**Stream 2.2: Novel winemaking processes to stabilise and package wine and deliver it to the consumer in optimum condition whilst maintaining or improving quality, value and sustainability**

## 1. Abstract:

This stream addressed the need to establish alternatives to bentonite fining by i) developing novel processes for protein haze control and ii) determining the feasibility of such treatment(s) and their effects on wine composition and sensory properties. In parallel, research determined the relative importance of winemaking practices, packaging choices and transport and storage conditions on oxygen ingress into wines and linked this to wine development in bottle.

From benchmarking a number of current bentonite fining practices, the best way to use bentonite was proven to be its addition during white wine fermentation. The main aspects of the mechanism of haze formation in wine were determined; this required isolation, identification and comprehensive characterisation of key proteins, including solving their crystal structure. Ultimately, critical new knowledge of the temperature required to unfold the haze forming proteins was gained. This led to a novel strategy for protein stabilisation of white wines, involving juice flash pasteurisation to unfold haze proteins so that they can be more easily broken down by food grade proteolytic enzymes. A 20% reduction in bentonite usage is reasonably achievable from this research, leading to approximately $10 million in potential annual savings for Australian wine producers.

The project also proved that managing 'stinky sulfur'-type aromas can be achieved using oxygen during fermentation and that this approach is more effective than attempts to remediate hydrogen sulfide after fermentation using copper or aeration. Importantly, additions of copper (or some other metals) at bottling was shown to promote the development of 'stinky sulfur'-type aromas in wine. In addition, new tools for total package oxygen management were created. Such knowledge and tools create value through enabling wineries to minimise premature development of wines, and increasing control of shelf-life and wine style development.

## 2. Executive summary:

Protein haze remains as one of the key instability risks in white wine production that requires costly treatment with potential for losses in overall quality. Work in this stream was structured to address the need for alternatives to bentonite fining by developing novel processes for protein haze control; determining the likely commercial feasibility of such treatment(s); and their effects on wine composition and sensory properties. This was achieved by developing a thorough understanding of the way that protein haze forms; using that knowledge to apply proteolytic enzymes and associated heat treatment approaches; as well as identifying and evaluating other alternative fining technologies.

The main aspects of the mechanism of haze formation in wines were determined after key proteins were isolated, characterised, crystallised and the role they and other compounds play in wine haze formation was elucidated. Several emerging and new alternatives to bentonite for the stabilisation of white wines were either evaluated or explored for the first time. Ultimately, the knowledge of the temperature required to unfold the haze forming proteins was used to develop a successful strategy for protein stabilisation of white wines. Juice flash pasteurisation was used to unfold the proteins so that they can be more easily broken down by a readily available, food grade mixture of enzymes (Aspergillopepsin I and II, 'AGP') commercially known as 'Proctase'. This is the first efficient and cost-effective alternative to bentonite treatment, and the most likely process to become adopted as a commercially viable application for the wine industry. For wineries choosing to retain bentonite fining, it was discovered that the best way to use bentonite was by addition during fermentation which led to faster fermentations using less bentonite. This process results in faster turnaround times for tanks , aiding process efficiency and scheduling but without additional capital investment. A 20%

reduction in bentonite usage is reasonably achievable from this research, potentially leading to an approximately $10 million annual saving in Australia.

At the outset of this stream, the industry also had few tools to predict shelf-life because the key controlling factors were poorly understood. Other work aimed to assess the relative importance of winemaking practices, packaging choices and transport and storage conditions on oxygen ingress into wines and to link this to wine development in bottle. This required research into, and application of, tools to estimate oxygen ingress into bottled wines.

It was also proved that managing 'stinky sulfur'-type aromas can be achieved using oxygen during fermentation and that this approach is more effective than attempts to remediate after fermentation using copper or aeration. Use of oxygen early in the fermentation can prevent the production of hydrogen sulfide (rotten egg aroma). Different oxygen levels during fermentation also lead to differing aroma and textures showing that oxygen exposure during ferment can be used to create different wine styles. Early or late post-ferment copper treatments are effective at reducing some undesirable sulfur compounds, but late copper additions increase the residual copper concentration. Post-ferment aeration treatments to remediate the undesirable aromas were not very effective. Thus, early oxygen exposure of crushed grapes plays a very important role in determining the 'stinky sulfur' aroma of a wine.

Research, with co-investment from a supplier, proved that copper additions at bottling can promote the formation of hydrogen sulfide post-bottling and that other metals affect the development of undesirable sulfur aromas in wine. Furthermore, while glutathione appears to protect the varietal sulfur compound responsible for tropical aroma (3-MH), copper is clearly detrimental to the concentration of this key aroma compound. This means that a wine's compositional characteristics such as glutathione, copper, varietal thiol profile and oxygen exposure all interact in a complex manner to define the sensory outcome after storage post-bottling. In red wine, oxygen exposure through micro-oxygenation or from transmission through the closure can modulate wine style. In addition, oxygen transmission through the closure can, in time, have a similar effect compared to oxygen pre-bottling through micro oxygenation. Such knowledge of wine style evolution post-bottling, and the closure performance parameters that underpin it, provides winemakers with greater influence over the style of their wine at the time it is purchased by consumers.

Validation and application of a new fluorescence-based oxygen measurement tool enabled direct quantification of total package oxygen (TPO) at bottling. Demonstration studies and comprehensive extension activities led to improved bottling line management by numerous wine businesses. Such new tools for TPO management also enabled wineries to minimise premature oxidation of wines; increase their control of shelf-life; and increase control over wine style development.

