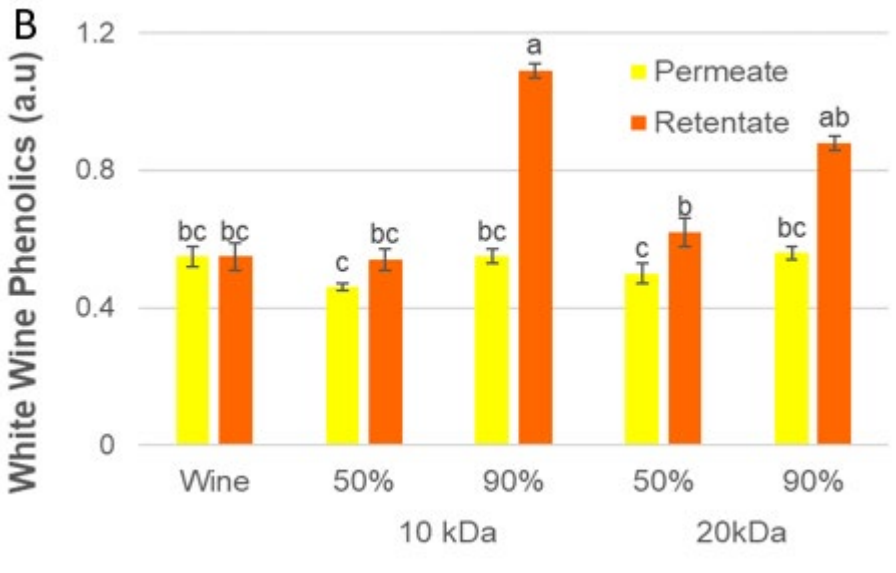


Figure 20. Compositional changes following UF fractionation of A) red wine, B) white wine

7.7.2 Surface properties of zeolites

The surface properties of six zeolites selected for benchmarking were investigated in detail using a range of characterisation techniques. The crystallinity of the samples was analysed by X-ray diffraction (XRD) measurements. According to the X-ray diffraction studies, the five Australian zeolites had a distinct crystalline structure, with patterns marked by characteristic peaks of clinoptilolite and mordenite. Indonesian zeolite was found to be a mixture of calcite and zeolite (calcite-mordenite-clinoptilolite) based on the XRD analysis. The X-ray photoelectron spectroscopy was used to measure the zeolites' surface composition, whereas the energy-dispersive X-ray spectroscopy was used to determine their bulk elemental composition. All zeolites had a Si:Al ratio of nearly 4:1, which classifies them as intermediate Si/Al zeolites. However, it is noteworthy that the Indonesian zeolite had the highest sodium and calcium content as measured by EDX. The XPS analysis showed that the Indonesian zeolite did not contain sodium ions on the surface. Scanning electron microscopy (SEM) was used to study surface morphology. The SEM indicated that natural zeolites are crystalline, which was also confirmed by XRD.

The average pore diameter, surface area and pore volume are important physical properties that influence the quality and utility of zeolites and are determined using the BET adsorption technique. The average pore size of the Indonesian and Australian zeolite was smaller (5 and 7 nm, respectively) than that of Orku, Grind and ZeoNatural (14, 14 and 11 nm, respectively). The BET surface area of the Indonesian zeolite was the highest at 59.95 m^2/g, resulting in a pore volume of 0.069 cm^3/g. In contrast, the lowest surface area was measured for Orku, Grind and ZeoNatural, with BET surface areas of 18.21, 17.20 and 17.39 m^2/g, and pore volumes of 0.062, 0.059 and 0.049 cm^3/g, respectively. The Aussie zeolite had a surface area of 26.02 m^2/g and the lowest pore volume of 0.044 m^2/g.

7.7.3 Surface geometry of plasma-coated surfaces

In plasma deposition, film grows independently of the substrate material used. For this reason, it was possible to directly transfer the surface modification technology from model silicon wafers to stainless steel mesh surfaces. This significantly increased (more than double) the substrate available surface area. It was also possible to coat both sides of the mesh sheet, which increased the number of surface functional groups. In addition, from a practical perspective, handling the mesh is much more convenient, especially in industrial applications.

Silicon wafers and mesh surfaces were investigated to evaluate surface geometry effects of plasma-coated surfaces on KHT removal from cold-unstable wines. As expected, mesh surfaces were more effective due to their larger surface area. After two weeks, they removed 78% of the KHT from white wine, while the silicon wafer removed only 40%. Mesh surfaces with different plasma coatings (allylamine - AA, oxazoline - POX, and acrylic acid - AcrA) were tested on 2021 vintage wines, Chardonnay Sauvignon Blanc and Pinot Grigio with results from this trial presented in section 7.6.1. In addition, the full paper detailing this work is currently under internal review.

7.8 Year 5 deliverable. Knowledge of how vineyard and winery production processes influence factors affecting extraction and retention of key compounds, wine clarity, stability and production efficiency

7.8.1 Phenolic extractability and maceration techniques

The AWRI continued its collaboration with Coriole Winery, to further explore the use of the DTMA maceration technique on Shiraz. The new study aimed to compare changes in 'phenolic potential' of their Shiraz vineyards during ripening, which is an assessment of both total and extractable tannin, anthocyanin and phenolics. It was found that extractable tannin and colour increased during ripening for all vineyards, while total tannin decreased gradually, and then remained constant.

From this assessment, two vineyards designated 'high phenolic potential' (HP) and 'low phenolic potential' (LP) were selected. The grapes from these two vineyards were harvested at commercial ripeness and either underwent standard crushing or DTMA. The maceration times were also altered, and were either a short maceration (four days) or a standard maceration (seven days). It was found that for wines prepared from the LP vineyard, DTMA maceration was very effective at increasing tannin extraction. This was evident for both tannin and colour (Figure 21), even after four days of maceration. For the HP vineyard, however, DTMA increased tannin extraction to four days, but after a seven-day maceration there was no difference from the control.

Interestingly, it was found that the DTMA-treated LP wines had an equivalent tannin concentration to the HP wines, and a higher colour after seven days of maceration. It was found for neither the HP or LP vineyards, that DTMA changed the composition or size of the tannin (data not shown), confirming previous reports that tannin is not selectively extracted from damaged seeds. It was interesting to find that for the LP vineyard, DTMA increased the extraction of grape pectins into the wine (result not shown), being unaffected for the HP vineyard. This may indicate that grapes which have high tannin extractability could also have relatively degraded and porous skins which facilitate the extraction of both tannin and pectins. For the LP vineyard, the use of DTMA may have increased skin breakage, facilitating the release of otherwise non-extractable tannin and polysaccharide from the grapes during maceration. Sensory analysis on the DTMA trial was conducted. Generally, DTMA was found to improve the visual (sensory) opacity of wines from the low-phenolic vineyard, independent of maceration time. For the high-phenolic vineyard, no improvement of opacity was found with DTMA. Changes in opacity closely reflected the results for wine colour and tannins.

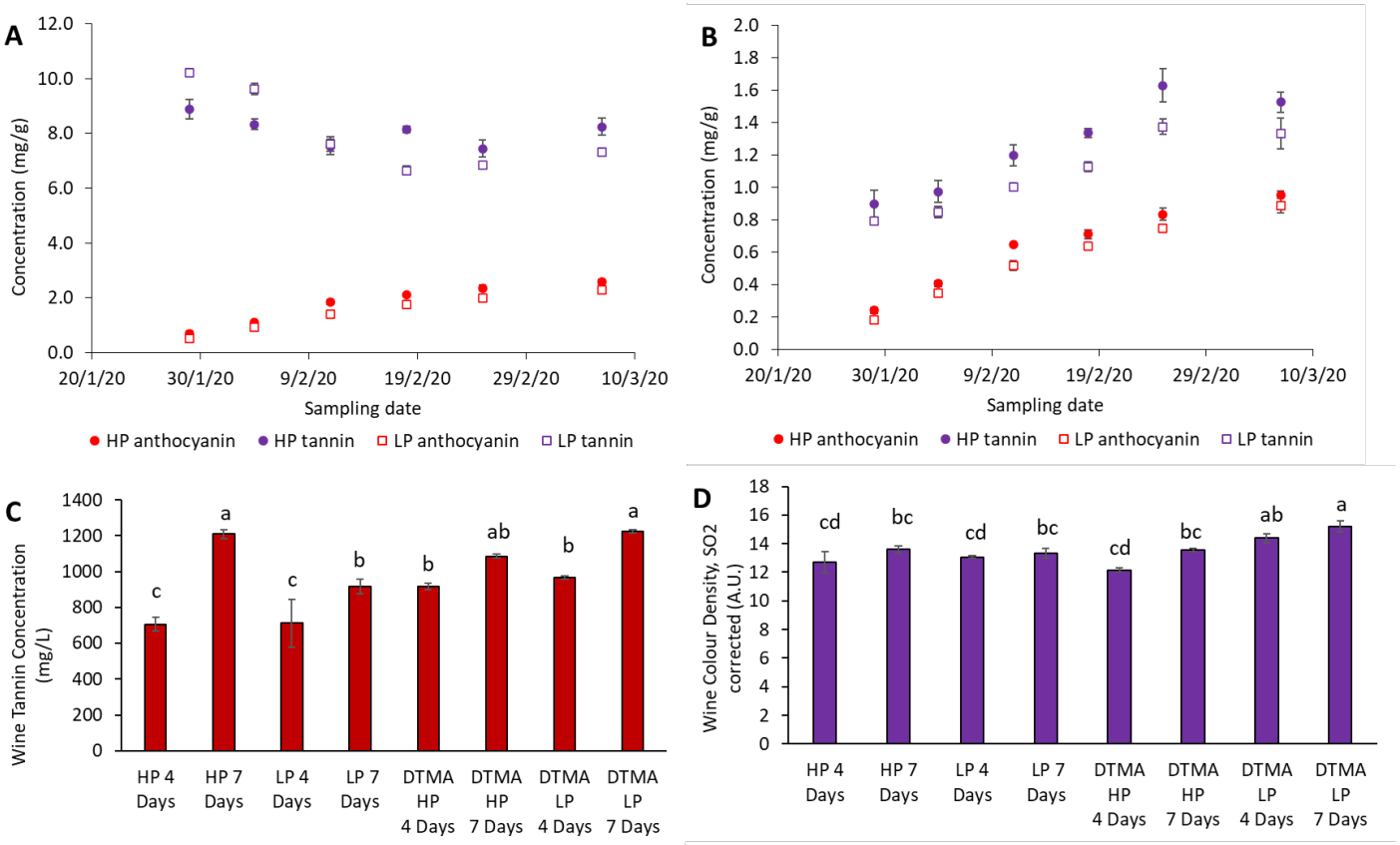


Figure 21. Tannin and colour measures determined for grapes and wines prepared from high phenolic potential (HP) and low phenolic potential (LP) vineyards, with or without accelerated maceration (DTMA) and with a 4 or 7 day maceration period, showing A. Total grape tannin and anthocyanin from LP and HP vineyards during ripening; B. Extractable grape tannin and anthocyanin from LP and HP vineyards during ripening; C. Tannin concentration in wines made from LP and HP vineyards; D. Corrected colour density in wines made from LP and HP vineyards. (Data are the mean ± standard error compared by one-way ANOVA, $P<0.05$, different letters indicate significant differences by Student's post-hoc T-test).

7.8.2 Interactions of red wine phenolic fractions with polyproline

To continue on from earlier work on the effect of aeration on the properties of wine tannin and its interactions (Section 7.5.4), a further study aimed to investigate the effect of aeration during red wine fermentation on the properties of Shiraz wine phenolic fractions and their reactivity with salivary proteins. A fractionation protocol was applied to isolate discrete classes of phenolics in red wine, and phenolic composition was characterised by LC-MS. This confirmed that the respective fractions contained primarily phenolic acids (fraction 1), monomeric flavan-3-ols (fraction 2), oligomeric flavan-3-ols (fraction 3), anthocyanins and low molecular weight derived pigments (fraction 4) and polymeric (including tannin and polymeric pigments) (fraction 5). The phenolic fractions were reacted with polyproline (as a model salivary protein) and monitored using isothermal titration calorimetry (ITC). Fraction 5 was also analysed for its hydrophobic binding properties (tannin activity).

For fraction 1, the ITC results showed that for both wines an exothermic reaction proceeded, and that both hydrophobic and hydrogen bonding were present (Figure 22). This demonstrated that a strong interaction between phenolic acids and polyproline occurred, but aeration was not found to affect the interaction. For fractions 2 and 3 containing monomeric and oligomeric flavan-3-ols respectively, no binding with polyproline was observed. For fractions 4 and 5, only the isolate from the aerated wine was found to interact with polyproline (Figure 23). Again, these reactions were found to be exothermic, spontaneous, and to involve both hydrophobic and hydrogen bonding.

The observation that polymeric tannins from the control wine did in fact not bind to polyproline, while those isolated from the aerated wine did, was contradictory to other published work (Watrelot et al. 2019). Since compositional differences between the tannin fractions isolated from the two treatments were minor, it was not possible to speculate what may have disrupted binding with protein in the control. Further analysis of the tannin fractions for their hydrophobic binding properties was carried out, revealing that the tannin activity of the control was -8.27 kJ/mol while that of the aerated treatment was -6.52 kJ/mol. This indicated that the hydrophobic binding of the control treatment (Watrelot et al. 2019) was greater than that of the oxygen treatment, in agreement with earlier work. It would therefore have been expected that the tannin fraction prepared from the control wine would have had a strong binding interaction with polyproline.

Despite the unexpected results for tannins, these results have shown for the first time that wine-derived phenolic acids have a strong binding interactions with polyproline, and may play an important role in modulating wine astringency. Their relevance in red wine have been previously overlooked. Ongoing work using mass spectrometry will aim to further elucidate compositional differences between the fractions isolated from the treatments.

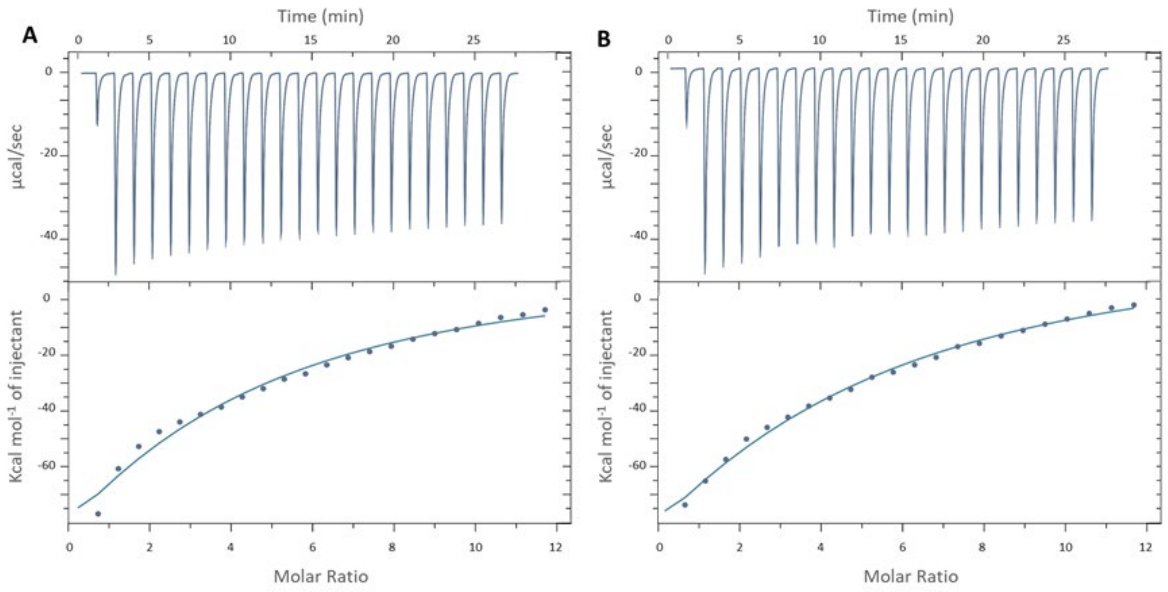


Figure 22. Titration plot of Fraction 1 (phenolic acids) with polyproline in the control wine (A) and aerated wine (B) by isothermal titration calorimetry

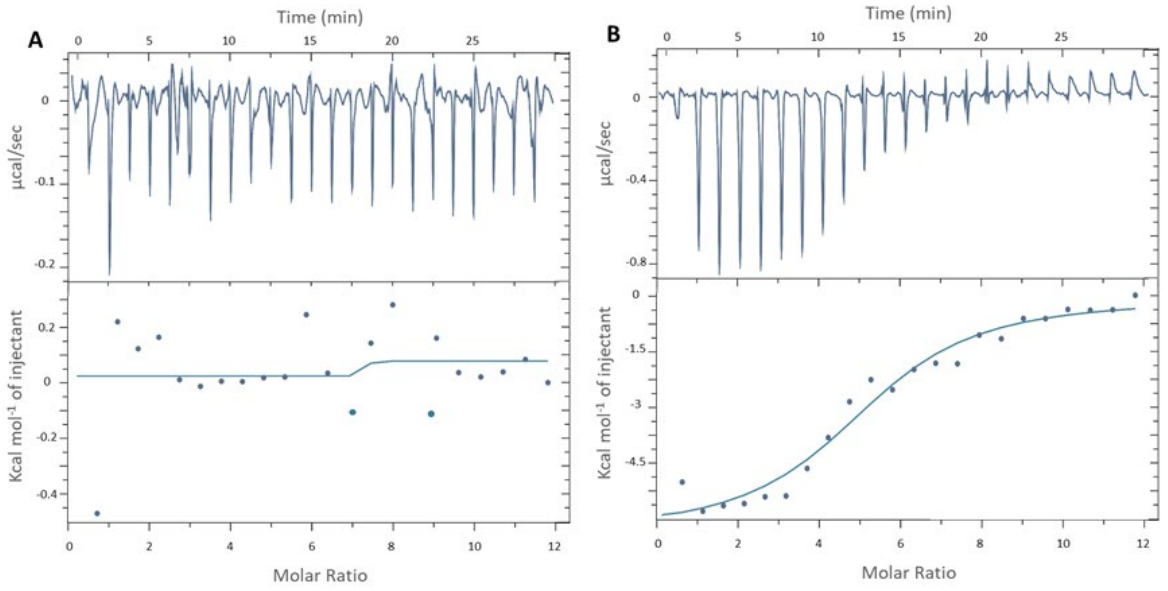


Figure 23. Titration plot of Fraction 5 (polymeric pigments and tannins) with polyproline in the control wine (A) and aerated wine (B) by isothermal titration calorimetry