It is recommended that the pursuit of regulatory approval of Proctase by Food Standards Australia and New Zealand (FSANZ) and the Organisation Internationale de la Vigne et du Vin (OIV) is continued. Further opportunities can be realised from the knowledge of the haze formation mechanism; for example, future research could target proteases that degrade grape proteins at fermentation temperature ranges (which would remove the need for the protein unfolding step through pasteurisation as used in the current Proctase strategy). Continued research is required into the role of metals and the effects of metal management on reductive aroma formation, and the role of pre-fermentation oxygen and introduction of oxygen during fermentation in shaping wine style.

| **Affiliation** | **Area of support/contribution** |
|---|---|
| University of Queensland | Thermal aggregation of purified grape and wine proteins |
| INRA, Montpellier, France | Study of protein aggregation behaviour by Dynamic Light Scattering |

| Affiliation | Area of support/contribution |
|---|---|
| Università degli Studi di Padvoa | Study of the mechanism of haze formation in white wines |
| Macquarie University | Proeomic analysis on wine proteins |
| Treasury Wines Estates | Carrageenan for white wine stabilisation |
| Flinders University | Crystal structure of haze proteins |
| Nomacorc | Oxygen management |
| Orlando Wines | Novel winemaking processes |

## 3. Background:

Protein haze is one of the key instabilities in white wine production which requires costly treatment with associated wine losses and potential quality downgrades. Most of these costs are associated with the quality downgrading of wine recovered from the bentonite lees. Work in this stream addressed the need to develop alternatives to bentonite fining by discovering novel proteolytic enzymes; developing new heat treatment regimes; and through a thorough understanding of the mechanisms that lead to haze formation.

In addition to protein haze formation after bottling, wine can also be placed at risk of oxidative or reductive spoilage and premature development through packaging, transport and storage decisions. At the start of this stream, the industry had little in the way of tools to predict shelf-life because the factors likely to impact on it were poorly understood. In particular, information was lacking or incomplete on what has the greatest influence on oxygen ingress and it was difficult to accurately quantify the amount of oxygen that was introduced into bottles during packaging and wine transfer operations.

Other work in this stream aimed to assess the relative importance of winemaking practices, packaging choices and transport and storage conditions, on oxygen ingress into wines and to link this to wine development in bottles and oxidative spoilage. This information is required to understand development of wine style throughout various stages of a bottled wine's life.

The expected outcomes of benefit to the Australian wine industry were:

- The ability to utilise innovative processes to stabilise wines against protein haze formation, and knowledge of the impact of these processes on wine quality.
- An enhanced ability to tailor wine packaging to wine style and development potential, and reduce the incidence of oxidative spoilage in bottled wines.

## 4. Stream objectives:

The stream objectives were to:

- develop novel processes for protein haze control and determine the likely commercial feasibility of such treatment(s) and their effects on wine composition and sensory properties;
- develop a tool to estimate oxygen ingress into bottled wines and gain an understanding of the main parameters influencing oxygen ingress into bottled wine;
- understand the relative impact of oxygen ingress, varying temperature, varying $SO_2$ and ascorbic acid ratios and other wine compositional factors, including variety, on wine development and oxidative spoilage; and
- develop and validate algorithms and models predicting shelf-life of bottled wines.

The main focus of the third objective was changed from 'temperature, $SO_2$ and ascorbic acid ratios' to the 'relative impact of oxygen ingress' component. This was because preliminary research indicated that the greatest value was likely to be returned from this shift of the main focus. In addition, both oxidative and reductive spoilage were addressed.

The fourth objective 'develop and validate algorithms and models predicting shelf-life of bottled wines' was addressed through an additional project 'The role of oxygen ingress at bottling and oxygen transmission during storage on wine development' that attracted co-investment from a supplier. The objective of this project became:

- Understanding the role of oxygen ingress at bottling and oxygen transmission during storage on wine development.

## 5. Methodology:

In order to elucidate the mechanism of haze formation and obtain information required for trialling new prevention strategies, proteins from wines had to be purified and characterised, and their aggregation behaviour analysed in reconstitution experiments and in combination with other wine components. Experimental detail is provided in cited publications. Isolation of proteins from juice and wine was achieved by developing a two-step chromatographic method based on strong cation exchange (SCX) and hydrophobic interaction chromatography (HIC) (Van Sluyter et al. 2009). For protein characterisation, the purity of isolated proteins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reverse phase-high performance liquid chromatography (RP-HPLC) and their identity was confirmed by peptide nano liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) (Marangon et al. 2009). Unfolding temperature of purified proteins was measured by differential scanning calorimetry (DSC) (Falconer et al. 2010). Purified thaumatin-like proteins (TLPs) were crystallised using a high-throughput screening method (Van Sluyter et al. 2009), and the obtained crystals produced high resolution X-ray crystallography data (see RCSB protein databank links in references) that allowed their 3D structure to be determined. Reconstitution experiments were also used, whereby key components likely to be involved in haze formation were purified and added back to wines or model wines. The aggregation behavior upon heating of samples prepared with different combinations of purified proteins and other wine components was analysed by nephelometry (Marangon et al. 2011c), Dynamic Light Scattering (Marangon et al. 2011b), and Scanning Ion Occlusion Sensing (Gazzola et al. 2012).