7.8.3 Zeolite benchmarking for protein and cold stability

Chardonnay, Sauvignon Blanc and Muscat Gordo wines were treated with five different zeolites for benchmarking purposes. Analysis of volatiles and monoterpenes was performed to determine potential stripping by zeolites. There was a significant difference between the wines derived from treatment with Indonesian zeolite and four Australian zeolites. In the case of Chardonnay wine, Indonesian zeolite removed 50.5% of ethyl octanoate and 54% of ethyl decanoate (sweet, soap), whereas Australian zeolites did not alter these volatiles. In addition, Indonesian zeolite removed 41% of 2-phenylethyl acetate (floral, rose), while Australian zeolites removed 10% or none. In general, Chardonnay wine was the least affected wine after treatment with zeolites (Table 5 in Appendix 5).

There was also a significant difference between the wines treated with zeolites and bentonite. Zeolites mainly removed unpleasant volatiles (3-methylbutanol (harsh, nail polish), decanoic acid (fatty), hexanoic acid (cheese, sweaty), octanoic acid (rancid, harsh) from Chardonnay wine. On the other hand, bentonite removed 8% of ethyl acetate (VA, nail polish) and acetic acid (VA, vinegar) from Chardonnay wine but also 10% banana and fruity aroma (e.g. ethyl propanoate (fruity), ethyl-2-methylpropanoate (fruity), 2-methylpropyl acetate (banana, fruity), 3-methylbutyl acetate (banana), 2-methylbutyl acetate (banana). In addition, bentonite altered concentrations of ethyl-3-methylbutanoate (berry) and ethyl hexanoate (green apple).

Similar trends were observed for Muscat Gordo wine (Table 6 in Appendix 5). Zeolites removed mainly volatiles associated with nail polish, cheese, sweet soap, and rancid, harsh characters. In comparison, bentonite removed volatiles associated with banana and fruity aromas. All zeolites and bentonite removed a considerable amount of decanoic acid (zeolites between 19-36% and bentonite 36%) and ethyl decanoate (sweet, soap) (Australian zeolites between 35-50%, Indonesian zeolite 78%, and bentonite 34.5%). As with the first wine, a significant difference was found between the wines treated with Indonesian zeolite and four Australian zeolites. For example, Indonesian zeolite removed 87% 2-phenylethyl acetate (floral, rose), 21% ethyl propanoate (fruity), 20% 2-methylpropyl acetate (banana, fruity), and 64% hexyl acetate (sweet, perfume), while Australian zeolites removed few or none of these volatiles.

Sauvignon Blanc was the most altered wine after treatment with zeolites and bentonite. As with the Chardonnay and Muscat Gordo wines, the Indonesian zeolite had a much stronger effect on volatiles in Sauvignon Blanc wine than Australian zeolites (Table 7 in Appendix 5). Indonesian zeolite removed 76% 2-phenylethyl acetate (floral rose), 34% 3-methylbuthyl acetate (banana), 38% 2-methylpropyl acetate (banana, fruity), 50% hexyl acetate (sweet, perfume) and 40% ethyl hexanoate (green apple), while Australian zeolites removed much fewer of these volatiles. Once again, all zeolites and bentonite removed a considerable amount of decanoic acid (fatty) (zeolites between 23-36% and 13.5% bentonite). Whereas zeolites removed 14-23% octanoic acid (rancid, harsh), 9-17% hexanol (green grass), 13.5-19% ethyl-3-methylbutanoate (berry) and 7-15% 2-phenylethanol (floral, rose) from Sauvignon wine, bentonite did not.

Furthermore, the results from this trial showed that the monoterpenes of Sauvignon Blanc were not affected after treatment with zeolites. Chardonnay and Muscat Gordo wines showed some changes, but only in the concentration of α-terpineol after treatment. The other 15 tested monoterpenes were not affected by zeolites in any of the wines tested. In Chardonnay wine, the concentration of α-terpineol was increased by 9% after treatment with Orku zeolite and by 12.5% after treatment with Aussie zeolite. The other three zeolites had no influence on the monoterpenes. Muscat Gordo was the only wine that showed a slight decrease in α-terpineol concentration after treatment with all zeolites. Aussie, Indonesian, and ZeoNatural showed a decrease of 11%, and Grind and Orku 9%.

In a proof-of-concept winemaking trial, Indonesian zeolite was used during the fermentation of a Chardonnay juice and a blend of 83% Sauvignon Blanc/17% Riesling juice from the 2022 vintage. In parallel, these wines were treated with bentonite for comparison purposes. The Indonesian zeolite was used because it had the lowest aluminium concentration in the wines after treatment. Furthermore, when Indonesian zeolite was selected, the project team was not yet aware that heat treatment of zeolites prior to their use in wine leads to a significant reduction in aluminium uptake by the wine. Indonesian zeolite reduced the protein concentration in Chardonnay wine by 37% and in SAB/Riesling by 21% but did not protein-stabilise the wines at bottling. In comparison, bentonite treatment resulted in protein stabilisation of these wines. In terms of cold stability, neither Indonesian zeolite nor bentonite resulted in the cold stabilisation of the wines. Regarding Indonesian zeolite, we can conclude that it does not work when added during fermentation and must be added to the finished wine if cold and protein stabilisation are to be achieved. The results of this experiment are not conclusive. As we have shown, Indonesian zeolite is very different from

Australian zeolites. Therefore, the result of this trial cannot be applied to all natural zeolites and further research is needed to include Australian zeolites.

Zeolites have the potential to induce both heat and cold stability in a single treatment, which would be a considerable advantage over conventional bentonite fining to achieve heat stability and refrigeration for cold stability. Some of the disadvantages of zeolite treatment are the relatively high dosage required, high aluminium content in the wine after treatment, and that some zeolites are less effective than others at protein removal. The possibility for the easy regeneration of zeolites (for example, by treatment with concentrated (10%) NaCl solution) somewhat reduces the negative aspects of the high dose requirements, because the material can be reused many times. Also, the possible reduction of aluminium by heat treatment of zeolite before adding it to the wine reduces this negative aspect. In addition, based on structure-activity studies, it was found that natural zeolites with a low calcite content had a better ability to remove haze-forming proteins in wines, making these zeolites more attractive for use as they induce both heat and cold stability in a single treatment. These findings indicate that using zeolite to achieve wine cold and heat stability is feasible and promising.

7.8.4 Collaborative studies on macromolecule extraction and composition

7.8.4.1. Flash détente

A study was undertaken in collaboration with the University of Adelaide and E. & J. Gallo. The aim of this study was to investigate outcomes of flash détente processing on wine composition and sensory properties. AWRI's contribution to this work was to perform detailed tannin and polysaccharide analysis. More information on this study can be found in Ntuli et al. (2022a and 2022b).

7.8.4.2. The role of amino acids in red wine texture and taste

An internal collaboration was conducted with Project 3.1.1 to investigate the influence of amino acids, in particular proline and glutamic acid, on red wine sensory properties. Results from this work are included in the Final Report for that project. Amino acid profiling and tannin analysis were performed as part of this project. Briefly, proline was found to increase sweetness, viscosity and red fruit flavour, and decrease bitterness and astringency; while glutamic acid imparted savoury/umami taste to the wine. The work was then extended to explore the impact of blending wines with different proline and tannin concentrations, showing that proline increased sweetness, viscosity, fruit flavour while decreasing bitterness and astringency. More information on this study can be found in Espinase Nandorfy et al. (2022).

7.9 Year 5 deliverable. Practical advice and extension materials for winemakers

This project undertook some key extension and communication activities during the project period (see Appendix 10.2 for details).

Work on the updated heat test was developed into an extension presentation and was presented as an AWRI webinar entitled 'Predicting heat stability of wine: the heat test revisited'. This webinar has been viewed 3,527 times on YouTube since 17 August 2017. The work was also prepared as a shorter version entitled 'Hot news in heat stability' for AWRI roadshows, and was selected once for presentation in the Langhorne Creek region in September 2018. The heat stability roadshow presentation was also updated to include newer work on carrageenan and zeolites, but was not selected by any of the regions during the project period.

Work on red wine macromolecule extraction built on previous research, and highlighted multiple techniques that can modulate wine macromolecules, wine texture, flavour and aroma. This work

was first consolidated into two AWRI roadshow topics 'How can I predict tannin and colour in the vineyard?', which was presented five times, and 'How to maximise the phenolics of grapes through innovative winemaking?'. The latter seminar was reworked to be the focus of a workshop at the 17th AWITC in July 2019 on 'Using maceration techniques to tailor red wine styles', which was sold out. The topic was shortened to a roadshow seminar, and has been presented eight times, as an AWRI webinar and roadshows, and as invited talks for USA audiences (E. & J. Gallo, Texas ANU, Oregon Wine Symposium). Besides attendees at the original session, the webinar version of this presentation received 1,300 views on YouTube within its first year, and 2,839 views since October 2019. An adapted version of this seminar was also presented as part of the ASVO's seminar series on phenolics in October 2020 entitled 'Balancing the extraction and loss of phenolics'.

Work on whole bunch fermentation and pectolytic enzymes was included in the maceration roadshow seminar. In addition, Wine Tasmania requested invited talks on 'Enzymes, do they work?' as well 'Whole bunch fermentation – a focus on Pinot Noir' for Winemaker Symposia held in November 2019 and October 2021, respectively.

A presentation on outcomes of the AWRI water addition studies, as well as others conducted within Australia, was developed in October 2020. This was presented as an AWRI webinar, with the recording receiving 859 views since its release. This seminar was also presented as part of a workshop 'Fermentation 101' at the 18th AWITC in June 2022.

Work on the role of wine composition in cold stability was prepared as a roadshow presentation 'What makes a wine cold unstable?' and was presented once in June 2022. This topic also formed part of a focused AWITC workshop entitled 'Predict and prevent – improving wine cold stabilisation across the value chain' in June 2022. This workshop also included updates on cold stability testing for producers, as well as alternative approaches to cold stabilisation such as potassium polyaspartate.

Collaborative research demonstrating the possibility of regenerating magnetic nanoparticles used for protein stabilisation of white wine was promoted with a media release distributed by the University of South Australia on 11 May 2020 entitled 'Raise your glass: new nanotech clears haze from white wine'. This media release generated substantial interest from journalists, including three radio interviews, documented in Appendix 10.1.

The outcomes of this project have resulted in ten poster and/or conference presentations (Appendix 10.3). There were also 21 peer-reviewed manuscripts and 6 technical or proceedings articles published. These are documented in Appendices 10.4 and 10.5 respectively.

8. Outcomes and conclusions

8.1 New approaches to predict and manage heat instability in white wine

Novel magnetic separation technology for the rapid and selective removal of pathogenesis-related proteins from white wine was developed. The technology is based on using acrylic acid (AcrA) plasma polymer-coated magnetic nanoparticles. The new separation method is simple and very effective in removing both pathogenesis-related protein classes (TLPs, and chitinases) from white wines, even when the wine contains an extremely high concentration of proteins. This new technology may become an alternative to conventional bentonite treatment. One advantage of this method is that functionalised surfaces are classified as processing aids rather than additives. Another advantage of this technology over bentonite fining is that magnetic nanoparticles can be regenerated and reused. Also, in this project, for the first time, the use of natural zeolites as an alternative to bentonite to remove the proteins responsible for white wine haze was evaluated. Zeolites have been widely used as catalysts, separators, water softeners and desiccants, but importantly from a wine production standpoint meet the requirements for protein adsorption.

Zeolites are also easy to use and cheap compared to many other types of commercially available adsorbents. For zeolites, a lack of swelling behaviour can reduce lees production relative to a number of commercial bentonites. The results of this study indicated that using natural zeolites to achieve wine cold and heat stability is feasible and promising.

This project also supported the implementation of new techniques to study and analyse proteins and their interactions in wine. The application of FCS was demonstrated in real wines for the first time to investigate the problem of protein haze formation, through live monitoring of heat-induced aggregation in wine. This study pointed to further opportunities provided by FCS to more accurately and selectively study the complex phenomena occurring in wines in a novel way. Further, in this project, an efficient and rapid fluorescence-based technology to detect haze-forming proteins in white wines was developed. A fluorescent compound was synthesised to selectively bind haze-forming proteins. The developed method was selective for haze forming proteins, provided a linear detection range and had a low detection limit of 2 mg/L. In the future, the technology developed could potentially offer winemakers an alternative to the commonly used heat test, enabling faster, cheaper, and more accurate screening of potentially protein-unstable white wines.

8.2 New knowledge on the role of the wine macromolecules as crystallisation inhibitors and novel approaches to achieve cold stability identified

Significant progress was made within different facets of the work on cold stability. The project initially set out to optimise the methodology used to measure cold instability, as it was recognised that the existing tests were limited either by a lack of a quantifiable response, or were poorly reproducible and inaccurate. Two improved methods were developed, firstly an updated version of the three-day cold test which quantified KHT crystal formation as reconstituted tartaric acid by HPLC. Secondly, micronised KHT was used to replace standard analytical grade KHT in a shortened (4 min) version of the mini-contact test, with a high level of reproducibility and accuracy achieved in both a standard and a 30 mL format. These tests were then used to determine the impact of wine macromolecules on KHT crystal formation. Key drivers of instability in white and red wines were highlighted through the compositional survey of commercial wines over two seasons.

In the completed study, it was shown that wine polysaccharides did not have an impact on cold stability within the range found in white or red wines, contrary to what has been proposed in the literature (Gerbaud et al. 1996, Gerbaud et al. 1997). For white wines, macromolecules (including protein and phenolics) had only a limited impact in conferring cold stability, and the ability of a white wine to resist KHT crystallisation was driven primarily by a high potassium concentration relative to a low tartaric acid concentration. Hence, strategies that address the removal of potassium through adsorbents or membrane technology appear to be the most promising approach to improve the process of cold stabilisation by reducing energy inputs. In red wines, monomeric anthocyanins were found to be primarily responsible for the intrinsic cold stability differences between white and red wines. The prediction of anthocyanin conversion and loss has potential to be an important tool in the management of red wine cold stability in the future.

Further, work on the development of alternative cold stabilisation strategies has taken an important leap forward. From the observation that potassium removal is critical to improve white wine cold stability, zeolites were identified as a potential means to address this issue, as they remove a substantial quantity of potassium. Multiple zeolites were shown to be effective in reducing wine potassium and initiating KHT crystallisation without chilling, and could effectively cold stabilise white wines. With regard to the use of natural zeolites, it was found that low calcium zeolites have the potential to provide both heat and cold stability in a single treatment, which is a significant advantage over conventional approaches currently used in Australia. Further work is needed on these alternative strategies to ensure that they do not negatively affect wine sensory properties nor introduce contaminants, in particular aluminium.

In this project a further alternative to cold stabilisation was explored, harnessing the potential to deploy tailored surface coatings obtained by plasma polymerisation for the first time. Importantly, this technology was effective at cellar temperature, and could have a positive impact on wine production in terms of cost and energy efficiency.

Differences were found in the effectiveness of plasma polymerised coatings depending upon whether a wine was heat stable or not. Coatings containing amine functional groups were most efficient at initiating KHT crystallisation in heat-unstable wines while surfaces enriched with carboxyl acid groups performed best for heat stabilised wines. The results indicate the potential for cold stabilisation to be induced at higher temperatures, reducing the need for cooling facilities.

8.3 Knowledge of how to modulate extraction and retention of key compounds for given wine styles

Three core projects were completed which aimed to understand the effect of maceration conditions on macromolecule extraction.

The first project explored phenolic outcomes in wines obtained by delaying Shiraz harvest date, or applying water addition to the highest sugar (15.5 °Baumé) must. A strong increase in wine tannin, non-bleachable pigments (bisulfite resistant colour) and wine colour density was found with the transition of harvest date from 13.5 to 15.5 °Baumé. Water addition consistently reduced wine tannin concentration, non-bleachable pigments and colour density compared to the 15.5 °Baumé control wines. However, it was interesting to observe that neither the method of water addition (direct addition versus run off/replace) nor the quantity of water added had a significant impact on tannin and colour. This indicates that the reason for the reduction in phenolics extraction was not simply due to a change in the solids:must ratio.

Further research was undertaken to try to find approaches to overcome the losses in phenolics observed with dilution in Shiraz by applying DTMA. However, in that study it was found that water addition did not affect Shiraz wine colour or tannin concentration. Variable outcomes have been reported with respect to water addition in Shiraz (Schelezki et al. 2020 a, b, Teng et al. 2020 a, b) and a literature review should be conducted to consolidate the results of the body of research which has been completed. Based on this review, further research may be needed on water addition to make firm recommendations to producers.