In order to assess the viability of alternatives to bentonite, new processing aids were first trialled at laboratory-scale to define the optimal conditions. If successful, these conditions were scaled up in small-scale winemaking experiments. Experimental wines were prepared with different treatments (as carrageenan, pectin, zirconia, flash pasteurisation with proctase, bentonite), and characterised through chemical and sensory analysis (Marangon et al. 2012b; Marangon et al. 2012a; Marangon et al. 2011a).

Oxygen ingress measurements were carried out using a PreSens Fibox 3 trace v3 oxygen meter as described by Ugliano et al. (2011). The mid infra-red (MIR) spectra of wines were recorded as described by Cozzolino and Curtin (2012). Wine color was measured using a Varian/Cary 300 Spectrophotometer, as described by Skouroumounis et al. (2005). Gas chromatography (GC) coupled to sulfur chemiluminescence detection was used to analyse low molecular weight sulfur compounds (LMWSC). Wines were analysed for their LMWSC profiles using an Agilent 355 SCD sulfur chemiluminescence detector coupled to an Agilent 6890A gas chromatograph as described by Siebert et al. (2010). The varietal thiol 3-mercaptohexanol (3-MH) was quantified as the pentafluorobenzyl derivative using a stable isotope dilution assay, by means of solid phase microextraction (SPME)

coupled with GC-MS as described by Ugliano et al. (2011). Wines and stock solutions were analysed for their metal concentrations by Flinders Analytical, Flinders University (Adelaide, Australia) using an Agilent 7500 cx inductively coupled plasma mass spectrometers (Agilent Technologies, Tokyo, Japan) as described in Thiel et al. (2004).

Glutathione was measured as described by Du Toit (2007) and the concentration of methionine was determined by the Australian Proteome Analysis Facility (Macquarie University, Sydney, Australia). A more specialised method analysing 22 amino acids including cysteine was developed and implemented by the AWRI, as described by Seiwert and Karst (2007).

Red and white winemaking was undertaken on various scales including 10 mL , 1 L, 2 L, 20 L, 80 L, 900 L in rotary tanks, static tanks, coffee plungers, plastic containers, test tubes and lab equipment. Sensory descriptive analysis of wines was also performed.

## 6. Results and discussion:

### Novel processes for protein haze control, the likely commercial feasibility of such treatment(s) and their effects on wine composition and sensory properties

*Mechanism of haze formation in wines*

Protein haze formation in white wines is a serious quality defect because consumers perceive hazy wines as faulty. Protein haze is caused by the presence of residual grape pathogenesis-related (PR) proteins in wines after bottling, in particular thaumatin-like proteins (TLPs) and chitinases. It is established that protein haze formation in wine is associated with the elevated temperatures that the wines can be exposed to during storage or transportation, and this can affect the stability of the PR proteins resulting in their aggregation into particles visible to the naked eye. Hence PR proteins need to be removed, and current industry practice to achieve this involves fining with bentonite clay. Research into alternatives to bentonite has been pursued because this fining method has several drawbacks. In order to find a valid substitute for bentonite, the mechanisms of protein haze formation were studied in detail. Research outputs have established that protein instability in wines is a two-step phenomenon: protein unfolding (a temperature mediated step) is followed by growth of particles (colloidal aggregation) due to the unfolded proteins sticking together. Key findings underpinning this understanding included:

- The development of protein purification methods to gain access to large amounts of purified grape proteins. This was critical to enable all subsequent aspects of the project, and was the first time such large-scale protein purification from wine was achieved (Van Sluyter et al. 2009).
- An advanced characterisation of critical proteins using proteomic and biochemistry techniques (Marangon et al. 2009).
- The solving of the crystal structure of a thaumatin-like protein. This detailed structural model provided novel insights into behaviour, mechanism and solutions to managing aggregation in wine.

Purified grape proteins were used to investigate the mechanism of haze formation in model and real wines by reconstituting samples with different components. Critically, chitinases were identified as mainly responsible for wine hazing and the small molecule sulfate was identified as a critical factor in modulating haze formation. The main aspects of the mechanism are now well established and represent a significant advancement. Key activities leading to this outcome were:

- Elucidation of the aggregation behaviour of purified proteins using different techniques and investigating different matrix effects (Gazzola et al. 2012; Marangon et al. 2011b, 2011c).
- Establishing the melting point of the main wine proteins (Falconer et al. 2010) which was critical to develop the Proctase management strategy (detailed below).
- Demonstration that chitinases easily unfold and aggregate while thaumatins were established as

more robust and with a reduced tendency to aggregate (Marangon et al. 2011b, 2011c

*Novel processes for protein haze control and their commercial feasibility*

Study of the timing of bentonite addition during processing and its impact on wine stability and sensory characteristics led to recommendations about the most effective use of bentonite in typical wine production settings. The optimal approach is to use a large addition of 50-70% of the estimated total bentonite required before or during fermentation, and then repeat the final bentonite treatment on the wine (Pocock et al. 2011). Key advantages of using this strategy during the production process include increasing the fermentation rate; easier removal of the bulk of the bentonite along with the yeast lees at the end of fermentation; and less sensory impacts due to reduced amounts of bentonite required in wine to finalise stabilisation.

The viability of zirconia and surface engineered silica (SES) as protein adsorbents and alternatives to bentonite was also assessed. Zirconia in pellet form produced wines that were fully heat stable with minimal loss of wine as lees, with the advantage that zirconia can be easily regenerated. However, since significant amounts are required, the economic feasibility remains to be established (Marangon et al. 2011a). Proof of concept experiments demonstrated that SES binds proteins (Lucchetta et al. 2013); however the sensory effects and the potential for regeneration of the SES material remain to be investigated in detail.

Carrageenan and pectin were assessed for protein removal from wine and carrageenan showed more promise than pectin (Marangon et al. 2012a). A follow-up vintage trial confirmed that carrageenan was capable of fully removing wine proteins at low addition rates and without significant sensory effects, compared to bentonite fining as typically used by the wine industry. Some technical issues remain to be investigated though, including management of foaming risk; a potentially reduced filterability; and possible haze formation in treated wine from residual carrageenan (Marangon et al. 2013).

A protease from *Botrytis cinerea* (BcAP8) was identified with potential to degrade wine proteins and methods were established for production of larger quantities of BcAP8 required for further testing (van Sluyter et al. 2013).

A yeast mannoprotein that acts as haze protective factor (HPF) was produced through overexpression in genetically modified yeast and its addition to wine was shown to improve wine stability (Schmidt et al. 2009). However, HPF addition only resulted in partial stabilisation and large quantities of HPF were required. Thus, HPF addition does not appear to be a feasible approach for stabilising wine on a commercial-scale.

One of the most exciting and practical outcomes from this work builds on the fundamental knowledge that had been generated regarding the mechanism of haze formation, especially the role of heat treatments and temperature in folding and un-folding haze proteins. This led to the development and application of a practically and economically viable stabilisation method. This method was based on flash pasteurisation of juice in the presence of small amount of a commercially available protease known as Proctase (Marangon et al. 2012b), which is further reported under Streams 2.3 and 4. The outcomes from research into this novel process for protein haze control are:

- The ability to produce fully haze stabilised wine, without bentonite fining, that is not different in its sensory or chemistry attributes compared to bentonite fined wine (using standard practices), with no wine loss through lees (as with bentonite fining) and demonstrated positive economic viability especially at larger production volumes (Marangon et al. 2012b).
- Successful industry trials with a number of collaborators on large volumes (Robinson et al. 2012).
- An application to FSANZ seeking regulatory approval for Proctase use in Australian wine production.

**Tools to estimate oxygen ingress into bottled wines and to gain an understanding of the main parameters influencing oxygen ingress into bottled wine**

At the outset of this research, oxygen measurement in wine and wine bottles was a challenging undertaking which had limited advancements in measuring and managing the impacts of oxygen in wine. The objectives were to develop an analytical tool to estimate oxygen ingress into bottled wine and to gain an understanding of the main parameters influencing this oxygen transmission rate (OTR). Initially, research focused on development and application of a dye Bis-9,10 anthracene-(4-trimethylphenyammonium) dichloride (BPAA) that responded to oxygen exposure and allowed non-destructive quantification of oxygen ingress into wine bottles (Skouroumounis and Waters 2008). While the BPAA approach was successful for model solutions, several fluorescence-based oxygen measurement technologies (e.g. PreSens, NomaSense) became widely available and this technology was subsequently utilised to quantify oxygen during winemaking, bottling and ageing of wine.

Capitalising on the Nomasense technology, a non-destructive and *in situ* OTR measurement methodology was developed with applicability to all closure technologies. The method involves fitting a bottle with two oxygen sensors, to measure dissolved and headspace oxygen, placing the sealed bottle or packaged wine inside a sealed container with nitrogen or storing in air, and calculating the OTR from the repeat oxygen measures, taking into account the amount of oxygen from closures (Ugliano et al. 2011).

Winemaking doesn't finish once the product has been bottled, and this work has improved the technical understanding of the role of oxygen on the 'in-bottle maturation' of wine (Ugliano et al. 2010). In particular, it has resulted in improved understanding of the relative contributions of DO, headspace oxygen, TPO and closure OTR to wine development post-bottling (Ugliano et al. 2011). Key findings include that:

- in the first six months after bottling, the contribution of the oxygen trapped in cylindrical closures and in the headspace is relevant to wine development and should not be ignored; and
- after six months bottle storage, ingress through the closure is generally more important than headspace oxygen or oxygen entrained in the closure.

*The relative impact of oxygen ingress and other wine compositional factors, including variety, on wine development*

Wine composition has an important part to play in how oxygen exposure will shape a wine's development. The formation and degradation of key compounds results in a system that undergoes constant change (Waters et al. 2011). For example, this work demonstrated in Sauvignon Blanc that oxygen exposure, copper addition and glutathione all significantly affected the concentration of the key tropical aroma compound 3-mercaptohexanol (3-MH). Increased glutathione levels generally resulted in wines with increased 3-MH. Conversely, increased copper concentration at bottling generally led to decreased 3-MH concentration and most of the effect from increased copper concentration occurred at bottling. Oxygen exposure also had a significant influence on 3-MH, with lower exposure throughout post-bottling storage resulting in less loss of 3-MH (Ugliano et al. 2011).

Significant differences in the final concentration of hydrogen sulfide ($H_2S$), the compound responsible for rotten egg aroma, were also observed in response to changes in glutathione, copper and oxygen concentrations in Sauvignon Blanc wine (Ugliano et al. 2011). In general, wines with added glutathione always exhibited higher $H_2S$ concentrations than their corresponding untreated samples. Given the antioxidant capacity of glutathione, this higher accumulation of $H_2S$ could be due to a lower degree of oxidation occurring in these samples; alternatively glutathione might act as a direct precursor to $H_2S$. Oxygen exposure determined large variations in $H_2S$ content, with the highest $H_2S$ concentrations generally observed in wines with the lowest oxygen exposure. This is consistent with the generally accepted idea that extremely low oxygen exposure, such as that achieved by using certain screwcap closures, can sometimes result in conditions favouring the development of unwanted reductive aromas.