A further project took a comprehensive look at whole bunch addition to Shiraz and Pinot Noir fermentations. Important differences in the perception of ‘green capsicum’ flavour and aroma were observed with whole bunch addition in both varieties, and are reported separately (see Final Report for Project 3.1.1 and Day et al. 2020). For Shiraz, the concentration of tannin in wine and the perception of astringency were highly correlated with the proportion of whole bunch inclusion, while for Pinot Noir, tannin concentration of tannin in wine was variably affected. However, for Pinot Noir, whole bunch inclusion introduced an important transition in the sensory perception from a brown to a red hue, which was related to a small reduction in the analytical measures for hue (420/520 nm). This study clearly demonstrated that whole bunch inclusion brings about meaningful changes in wine composition and stylistic outcomes. However, advice to producers remains cautionary, particularly as the level of whole bunch inclusion approaches 75 to 100%, due to the obvious impartation of ‘green capsicum’ attributes due to isobutyl methoxypyrazine extraction from stems, as well as for Shiraz, increases in astringency due to stem tannin extraction to the wine.

The last area of research which explored maceration techniques was to continue the study of DTMA, with a view to understanding its impact depending upon the phenolic potential of the grapes being fermented. This work provided some practical insights into the changes in phenolic extractability occurring during grape ripening, showing that tannin extractability increases during ripening. Conversely, total grape tannin (which includes that from both skin and seed) decreased during

ripening and then remained constant. Although previous studies have demonstrated increases in phenolic extractability during ripening (Teng et al. 2020a; Schelezki et al. 2018) it has not been possible to separate differences associated with ripening from the concentration effect introduced by berry shrivel. In this work, tannin extractability was studied over a wider period of time, and for multiple vineyards, showing that the trend of increasing extractability during ripening was consistent, and also independent of berry size. The outcomes of the DTMA maceration were interesting, showing that tannin extraction was enhanced by the technique when the phenolic extractability of the grapes was limiting. For grapes with a high phenolic potential, the DTMA technique was an accelerated extraction of tannin for a short maceration time, but this effect was lost as maceration time was extended. This result indicates that the DTMA technique may show promise to overcome issues with poor grape phenolic development experiences in warm, irrigated regions, or with low phenolic varieties.

9. Recommendations

Overall, this project provided potential alternatives to bentonite fining, the main method for managing the heat stability of white wines. One of the alternative methods is based on the application of a thin plasma polymer coating on a surface of magnetic nanoparticles, while the second method uses zeolites. For the first approach, pathways for commercialisation need to be further explored, and it is expected this will be done through an open innovation approach. If commercialisation routes are identified, scale-up trials are required to apply the technology on an industrial scale.

For the second approach identified, using natural zeolites as a dual heat- and cold-stabilising agent, benchmarking work will need to be repeated once zeolites have undergone dealumination through heating. Once zeolite sources are identified which can be consistently used for heat and/or cold stabilisation, further research will still be required to assess the level of variation within the source material, and whether this might limit the effectiveness of the application. Sensory evaluation of different wine varieties after treatment with zeolites is also required, with associated analysis of wine volatiles.

The application of nanoparticles and zeolites in wine production appears very promising, but these technologies are still at a very early stage. At present, there are regulatory barriers that have to be considered since neither magnetic nanoparticles nor zeolites are currently approved as wine additives in Australia. Obtaining regulatory approval for the use of zeolites in white winemaking has been identified as a priority, but further study of the natural zeolites available on the market is required due to concerns about possible aroma scalping or aluminium deposition. The price competitiveness of zeolites compared to traditional or alternative stabilisation methods will also need to be evaluated through commercial trials. Producers may realise commercial benefits in the form of increased wine recovery following stabilisation and potentially reduced production costs due to the ability to regenerate and reuse zeolite.

Approaches for the introduction of functionalised surfaces to wines for cold stabilisation showed promise and will require further development before commercialisation can be considered. After the surface is deployed to an unstable wine, and crystallisation has been completed, it will be required for the surface to be removed and regenerated. Strategies to deploy, remove and regenerate plasma polymerised surfaces in wine will need to be further explored in consultation with a commercial partner. Importantly, this approach was found to be effective at cellar temperatures, showing promise that cold stabilisation could become more sustainable, avoiding the need for refrigeration facilities.

Based on the outcomes of the study on the role of wine composition on cold stability, an important finding was that ionised anthocyanins play a protective role in red wine, limiting KHT crystallisation.

Since Australia's warm, irrigated regions can experience both poor colour and higher pH values, future research should aim to support these producers preserve colour and reduce the inputs required for cold stabilisation. Future work should also aim to characterise the mechanism by which ionised red wine pigments limit KHT crystallisation, to better control pigment composition, and hence red wine cold stability as pigment profiles become modified during wine processing and ageing.

In this work a number of new methods were developed and validated. The first example of a fluorescence-based sensing approach for detection of haze-forming proteins in wine has been presented, potentially offering an alternative approach to the commonly used heat test. If commercialised, the test could enable fast, cheap and accurate screening of haze-forming proteins in white wines. However currently, calibration of the protein screen can only occur against the existing heat test, and as such may result in overfining of wines. A new, less stringent heat test will need to be developed to determine whether a higher protein concentration threshold for heat stability is acceptable in white wine.

Fluorescence correlation spectroscopy (FCS) was found to be an innovative approach to studying the complex interactions between proteins, polysaccharides and tannins in both model or real wines *in situ*, with high sensitivity and selectivity. This breakthrough work provided strong experimental evidence that interactions between wine macromolecules can be successfully studied with FCS. These experiments showed that FCS is well suited to study the diffusion (mobility) of proteins, polysaccharides or polyphenols in complex, heterogeneous solutions such as wine. FCS also allows for characterisation of size, polydispersity, concentration and aggregation of the above-mentioned wine colloids. There is an extensive opportunity to apply this technology within other fields of wine research.

From the studies on water addition to Shiraz musts, this appeared to be an effective way to manage high sugar concentrations associated with very ripe fruit, although some losses in phenolics could be expected. The mode of water addition (i.e. if juice was run off prior to the addition of the water or water was added directly) had a relatively small impact wine phenolics, but had a larger effect on wine sensory properties, such that it is recommended that water be applied moderately. Given that outcomes for Shiraz have been variable when water addition was applied to different grape sources, a literature survey of published studies is needed to make clear recommendations to the industry.

From the study on whole bunch addition, recommendations can be made for the application of this technique in both Shiraz and Pinot Noir ferments. In this study, additions of whole bunches were high, ranging from 25% to 100%, so the results cannot be extended to cases where only a small proportion is used. For Shiraz, clear impacts of whole bunch inclusion on increasing colour, tannin extraction and astringency were observed, with benefits observed even at 25% inclusion. For Pinot Noir there was some evidence that whole bunch addition could improve the visual perception of red colour, despite only small differences in spectral colour measures. Despite potential benefits on phenolic outcomes, it may be important to avoid high levels of whole bunch addition due to the introduction of unwanted increases in 'green capsicum' flavour and aroma. A cautionary approach is therefore recommended when higher proportions of whole bunch fermentation are applied.

The outcomes of the DTMA maceration trials on Shiraz demonstrated that the technique has the potential to overcome poor phenolic extraction in varieties commonly known to have lower phenolics such as Pinot Noir, but more importantly for warm, irrigated regions which experience issues with low colour and tannin. The trial did not show a substantial benefit for the reduction of maceration time in this variety, and it appears to be a gentler approach from that initially prototyped by the Tasmanian Institute of Agriculture. Future work should explore the potential benefit of DTMA in warm, irrigated regions, in particular to reinvestigate vineyard phenolic variability.

10. Appendix 1: Communication

10.1 Media interviews

Collaborative research demonstrating the possibility of regenerating magnetic nanoparticles used for protein stabilisation of white wine was promoted with a media release distributed by the University of South Australia on 11 May 2020 entitled 'Raise your glass: new nanotech clears haze from white wine'. This media release generated substantial interest from journalists, including three radio interviews:

1. K.A. Bindon, Nanotechnology for protein stabilisation. Interviewer Belinda Willis, The Lead SA, 11 May 2020
2. K.A. Bindon, Nanotechnology for protein stabilisation. Interviewer Bridget Hermann, ABC South East, 12 May 2020
3. K.A. Bindon, Nanotechnology for protein stabilisation. Interviewer Rita Erlich, Travel Writers Radio, 13 May 2020

10.2 Industry presentations at roadshow seminars or workshops

1. Bindon., K.A. Managing phenolics in the vineyard and winery. Michigan State University opportunity for vineyard and winery managers. 5 September 2017, Northwest Michigan Horticulture Research Center (NWMHRC), 6686 S Center Hwy, Traverse City, MI 49684.
2. Bindon, K.A. How can I predict wine tannin and colour in the vineyard? AWRI Orange Seminar. 1 November 2017, Hotel Canobolas, Media Room, 1st Floor, 248 Summer Street, Orange, NSW.
3. Bindon, K.A. How can I predict wine tannin and colour in the vineyard? AWRI Canberra Seminar. 3 November 2017, Lerida Estate, 87 The Vineyards Road, ACT.
4. Bindon, K.A. How to maximise the phenolics of grapes through innovative winemaking? AWRI Canberra Seminar. 3 November 2017, Lerida Estate, 87 The Vineyards Road, ACT.
5. Bindon, K.A. How to maximise the phenolics of grapes through innovative winemaking? AWRI Robe Seminar. 14 February 2018, Robe Bowls Club, Robe, SA.
6. Bindon, K.A. How to maximise the phenolics of grapes through innovative winemaking? AWRI Langhorne Creek Seminar. 20 September 2018, The Winehouse, 1509 Langhorne Creek Road, Langhorne Creek, SA.
7. McRae, J.M. Hot news in heat stability. AWRI Langhorne Creek Seminar. 20 September 2018, The Winehouse, 1509 Langhorne Creek Road, Langhorne Creek, SA.
8. Bindon, K.A. Using maceration techniques to tailor red wine styles. Seminar presented at E. & J. Gallo Winery Statewide Short Course, 5 June 2019, Wine and Roses Hotel, Lodi, CA, USA.
9. Bindon, K.A. The secret life of wine macromolecules. Webinar presented to staff and students of the Midwest Grape and Wine Industry Institute, Iowa State University and the College of Agriculture and Life Sciences, Cornell University, 26 June 2019, 2573 Food Sciences Building, 536 Farm House Lane Ames, Iowa, USA.
10. Bindon, K.A. 'Using maceration techniques to tailor red wine styles' Workshop Presenter and Convenor at 17th AWITC Conference, Adelaide Convention Centre, 21 July 2019.
11. Bindon, K.A. Using maceration techniques to tailor red wine styles. AWRI Webinar Series, 3 October 2019.

12. Bindon, K.A. How can you predict wine tannin and colour in the vineyard? AWRI Orange Seminar. 15 October 2019, Hotel Canobolas, 248 Summer Street, Orange, NSW.
13. Bindon, K.A. How can you predict wine tannin and colour in the vineyard? AWRI Southern Highlands Seminar. 16 October 2019, Wingecarribee Shire Council Theatrette, 68 Elizabeth Street, Moss Vale, NSW.
14. Bindon, K.A. How can you predict wine tannin and colour in the vineyard? AWRI Canberra Seminar. 17 October 2019, Quality Hotel Dickson, Speedwell Room, Cnr Badham Street & Cape Street, Dickson, ACT.
15. Bindon, K.A. How can you maximise the phenolic potential of grapes through innovative winemaking? AWRI Canberra Seminar. 17 October 2019, Quality Hotel Dickson, Speedwell Room, Cnr Badham Street & Cape Street, Dickson, ACT.
16. Bindon, K.A. Enzymes, do they work? Extraction vs Clarification. Wine Tasmania Winemaker Symposium, 5 November 2019, C3 Convention Centre, 64 Anglesea St, South Hobart.
17. Bindon, K.A. Water addition to must: when to use it and how much? AWRI Webinar Series, 1 October 2020.
18. Bindon, K.A. Using maceration techniques to tailor red wine styles. Texas A&M University Enology Webinar Series, 2 October 2020.
19. Bindon, K.A. (presented remotely). Balancing the extraction and loss of phenolics. ASVO Phenolics and Texture Webinar Series, 20 October 2020.
20. Bindon, K.A. How to improve fruit set in cool climates? AWRI Limestone Coast Seminar 28 October 2020, Coonawarra Soldiers Memorial Hall, 11 Memorial Drive, Coonawarra, SA, 5263.
21. Bindon, K.A. Using maceration techniques to tailor red winemaking styles. AWRI Limestone Coast Seminar 28 October 2020, Coonawarra Soldiers Memorial Hall, 11 Memorial Drive, Coonawarra, SA, 5263.
22. Bindon, K.A (presented remotely). Using maceration techniques to tailor red winemaking styles. AWRI Hobart Seminar, 9 February 2021, The Old Woolstore, 1 Macquarie Street, Hobart, TAS, 7000
23. Bindon, K.A (presented remotely). Using maceration techniques to tailor red winemaking styles. AWRI Launceston Seminar, 10 February 2021, Tailrace Centre, 100 Lower Paper Mills Rd, Launceston, TAS, 7250.'
24. Bindon, K.A. (presented remotely). Review of Grape Phenolics and Effects of Maceration Techniques. Oregon Wine Symposium, 18 February 2021.
25. Bindon, K.A. and Bilogrevic, E. (presented remotely). Whole Bunch Fermentation – a focus on Pinot Noir. 4th Winemaker Symposium 'Pinot Noir, You're A Star', 13 October 2021, Tailrace Centre, 100 Lower Paper Mills Rd, Launceston, TAS, 7250.'
26. Bindon, K.A. Using maceration techniques to tailor red winemaking styles. AWRI Hunter Valley Seminar, 1 June 2022, Hunter Valley Resort, Corner Hermitage Road and Mistletoe Lane, Polkobin, NSW, 2320.
27. Bindon, K.A. What makes a wine cold unstable? AWRI Mudgee Seminar, 2nd June 2022, The Pavillion at Lowe, Tinja Lane, Eurunderee, NSW, 2850.
28. Bindon, K.A. Using maceration techniques to tailor red winemaking styles. AWRI Mudgee Seminar, 2 June 2022, The Pavillion at Lowe, Tinja Lane, Eurunderee, NSW, 2850.
29. Bindon, K.A. Predict and prevent – improving wine cold stabilisation across the value chain. Workshop Presenter and Convenor at 18th AWITC Conference, Adelaide Convention Centre, 26 June 2022.

30. Bindon, K.A. Water addition to must: when to use it and how much? Presenter in 'Fermentation 101' Workshop at the18 AWITC Conference, Adelaide Convention Centre, 26th June 2022.

10.3 Posters and conference presentations

1. Bindon, K., Kassara, S., Curtin, C., Li, S., Hixson, J., Teng, B., Wilkinson, K., Smith, P. Cap on red wine macromolecules? Updates on how winemaking interventions influence tannin and polysaccharide composition in Shiraz wines. ACS Conference Session 'From Fermentation to Fume Hood: The Chemistry of Wine', August 20th 2017, Walter E. Washington Convention Center, Washington DC, DC, USA.
2. Bindon, K.A. Mierczynska-Vasilev, A., Li, S., Watrelot, A., Teng, B., Bekker, M., Smith, P. The Secret Life of Wine Macromolecules. Paper presented at the 70th ASEV National Conference, Marriot Hotel, Napa Valley, CA, USA, 21 June 2019.
3. Bindon, K.A., Teng, B., Smith, P.A., Espinase Nandorfy, D., Solomon, M. and Petrie, P.R. Winemaking with high Baume juice: optimising fermentation and extraction. Paper presented at the 17th AWITC Conference, Adelaide Convention Centre, Adelaide, SA, Australia, 23 July 2019.
4. Day, M., Capone, D., Kassara, S., Bilogrevic, E., Espinase Nandorfy, D., Watson, F., Francis, I.L., Bindon, K. Whole bunch fermentation of Shiraz and Pinot Noir: influence on 'green' characters and astringency. Paper and poster presented at the 17th AWITC Conference, Adelaide Convention Centre, Adelaide, SA, Australia, 24th July 2019.
5. Miercyzska-Vasilev, A.M., Debare, R., Reilly, T.G. Wahono, S., Vasilev, K., Bindon K.A New solutions to tartrate instability in white wines. Paper and poster presented at the 18th AWITC Conference, Adelaide Convention Centre, Adelaide, SA, Australia, 28th June 2022.
6. Espinase-Nandorfy, D., Watson, F.T., Likos, D., Siebert, T.E., Bindon, K.A., Kassara, S., Qi, S., Pearson, W.P., Shellie, R. Keast, R., Francis , I.L.The influence of amino acids, and their interactions, on the sensory properties and consumer acceptance of dry red wine. Paper and poster presented at the 18th AWITC Conference, Adelaide Convention Centre, Adelaide, SA, Australia, 29th June 2022.
7. Reilly, T., Schulkin, A., Wilkes, E.N., Bindon, K.A. New insights on how white and red wine composition affects cold instability. Poster presented at the 18th AWITC Conference, Adelaide Convention Centre, Adelaide, SA, Australia, 26-29 June 2022.
8. Espinase-Nandorfy, D., Watson, F.T., Teng, D., Likos, D., Lewin, S., Gledhill, J.A., Siebert, T.E., Barter, S.R., Bekker, M.Z., Kulcsar, A.C., Bindon, K.A., Kassara, S., Shellie, R., Keast, R., Francis I.L. Using a new class of compositional targets with wine blending to improve warm inland Cabernet Sauvignon wine flavour. Poster presented at the 18th AWITC Conference, Adelaide Convention Centre, Adelaide, SA, Australia, 26-29 June 2022
9. Ntuli, R. Saltman, Y., Ponangi, R., Jeffery, D.W., Bindon, K.A., Wilkinson, K.L. An innovative approach to differentiated Cabernet Sauvignon wine styles using flash détente. Poster presented at the 18th AWITC Conference, Adelaide Convention Centre, Adelaide, SA, Australia, 26-29 June 2022
10. Angela, S. Wollan, D., Muhlack, R., Bindon, K., Wilkinson, K. Compositional consequences of ultrafiltration treatment of red and white wine. Poster presented at the 18th AWITC Conference, Adelaide Convention Centre, Adelaide, SA, Australia, 26-29 June 2022