In a further study, this time with Shiraz wine, oxygen exposure post-fermentation but pre-bottling, through micro oxygenation (MOX), was compared to oxygen exposure post-bottling, through ingress through the closure (Ugliano et al. 2012a). The impact of low oxygen exposure on the development of 'reduced' characters was demonstrated, and it was observed that oxygen added before bottling to red wine through MOX might play a role in wine development that is similar to oxygen 'added' post-bottling through the closure. In summary, both the Sauvignon Blanc and Shiraz studies (Ugliano et al. 2011, 2012a) demonstrated that:

- the effects of oxygen ingress into wine are modulated by wine composition;
- glutathione protects thiols responsible for varietal character in Sauvignon Blanc from oxidative losses;
- copper addition at bottling reduces the concentration of 'tropical' thiols and does not necessarily prevent $H_2S$ accumulation over time; and
- copper addition at bottling might enhance $H_2S$ accumulation over time and thus precautionary copper additions at bottling might not prevent reductive characters developing post-bottling.

Overall, it was confirmed that oxygen exposure post-bottling influences wine development; copper can act as a pro-oxidant (stimulating oxidation); and, as expected, glutathione can act as an anti-oxidant (preventing oxidation). The observation that copper additions at bottling can result in the evolution of some volatile sulfur compounds (VSCs) post-bottling, such as $H_2S$ and methanethiol (MeSH), led the last stage of this work to focus on reductive aroma formation in anaerobic environments (i.e. conditions that are typical for wine bottled with screwcap closures).

*Fate and formation of reductive aromas*

Aromas caused by some sulfur compounds can be positive (e.g. passion-fruit, grapefruit notes), while others can impact negatively on the aroma of wine. Boiled or rotten egg, sewage and rubber are descriptors associated with these negative impact low molecular weight sulfur compounds (LMWSC). The identity of several of these molecules (hydrogen sulfide, methanethiol and dimethylsulfide) is known, but debate continues over their source and the best ways to manage them.

Following on from the observation that copper additions at bottling can promote the accumulation of $H_2S$ post-bottling, the role of copper and other transition metals in the development of LMWSCs in wine was studied in greater detail (Viviers et al. 2013). Dissolved oxygen (DO) levels in a bottled wine were monitored over time to evaluate the impact of oxygen and metal ions on the production of LMWSCs. Results show that copper initially, and at a time when DO concentrations are still above the limit of quantification, reduces the concentration of $H_2S$. However, after four months of anaerobic storage during which time the wine had used up all the DO, the $H_2S$ concentration increased due to the presence of copper. This effect was observed to a lower extent with other metals, and a similar kinetic profile was noted for methanethiol (MeSH). Thus, copper effects vary depending on the aerobic state of the wine, and that the use and timing of copper fining pre-bottling should be carefully considered.

Early oxygen exposure in must, which occurs from the point of harvesting and crushing until bottling, has been shown to play an important role in shaping wine flavour, particularly with regard to the fate and formation of reductive aromas. Anecdotal evidence from winemakers suggested that making red wine in closed tanks might potentially preserve fruit characters, yet this approach can frequently lead to the development of 'stinky sulfur' aromas. To manage these undesirable aromas, oxygen can be introduced at different stages of the winemaking process, but the underlying principles and ideal quantities of such oxygen treatment are not well understood, nor are the post-bottling effects of earlier oxygen exposure. Commonly used options for introducing oxygen into an active red fermentation are the use of pump-overs or active aeration through fixed or removable sinters inside the tank. To remove any residual post-ferment reductive aromas, aerial racking (or splashing) and/or copper additions are

frequently employed. However, it is unclear whether the splashing involved in these operations is physically displacing (i.e. sparging) any $H_2S$, or whether $H_2S$ is reacting chemically with oxygen in air to create other types of sulfur molecules, which might possibly re-emerge as negative aromas post-bottling. Through winery trials (Day et al. 2013), it was shown that stinky sulfur compound formation can be stopped much earlier through the injection of air during red wine fermentations. This effect is not the consequence of physical stripping of the undesirable compound, but rather due to chemical reactions. The effect on fruity, yeast derived esters appears to be minimal. As such, the use of oxygen injections during fermentation may minimise the concentration of $H_2S$ and some stinky sulfur compounds in young wine, although its impact on the long-term development of a wine remains to be established.

## 7. Outcome and Conclusion:

The AWRI has successfully discovered the main aspects of the mechanism of haze formation in wine; developed novel processes for protein haze control; and determined the likely commercial feasibility of such treatment(s) and their effects on wine composition and sensory properties. In particular, the main aspects of the mechanism of haze formation in wines were determined after key proteins were isolated, characterised, crystallised and used to determine the role they and other compounds have in wine hazing. Several alternatives to bentonite for the stabilisation of white wine were either tested or developed. Ultimately, the knowledge generated by this work in recent years, in particular the detailed understanding of the temperature required to unfold the haze forming proteins (Falconer et al. 2010) was used to develop a novel strategy for protein stabilisation of white wine. Juice flash pasteurisation was successfully adopted to unfold the proteins to break them down by a food grade mixture of enzymes (Aspergillopepsin I and II, AGP) commercially known as Proctase. This is the first efficient and cost-effective alternative to bentonite treatment, and most likely to become adopted as a commercially viable application by the wine industry.