10.4 Published peer-reviewed manuscripts

1. McRae, J.M., Barricklow, V., Pocock, K.F., Smith, P.A. 2018. Predicting protein haze formation in white wines. *Aust. J. Grape Wine Res.* 24(4): 504-511.
2. Teng, B.; Hayasaka, Y.; Smith, P., Bindon, K. 2019. Effect of grape seed and skin tannin molecular mass and composition on the rate of reaction with anthocyanin and subsequent formation of polymeric pigments in the presence of acetaldehyde. *J. Agric. Food Chem.* 67 (32): 8938-8949.
3. Kassara, S., Li, S., Smith, P., Blando, F., Bindon, K. 2019. Pectolytic enzyme reduces the concentration of colloidal particles in wine due to changes in polysaccharide structure and aggregation properties. *Int. J. Biol. Macromol*. 140: 546-555.
4. Watrelot, A.A., Day, M.P., Schulkin, A., Falconer, R.J., Smith, P., Waterhouse, A.L., Bindon, K.A. 2019. Oxygen exposure during red wine fermentation modifies tannin reactivity with poly-L-proline. *Food Chem.* 297:124923.
5. Mierczynska-Vasilev, A.M., Wahono, S.K., Smith, P., Bindon, K.A. Vasilev, K. 2019a. Using zeolites to protein stabilize white wines. *ACS Sustain. Chem. Eng.* 7(14): 12240−12247.
6. Mierczynska-Vasilev, A., Qi, G., Smith, P., Bindon, K., Vasilev, K. 2019b. Regeneration of magnetic nanoparticles used in the removal of pathogenesis-related proteins from white wines. *Foods.* 9(1):1.
7. Li, S. Wilkinson, K. Mierczynska-Vasilev, A., Bindon K. 2019. Applying nanoparticle tracking analysis to characterize the polydispersity of aggregates resulting from tannin–polysaccharide interactions in wine-like media. *Molecules* 24(11): 2100.
8. Mierczynska-Vasilev, A.; Mierczynski, P.; Maniukiewicz, W., Visalakshan, R. M.; Vasilev K., Smith, P. A. 2019c. Magnetic separation technology: Functional group efficiency in the removal of haze-forming proteins from wines. *Food Chem.* 275: 154-160.
9. Teng, B., Petrie, P., Smith, P., Bindon, K. 2020a. Comparison of water addition and early-harvest strategies to decrease alcohol concentration in *Vitis vinifera* cv. Shiraz wine: impact on wine total phenolics, tannin and colour properties. *Aust. J. Grape Wine Res.* 26(2):158-171.
10. Kang, W., Bindon, K.A., Wang, X., Muhlack, R.A., Smith, P.A., Niimi, J., Bastian, S.E. 2020. Chemical and sensory impacts of accentuated cut edges (ACE) grape must polyphenol extraction technique on Shiraz wines. *Foods*. 9(8): 1027.
11. Carew, A.L., Kerslake, F.L., Bindon, K.A., Smith, P.A., Close, D.C., Dambergs, R.G. 2020. Viticultural and controlled phenolic release treatments affect phenolic concentration and tannin composition in Pinot Noir wine. *Am. J. Enol. Vitic.* 71(4): 256-265.
12. Teng, B., Petrie, P.R., Espinase Nandorfy, D., Smith, P., Bindon, K. 2020b. Pre-fermentation water addition to high-sugar Shiraz must: Effects on wine composition and sensory properties. *Foods*. 9(9): 1193.
13. Bekker, M.Z., Espinase Nandorfy, D., Kulcsar, A.C., Faucon, A., Bindon, K., Smith, P.A. 2021. Comparison of remediation strategies for decreasing 'reductive' characters in Shiraz wines. *Aust. J. Grape Wine Res*. 27(1): 52-65.
14. Mierczynska-Vasilev, A., Bindon, K., Gawel, R., Smith, P., Vasilev, K., Koynov, K. 2021. Fluorescence correlation spectroscopy to unravel the interactions between macromolecules in wine. *Food Chem*. 352: 129343.
15. Varela, C., Bartel, C., Espinase Nandorfy, D., Bilogrevic, E., Tran, T., Heinrich, A., Balzan, T., Bindon, K., Borneman, A. 2021. Volatile aroma composition and sensory profile of Shiraz and Cabernet Sauvignon wines produced with novel *Metschnikowia pulcherrima* yeast starter cultures. *Aust. J. Grape Wine Res*. 27(3): 406-418.

16. Day, M.P., Espinase Nandorfy, D., Bekker, M.Z., Bindon, K.A., Solomon, M., Smith, P.A., Schmidt, S.A. 2021. Aeration of *Vitis vinifera* Shiraz fermentation and its effect on wine chemical composition and sensory attributes. *Aust. J. Grape Wine Res*. 27(3): 360-377.
17. Teng, B., Hayasaka, Y., Smith, P.A., Bindon, K.A. 2021. Precipitation of tannin–anthocyanin derivatives in wine is influenced by acetaldehyde concentration and tannin molecular mass with implications for the development of nonbleachable pigments. *J. Agric. Food Chem.* 69(16): 4804-4815.
18. Ntuli, R.G., Saltman, Y., Ponangi, R., Jeffery, D.W., Bindon, K., Wilkinson, K.L. 2022a. Impact of fermentation temperature and grape solids content on the chemical composition and sensory profiles of Cabernet Sauvignon wines made from flash détente treated must fermented off-skins. *Food Chem*. 369: 130861.
19. Mierczynska-Vasilev, A., Vasilev, A., Reilly, T., Bindon, K., Vasilev, K. 2022. Fluorescence sensing technology for the rapid detection of haze-forming proteins in white wine. *Food Chem*. 374: 131770.
20. Kassara, S., Norton, E.L., Mierczynska-Vasilev, A., Sacks, G.L., Bindon, K.A. 2022. Quantification of protein by acid hydrolysis reveals higher than expected concentrations in red wines: Implications for wine tannin concentration and colloidal stability. *Food Chem.* 385: 132658.
21. Espinase Nandorfy, D., Watson, F., Likos, D., Siebert, T., Bindon, K., Kassara, S., Shellie, R., Keast, R., Francis, I.L. 2022. Influence of amino acids, and their interaction with volatiles and polyphenols, on the sensory properties of red wine. *Aust. J. Grape Wine Res*. (in press).
22. Ntuli, R.G., Saltman, Y., Ponangi, R., Jeffery, D.W., Bindon, K., Wilkinson, K.L. 2022b. Impact of Skin Contact Time, Oak and Tannin Addition on the Chemical Composition, Color Stability and Sensory Profile of Merlot Wines made from Flash Détente Treatment. *Food Chem* (in press).

10.5 Published technical articles and conference proceedings

1. Petrie, P.R., Teng, B., Dilution, Smith, P.A., Bindon, K.A. 2019. Managing high Baume juice using dilution, *Wine Vitic. J*. 34(1): 36-37
2. McRae, J., Mierczynska-Vasilev, A., Herderich, M., Johnson, D. 2019. AWRI report: Getting proactive about protein. *Wine Vitic J* 34(2): 33-34.
3. Day, M.P., Capone, D.L., Kassara, S., Bilogrevic, E.O., Espinase Nandorfy, D., Watson, F.T., Francis, I.L., Bindon, K.A. 2020. Whole bunch fermentation of Shiraz and Pinot Noir: influence on 'green' characters and astringency. Beames, K.S, Robinson, E.M.C., Dry, P.R., Johnson, D.L. (eds.). *Proceedings of the 17th Australian Wine Industry Technical Conference*, 21-24 July 2019, Adelaide, SA: 94-96.
4. McRae, J., Godden, P., Romanini, E. 2019. Effect of juice heating on the sensory profile of wine. *Technical Review*, 242: 6-10.
5. McRae, J.M., 2020. New techniques and technologies for wine protein stabilisation. Beames, K.S, Robinson, E.M.C., Dry, P.R., Johnson, D.L. (eds.). *Proceedings of the 17th Australian Wine Industry Technical Conference*, 21-24 July 2019, Adelaide, SA.: 125-126.
6. Bindon, K.A., Teng, B., Smith, P.A., Espinase Nandorfy, D., Solomon, M., Petrie, P.R. 2020. Winemaking with high Baume juice: optimising fermentation and extraction. Beames, K.S, Robinson, E.M.C., Dry, P.R., Johnson, D.L. (eds.). *Proceedings of the 17th Australian Wine Industry Technical Conference*, 21-24 July 2019, Adelaide, SA: 131-133.

11. Appendix 2: Intellectual property

Program IP has been communicated through presentations and publications and resides in the public domain. Copyright in any reports, posters or presentations is held by the AWRI. Copyright in

publications remains with the publisher. No patentable discoveries have been identified from the work.