A key outcome of this work stems from the increased value that can be captured by wine producers mainly due to reduced losses of wine and quality downgrades for wine recovered from lees. The discovery that the best way to use bentonite is through in-ferment addition, and that this in turn leads to faster fermentations using less bentonite, has resulted in the potential for tanks to be turned around faster. This aids process efficiency and scheduling, but without the capital investment required to adopt bentonite in-line dosing. Across all approaches that were developed and/or evaluated within this project, a 20% reduction in bentonite usage would be reasonably achievable, leading to an estimated $10 million annual saving for the Australian wine industry from this research.

From the research into the impact of oxygen exposure on wine flavour, early oxygen exposure in crushed grapes and during fermentation might play the most important role in determining the 'undesirable sulfur' aroma of a wine. Use of oxygen early in the fermentation has been demonstrated to stop the production of hydrogen sulfide ($H_2S$); at the same time nitrogen 'sparging' did not decrease $H_2S$, dispelling the notion that it is being physically stripped out of the wine by this process. Wines created with different oxygen levels also had differing aroma and textural attributes, confirming that oxygen exposure during fermentation can be used to create different wine styles. Early or late post-ferment copper treatments are effective in reducing LMWSCs, but late copper addition increases the residual copper concentration. Post-ferment aeration treatments to remediate the aroma were not very effective. This emphasises the importance of fermentation and post-fermentation practices to influence a wine's aroma stability post-bottling. In conclusion, managing 'stinky sulfur'-type aromas can be achieved using oxygen during fermentation and this approach is more effective than attempts to remediate undesirable compounds after fermentation using copper or aeration.

Over the last decade, research conducted by the AWRI has demonstrated that managing the amount of oxygen that ingresses through closures into wine after bottling can have a big effect on the aroma, texture and colour of the wine. If this 'oxygen transmision rate' (OTR) is low and the wine is also

bottled without much oxygen, then a wine can form a 'reductive' aroma characterised by 'stinky sulfur'-type aromas. This is quite variable though: one type of wine might form these undesirable aromas, while another type under similar conditions might not. Research in this stream proved that copper additions at bottling can promote the accumulation of $H_2S$ post-bottling and that other transition metals affect the development of 'stinky sulfur'-type aromas in wine. Furthermore, while glutathione appears to offer significant improvements in delaying tropical aroma (3-MH) degradation over time, copper is clearly detrimental to the concentration of this key aroma compound. At the same time, glutathione, particularly in combination with copper, also results in conditions favourable to the accumulation of powerful off-odour compounds such as $H_2S$. Oxygen exposure can be seen as a modulator of these competing pathways, with low oxygen exposure preserving 3-MH but also favouring accumulation of $H_2S$. This means that a wine's compositional characteristics such as glutathione, copper, varietal thiol profile and oxygen exposure, all interact in a complex manner to define the sensory outcome of post-bottling storage. Exposure of red wine to oxygen through either micro-oxygenation or the closure, can lead to manipulation of wine style. The impact of oxygen entering post-bottling through the closure can, in time, have a similar effect compared to oxygen added pre-bottling through micro oxygenation. In summary, wine oxygen exposure can be readily influenced by closure selection, while the use of copper to remove and/or prevent reductive off-odours prior to bottling warrants caution and appears problematic as a 'just in case' insurance policy. Such knowledge of wine style evolution post-bottling, and the closure performance parameters that underpin it, provides winemakers with greater control over the style of their wine at the time when it is purchased by consumers. In addition, it enables a reduction in lost wine value associated with write- offs due to oxidation issues or formation of in-bottle 'stinky sulfur' aromas.

The understanding of the impact of oxygen on the 'in-bottle maturation' of wine has improved significantly as a result of research associated with this stream. The oxygen transmission rate of packaging materials drives evolution of wine style post-bottling, and this can now be quantified non-destructively for any packaging technology. This was achieved through validation and application of a new fluorescence-based oxygen measurement tool that led to improvements in total package oxygen measurement. These technological advancements were widely disseminated through extension activities. Together with a commercially accessible method to easily quantify the TPO at bottling (O'Brien et al. 2009), bottling line benchmarking across Australian and New Zealand facilities, development of a proficiency testing program for TPO and DO measurements through the Inter Winery Analysis Group, and incorporation of TPO measurement specifications into the Winemakers' Federation of Australia's Packaging Guidelines for the Australian wine industry, this research led to improved bottling line management by numerous wine businesses. The concomitant adoption of new tools for total packaging oxygen management and the much extended knowledge of the roles played by oxygen, antioxidants and metals in defining wine flavour will continue to unlock extra value by enabling wineries to minimise premature oxidation of wines, improve shelf-life, and increase control over wine style development.

## 8. Recommendations:

This research project has identified several knowledge gaps and areas with potential to return high value through extending existing research. To facilitate the commercial use of alternatives to bentonite, it is recommended that support for obtaining FSANZ approval of Proctase in winemaking should be continued. Once formally approved, Proctase could be used for wine production in Australia, provided that the finished wine is destined for the domestic market. Wines treated with Proctase are not currently permitted for export to the European Union, but the AWRI anticipates working with the OIV to overcome this in the future (to be addressed in Project 2.2.4 of the AWRI's 2013-2018 R,D&E plan).

Research is recommended in the following areas to capitalise on the opportunities identified by this stream:

- Build on the knowledge of the haze formation mechanism through the identification of novel proteases that are active at ferment temperature ranges. This would remove the need for the protein unfolding step through pasteurisation used in the current Proctase strategy. Another opportunity is the validation of solid phase alternatives to bentonite that could be used in-line or in filter pads. While evaluating bentonite alternatives, a significant need has been identified for the development of a new predictive test for heat and hazing stability. These all have the potential to lead to a further decrease in bentonite use (to be addressed in Project 3.1.4 of the AWRI's 2013-2018 R,D&E plan).

- Continued research is required into the role of metals and their management on reductive aroma formation. This would also allow capitalisation on opportunities for modulating wine style through the introduction of oxygen during fermentation (to be addressed in Projects 3.3.2 and 3.5.3 of the AWRI's 2013-2018 R,D&E plan).

- To capture the full value of this research, continue to communicate, using a range of extension platforms, the opportunities for reducing bentonite use through addition during fermentation and using flash pasteurisation in combination with Proctase for wine stabilisation. Further extension activities would disseminate the knowledge generated through research into managing wine style through ferment oxygen exposure to modulate wine composition; best practice bottling line processes;, and closure selection that is appropriate to the anticipated shelf-life and style at point of consumption (to be addressed in Projects 4.1.1, 4.1.3, 4.1.4 and 4.3.1 in the AWRI's 2013-2018 R,D&E plan).

## 9. Budget reconciliation:

| Financial Year | Receipts / Income ❶ | Outgoings / Expenditure ❷ |
|---|---|---|
| *Year 1*: 2006/2007 | $541,207 | $541,207 |
| *Year 2*: 2007/2008 | $584,207 | $584,207 |
| *Year 3*: 2008/2009 | $588,731 | $588,731 |
| *Year 4*: 2009/2010 | $436,272 | $436,272 |
| *Year 5*: 2010/2011 | $545,722 | $545,722 |
| *Year 6*: 2011/2012 | $654,872 | $654,872 |
| *Year 7*: 2012/2013 | $777,220 | $777,220 |
| TOTAL | $4,128,231 | $4,128,231 |

❶ Note that the GWRDC – AWRI Investment Agreement budget was established and approved at an aggregate level, with variances to budget (i.e. annual overspends and underspends) reported and considered at that same aggregate (i.e. whole of agreement) level. The receipts / income relating to a Stream for any year therefore equate to the outgoings / expenditure within that Stream for that year, as any variances between total Investment Agreement funding received and total funds expended were considered at the whole of Agreement rather than individual Stream level.

❷ Includes a pro-rated share of Theme 5 *Executive management and administration*.

I hereby certify that this statement is true and accurate.

Signature of duly authorised representative.....

Chris Day — Group Manager – Corporate Services — 29/11/2013

*Name:* *Title:* *Date:*

## 10. References:

The AWRI's R,D&E plan 2013-2018; http://www.awri.com.au/about_the_awri/rde-plan/

Cozzolino, D., Curtin, C. (2012) The use of attenuated total reflectance as a tool to monitor the time course of fermentation in wild ferments. Food Control 26: 241-246.

Day, M.P., Viviers, M., Kassara, S., Smith, P.A. (2013) Post-bottling effects of early oxygen exposure during red winemaking. The 15th Australian Wine Industry Technical Conference, Sydney, New South Wales. The Australian Wine Industry Technical Conference Inc., Adelaide, South Australia.

Du Toit, W.J., Lisjak, K., Stander, M., Prevoo, D. (2007) Using LC-MSMS to assess glutathione levels in south African white grape juices and wines made with different levels of oxygen. Journal of Agricultural and Food Chemistry 55: 2765–2769.

Falconer, R.J., Marangon, M., Van Sluyter, S.C., Neilson, K.A., Chan, C., Waters, E.J. (2010) Thermal stability of thaumatin-like protein, chitinase, and invertase isolated from Sauvignon blanc and Semillon juice and their role in haze formation in wine. Journal of Agricultural and Food Chemistry 58: 975–980.

Gazzola, D., Van Sluyter, S.C., Curioni, A. et al. (2012) Roles of proteins, polysaccharides, and phenolics in haze formation in white wine via reconstitution experiments. Journal of Agricultural and Food Chemistry 60: 10666–10673.

Lucchetta, M., Pocock, K.F., Waters, E.J., Marangon, M. (2013) Use of zirconium dioxide during fermentation as an alternative to protein fining with bentonite for white wines. American Journal of Enology and Viticulture 64(3): 400-404.

Marangon, M., Van Sluyter, S.C., Haynes P.A., Waters E.J. (2009) Grape and wine proteins: their fractionation by hydrophobic interaction chromatography and identification by chromatographic and proteomic analysis. Journal of Agricultural and Food Chemistry 57: 4415–4425.

Marangon M., Lucchetta M., Waters E.J. (2011a) Protein stabilisation of white wines using zirconium dioxide enclosed in a metallic cage. Australian Journal of Grape and Wine Research 17: 28–35.

Marangon M., Sauvage F-X., Waters E.J., Vernhet A. (2011b) Effects of ionic strength and sulfate upon thermal aggregation of grape chitinases and thaumatin-like proteins in a model system. Journal of Agricultural and Food Chemistry 59: 2652–2662.

Marangon, M., Van Sluyter, S.C., Neilson, K.A., Chan, C. Haynes, P.A., Waters, E.J., Falconer, R.J. (2011c) Roles of grape thaumatin-like protein and chitinase in white wine haze formation. Journal of Agricultural and Food Chemistry 59: 733–740.