A commercialisation evaluation of zeolites as potential materials for achieving protein and cold stability was prepared for the WA-AWRI Investment Agreement Management Committee. Commercialisation opportunities will be further explored through an open innovation consultation process with producers and suppliers.

Plasma-coated surfaces have proven effective in initiating KHT crystallisation at cellar temperature and consequently could effectively cold stabilise white wines. Future research is required to investigate whether plasma-coated mesh surfaces affect the sensory properties of wine or introduce impurities.

12. Appendix 3: References

Bekker, M.Z., Espinase Nandorfy, D., Kulcsar, A.C., Faucon, A., Bindon, K., Smith, P.A. 2021. Comparison of remediation strategies for decreasing 'reductive' characters in Shiraz wines. *Aust. J. Grape Wine Res.* 27:52-65.

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Bosso, A., Motta, S., Petrozziello, M., Guaita, M., Asproudi, A., Panero, L. 2016. Validation of a rapid conductimetric test for the measurement of wine tartaric stability. *Food Chem.* 212:821-827.

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13. Appendix 4: Staff

- Dr Paul Smith (until September 2017)
- Dr Keren Bindon
- Dr Agnieszka Mierczynska-Vasilev
- Dr Jacqui McRae (until July 2019)
- Dr Bo Teng (until September 2018)
- Dr Yoji Hayasaka (until July 2020)
- Dr Alicia Jouin (from January 2020 until January 2022)
- Mrs Stella Kassara
- Mr Tim Reilly (from January 2019)
- Mr Luke Qi
- Ms Maddy (WenWen) Jiang
- Mr Damian Espinase Nandorfy
- Ms Eleanor Bilogrevic
- Ms Desiree Likos
- Mr John Gledhill

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

Table 5. Percentage of volatile compounds removed from Chardonnay wine after treatment with five zeolites and bentonite

Volatile compounds	Aussie	Grind	Indonesian	Orku	Zeo Natural	Bentonite
Ethyl acetate (VA, nail polish)	ns	ns	ns	ns	ns	8%
2-methylpropanol (Fusel, spiritous)	ns	10%	8%	7%	ns	ns
Acetic acid (VA, Vinegar)	ns	ns	ns	ns	ns	8.5%
Ethyl propanoate (Fruity)	ns	ns	ns	ns	ns	10%
Ethyl-2-methylpropanoate (Fruity)	ns	ns	ns	ns	ns	12%
3-methylbutanol (Harsh, nail polish)	10.5%	9%	12%	11.5	8%	ns
2-methylbutanol (Nail polish)	ns	ns	ns	ns	ns	ns
Decanoic acid (Fatty)	33%	35%	25%	36%	31%	ns
2-phenylethyl acetate (Floral, rose)	ns	10%	41%	10%	ns	ns
Propanoic acid (Vinegar)	8%	ns	ns	ns	ns	ns
2-methylpropyl acetate (Banana, fruity)	ns	ns	ns	ns	ns	10%
Ethyl butanoate (Acid fruit)	ns	ns	ns	ns	ns	10%
3-methylbutanoic acid (Blue cheese)	ns	9%	ns	7%	ns	9%
Ethyl-3-methylbutanoate (Berry)	ns	ns	ns	ns	ns	10%
3-methylbutyl acetate (Banana)	ns	ns	ns	ns	ns	9%
2-methylbutyl acetate (Banana, fruity)	ns	ns	ns	ns	ns	9%
Hexanol (Green, grass)	ns	9%	ns	10%	ns	ns
Ethyl hexanoate (Green apple)	ns	ns	ns	ns	ns	10%
Hexyl acetate (Sweet, perfume)	ns	ns	ns	ns	ns	ns
Hexanoic acid (Cheese, sweaty)	6%	13%	ns	13%	8%	ns
2-phenylethanol (Floral, rose)	7%	11%	10%	11%	7%	ns
Ethyl octanoate (Sweet, soap)	ns	ns	50.5%	ns	ns	13%

Octanoic acid (Rancid, harsh)	19%	18%	ns	21.5%	11%	10%
Ethyl decanoate (Sweet, soap)	ns	ns	54%	ns	ns	ns

Table 6. Percentage of volatile compounds removed from Muscat Gordo wine after treatment with five zeolites and bentonite

Volatile compounds	**Aussie**	**Grind**	**Indonesian**	**Orku**	**Zeo Natural**	**Bentonite**
Ethyl acetate (VA, nail polish)	22%	11.5%	19%	16%	13.5%	ns
2-methylpropanol (Fusel, spiritous)	18%	14%	18%	16%	17%	ns
Acetic acid (VA, Vinegar)	10%	11%	12%	12%	ns	ns
Ethyl propanoate (Fruity)	ns	ns	21%	ns	17%	ns
Ethyl-2-methylpropanoate (Fruity)	ns	ns	ns	ns	ns	ns
3-methylbutanol (Harsh, nail polish)	12%	15%	12%	15%	11%	ns
2-methylbutanol (Nail polish)	ns	ns	26%	ns	ns	14%
Decanoic acid (Fatty)	31%	30%	19%	35.5%	25%	36%
2-phenylethyl acetate (Floral, rose)	15%	17%	87%	17%	13%	ns
Propanoic acid (Vinegar)	6%	ns	ns	ns	ns	ns
2-methylpropyl acetate (Banana, fruity)	ns	ns	20%	ns	ns	9%
Ethyl butanoate (Acid fruit)	25%	12.5%	18%	ns	15%	10%
3-methylbutanoic acid (Blue cheese)	10%	ns	19%	32%	7.5%	ns
Ethyl-3-methylbutanoate (Berry)	ns	ns	ns	ns	ns	ns
3-methylbutyl acetate (Banana)	ns	ns	ns	ns	ns	12%
2-methylbutyl acetate (Banana, fruity)	ns	ns	ns	ns	ns	ns
Hexanol (Green, grass)	16%	13.5%	18%	ns	13%	ns
Ethyl hexanoate (Green apple)	34%	ns	46%	23.5%	22%	10%
Hexyl acetate (Sweet, perfume)	34%	ns	64%	ns	ns	17%

Hexanoic acid (Cheese, sweaty)	15%	16%	14%	13%	14%	ns
2-phenylethanol (Floral, rose)	12%	13%	17%	17%	11%	ns
Ethyl octanoate (Sweet, soap)	32%	ns	72%	22.5%	27%	14%
Octanoic acid (Rancid, harsh)	25%	25%	20%	28%	22%	ns
Ethyl decanoate (Sweet, soap)	50%	41%	78%	35%	50%	34.5%

Table 7. Percentage of volatile compounds removed from Sauvignon Blanc wine after treatment with five zeolites and bentonite

Volatile compounds	**Aussie**	**Grind**	**Indonesian**	**Orku**	**Zeo Natural**	**Bentonite**
Ethyl acetate (VA, nail polish)	14%	18%	18%	14%	13%	6%
2-methylpropanol (Fusel, spiritous)	11%	12%	14%	5%	6%	ns
Acetic acid (VA, Vinegar)	ns	ns	ns	ns	9%	ns
Ethyl propanoate (Fruity)	ns	17%	19%	13%	13%	7%
Ethyl-2-methylpropanoate (Fruity)	13%	17%	17%	16%	16%	ns
3-methylbutanol (Harsh, nail polish)	ns	ns	6%	ns	ns	8%
2-methylbutanol (Nail polish)	ns	11%	ns	ns	ns	14%
Decanoic acid (Fatty)	36%	36.5%	23%	30%	33%	13.5%
2-phenylethyl acetate (Floral, rose)	18%	16%	76%	12%	13%	9%
Propanoic acid (Vinegar)	ns	ns	ns	ns	ns	ns
2-methylpropyl acetate (Banana, fruity)	9%	11.5%	15%	11%	9%	10%
Ethyl butanoate (Acid fruit)	14%	16%	20%	15%	15.5%	8.5%
3-methylbutanoic acid (Blue cheese)	9%	13%	ns	ns	7.5%	ns
Ethyl-3-methylbutanoate (Berry)	13.5%	19%	24%	16%	17.5%	ns
3-methylbutyl acetate (Banana)	15%	17.5%	34%	16.5%	15%	10%
2-methylbutyl acetate (Banana, fruity)	ns	21%	38%	15.5%	ns	11%
Hexanol	14.5%	15%	17%	11%	9%	ns

(Green, grass)						
Ethyl hexanoate (Green apple)	24%	29%	40%	25%	25%	10%
Hexyl acetate (Sweet, perfume)	24.5%	29.5%	50%	27%	11.5%	8%
Hexanoic acid (Cheese, sweaty)	15%	14%	12%	12%	12%	ns
2-phenylethanol (Floral, rose)	12%	14.5%	15%	7%	9%	ns
Ethyl octanoate (Sweet, soap)	23%	32%	63%	27%	26%	7%
Octanoic acid (Rancid, harsh)	22%	23%	10.5%	16%	14%	ns
Ethyl decanoate (Sweet, soap)	34%	29%	65%	40%	37%	12.5%