Marangon, M., Lucchetta, M., Duan, D., Stockdale, V.J., Hart, A., Rogers, P.J., Waters, E.J. (2012a) Protein removal from a Chardonnay juice by addition of carrageenan and pectin. Australian Journal of Grape and Wine Research 18: 194–202.

Marangon, M., Van Sluyter, S.C., Robinson, E.M.C., Muhlack, R., Holt, H., Haynes, P.A., Godden, P.W., Smith, P.A., Waters, E.J. (2012b) Degradation of white wine haze proteins by Aspergillopepsin I and II during juice flash pasteurization. Food Chemistry 135: 1157–1165.

Marangon, M., Stockdale, V.J., Trethewey, T., Munro. P., Schulkin. A., Holt H.E., Smith, P.A., (2013) Addition of carrageenan at different stages of winemaking for white wine protein stabilization. Journal of Agricultural and Food Chemistry 61: 6516-6524.

O'Brien, V.T., Colby, C., Nygaard, M. (2009) Managing oxygen ingress at bottling. Australian and New Zealand Wine Industry Journal, 24 (1): 24-29.

Pocock K.F., Salazar F.N., Waters E.J. (2011) The effect of bentonite fining at different stages of white winemaking on protein stability. Australian Journal of Grape and Wine Research 17: 280–284.

Robinson, E., Scrimgeour, N., Marangon, M., Muhlack, R., Smith, P., Godden, P., Johnson, D. (2012) Beyond Bentonite. Wine & Viticulture Journal 27 (6): 20–26.

RCSB protein databank - see links:
http://www.rcsb.org/pdb/explore/explore.do?structureId=4L5H
http://www.rcsb.org/pdb/search/structidSearch.do?structureId=4JRU
http://www.rcsb.org/pdb/search/structidSearch.do?structureId=4MBT

Schmidt, S.A., Tan, E.L. Brown, S., Nasution, U.J., Pettolino, F., Macintyre, O.J., Lopes, M.D.B., Waters, E.J., Anderson, P.A. (2009) Hpf2 glycan structure is critical for protection against protein haze formation in white wine. Journal of Agricultural and Food Chemistry 57: 3308–3315.

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

Seiwert, B., Karst, U. (2007) Simultaneous LC/MS/MS determination of thiols and disulfides in urine samples based on differential labelling with ferrocene-based maleimides. Analytical Chemistry 79: 7131–7138.

Skouroumounis, G.K., Kwiatkowski, M.J., Francis, I.L., Oakey, H., Capone, D.L., Duncan, B., Sefton, M.A., Waters, E.J. (2005) The impact of closure type and storage conditions on the composition, colour and flavour properties of a Riesling and a wooded Chardonnay wine during five years ' storage. Australian Journal of Grape and Wine Research 11: 369-377.

Skouroumounis, G.K., Waters, E.J. (2008) Oxygen ingress into bottled wine. Practical Vineyard and Winery July/August 2008: 6-14.

Thiel, G., Geisler, G., Blechschmidt, I., Danzer, K. (2004) Determination of trace elements in wines and classification according to their provenance. Analytical and Bioanalytical Chemistry 378: 1630–6.

Ugliano, M., Kwiatkowski, M.J., Travis, B., Francis, I.L., Waters, E.J., Herderich, M.J., Pretorius, I.S (2010) Post-bottling management of oxygen to reduce off-flavor formation and optimize wine style. Australian and New Zealand Wine Industry Journal 30 (6): 14–21, 49.

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

Ugliano, M., Dieval. J.-B., Siebert, T.E., Kwiatkowski, M., Aagaard, O., Vidal, S., Waters E.J. (2012a) Oxygen consumption and development of volatile sulfur compounds during bottle aging of

two Shiraz wines. Influence of pre- and post-bottling controlled oxygen exposure. Journal of Agricultural and Food Chemistry 60: 8561-8570.

Van Sluyter, S.C., Marangon, M., Stranks, S.D., Neilson, K.A., Hayasaka, Y., Haynes, P.A., Menz, R.I., Waters, E.J. (2009) Two-step purification of pathogenesis-related proteins from grape juice and crystallization of thaumatin-like proteins. Journal of Agricultural and Food Chemistry 57: 11376–11382.

Van Sluyter, S.C., Warnock, N.I., Schmidt, S., Anderson, P., van Kan, J.A.L., Bacic, A., Waters, E.J. (2013) Aspartic acid protease from Botrytis cinerea removes haze-forming proteins during white winemaking. Journal of Agricultural and Food Chemistry 61: 9705-9711.

Viviers, M., Smith, M., Wilkes, E., Smith, P.A. ( 2013) Effects of Five Metals on the Evolution of Hydrogen Sulfide, Methanethiol and Dimethyl sulfide during Anaerobic Storage of Chardonnay and Shiraz wines. Journal of Agricultural and Food Chemistry, http://dx.doi.org/10.1021/jf403422x.

Waters, E., Ugliano, M., Kwiatkowski, M., Day, M., Bramley, B., Francis, L., Jung, R., Dimkou, E., Dieval, J-B., Aagaard, O., Nygaard, M., Vidal, S. (2011) Does winemaking continue after bottling? The influence of wine preparation for bottling and oxygen exposure during aging on wine composition. Chassagne, D, Gougeon, R.D. (eds) Wine active compounds 2011 : Proceedings of the Second Edition of the International Conference Series on wine active compounds. 157-158.

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